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ORIGINAL ARTICLE

Genome-wide identification, characterization and expression profiles of the *CCD* gene family in *Gossypium* species

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Abstract

Carotenoid cleavage dioxygenases (CCDs) are a group of enzymes that catalyze the selective oxidative cleavage steps from carotenoids to apocarotenoids, which are essential for the synthesis of biologically important molecules such as retinoids, and the phytohormones abscisic acid (ABA) and strigolactones. In addition, CCDs play important roles in plant biotic and abiotic stress responses. Till now, a comprehensive characterization of the *CCD* gene family in the economically important crop cotton (*Gossypium* spp.) is still missing. Here, we performed a genome-wide analysis and identified 33, 31, 16 and 15 *CCD* genes from two allotetraploid *Gossypium* species, *G. hirsutum* and *G. barbadense*, and two diploid *Gossypium* species, *G. arboreum* and *G. raimondii*, respectively. According to the phylogenetic tree analysis, cotton *CCD*s are classified as six subgroups including *CCD1*, *CCD4*, *CCD7*, *CCD8*, *nine-cis-epoxycarotenoid dioxygenase* (*NCED*) and z*axinone synthase* (*ZAS*) sub-families. Evolutionary analysis shows that purifying selection dominated the evolution of these genes in *G. hirsutum* and *G. barbadense*. Predicted *cis*-acting elements in 2 kb promoters of *CCD*s in *G. hirsutum* are mainly involved in light, stress and hormone responses. The transcriptomic analysis of *GhCCD*s showed that different *GhCCD*s displayed diverse expression patterns and were ubiquitously expressed in most tissues; moreover, *GhCCD*s displayed specific inductions by different abiotic stresses. Quantitative reverse-transcriptional PCR (qRT-PCR) confirmed the induction of *GhCCD*s by heat stress, salinity, polyethylene glycol (PEG) and ABA application. In summary, the bioinformatics and expression analysis of *CCD* gene family provide evidence for the involvement in regulating abiotic stresses and useful information for in-depth studies of their biological functions in *G. hirsutum*.

Keywords *CCD* genes · Cotton · Genome-wide analysis · Phylogenetic tree · Abiotic stresses · Expression analysis

Introduction

Cotton (*Gossypium* spp.) is the most important crop for natural textile fiber and one of the main sources for editable oil in the world (Chen et al. 2007). The genus *Gossypium* consists of about 45 diploid ($2n = 2\times = 26$) and five allotetraploid

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($2n = 4\times = 52$) species. Among them, two allotetraploid cotton, *G. hirsutum* (Upland cotton) and *G. barbadense* (Sea Island cotton) are two main domesticated cultivars that are used for fiber production. *G. hirsutum* dominates more than 90% of worldwide cotton production due to its high yield, and *G. barbadense*, with superior fiber quality, accounts for less than 10%. It is assumed that allotetraploid *Gossypium* arises 1–1.5 million years ago (MYA) through one hybridization event between one extant progenitor of *G. herbaceum* (Marion-Poll) or *G. arboreum* (A2) and another progenitor, *G. raimondii Ulbrich* (D5) (Endrizzi et al. 1985; Wendel 1989; Zhang et al. 2015). Cotton is a moderately salt-tolerant crop; however, it is usually planted on saline-alkali or dry land due to insufficient farm land. Abiotic stresses, such as high salt, drought, heat, seriously affect the productivity and quality of cotton fiber (Sexton and Gerard 1982). Thus, developing stress-resistant cotton varieties is important to improve cotton fiber quality and yield performance.

Carotenoids are a class of C_{40} isoprenoid pigments that play as accessory photosynthetic pigments and constituents of photosynthetic apparatus, and play essential roles in photosynthesis and photoprotection (Hashimot 2016). Apart from that, carotenoids act as precursors of a series of biologically important regulatory molecules in plants, such as β-cyclocitral (Ramel et al. 2012), the phytohormones abscisic acid (ABA) and strigolactone (Schwartz et al. 1997; Alder et al. 2012), and the recently identified plant regulatory metabolites, anchorene, iso-anchorene and zaxinone (Jia et al. 2019b, 2021; Wang et al. 2019). These derivatives are produced from the selective oxidative cleavage of carotenoids, mainly catalyzed by carotenoid cleavage dioxygenases (CCDs). CCDs are a family of non-heme Fe^{II}-dependent enzymes that cleave the conjugated C–C double bonds in carotenoids to produce different apocarotenoids. CCDs differ in their substrate specificity that covers the type of carotenoid, e.g., cyclic or acyclic, its stereo configuration (*cis*/*trans*) and the cleavage site, i.e., regiospecificity (Giuliano et al. 2003; Walter and Strack2011; Nisar et al. 2015; Jia et al. 2017). Moreover, several CCDs have been shown to cleave apocarotenoids rather than carotenoids (Alder et al. 2012; Nisar et al. 2015).

In Arabidopsis (*Arabidopsis thaliana*), there are five subfamilies of *CCDs*, which are designated as *CCD1*, *CCD4*, *CCD7*, *CCD8* and *nine-cis-epoxycarotenoid dioxygenase* (*NCED*) (Ohmiya 2009). Among them, CCD1 enzymes showed a wide substrate and relaxed double-bond specificity by cleaving various cyclic and acyclic all-*trans*-carotenoids as well as apocarotenoids in vitro, suggesting CCD1 enzymes are likely involved in scavenging unnecessary apocarotenoid metabolites (Ilg et al. 2009, 2014). In addition, *CCD1*s have been shown to be involved in the formation of flavor volatiles in different species (Sun et al. 2008; Ilg et al. 2009). CCD4 enzymes mainly cleave bicyclic carotenoids either at the C7′–C8′ to produce a C_{30} apocarotenal and C_{10} volatile, or at C9′–C10′ position to produce a C_{27} apocarotenal and C_{13} volatile, respectively (Ma et al. 2013; Rodrigo et al. 2013; Bruno et al. 2015; Zheng et al. 2019). The carotenoid cleavage activity of CCD4 determines carotenoid profiles in non-photosynthetic tissues of different plant species (Ohmiya et al. 2006; Gonzalez-Jorge et al. 2013; Zheng et al. 2019). *CCD7* and *CCD8* sub-families are mainly involved in the biosynthesis of strigolactones, a class of phytohormones that regulate plant architecture, as well as rhizospheric interactions with symbiotic arbuscular fungi and root parasitic plants (Alder et al. 2012; Al-Babili and Bouwmeester 2015; Jia et al. 2019a). The Arabidopsis *NCED* sub-family consists of five genes, including *NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9* (Tan et al. 2003). NCEDs mainly catalyze the stereospecific cleavage of 9-*cis*-epoxycarotenoids into apo-12′-epoxycarotenal (C_{25}) and the ABA precursor xanthoxin (C_{15}), which is a key rate-limiting step in ABA biosynthesis (Schwartz et al. 1997; Giuliano et al. 2003; Felemban et al. 2019). ABA plays a key role in plant response to various abiotic stresses, such as cold, heat, and drought (Nambara et al. 2010; Peleg and Blumwald 2011). In addition to the above-mentioned groups common present in Arabidopsis, rice (*Oryza sativa*) and other species contain a further *CCD* sub-family that has been recently identified and called *zaxinone synthase* (*ZAS*) (Wang et al. 2019). A rice ZAS was reported to catalyze the cleavage of 3-OH-β-apo-10′-carotenal into zaxinone, a novel carotenoid-derived plant growth regulator required for normal rice growth and a determinant of strigolactone content in both rice and Arabidopsis (Wang et al. 2019; Ablazov et al. 2020).

Albeit their importance for plant growth, development and response to environmental abiotic stresses, a comprehensive study on the *CCD* gene family in *Gossypium* spp. is still missing. Such a study has become possible through the recent release of high quality of third generation genome sequencing of diploid and allotetraploid *Gossypium* species (Wang et al. 2012; Li et al. 2014; Fang et al. 2017; Hu et al. 2019). Here, we performed genome-wide analysis to identify *CCD* family genes in two diploid and two allotetraploid *Gossypium* species, and comprehensively analyzed phylogeny, gene structure, gene duplications, *cis*-acting elements, molecular evolution and gene expression for the cotton *CCD* gene family. Our study demonstrates the role of cotton *CCDs* in regulating abiotic stress response and provides important insights for investigating their biological functions in the future.

Methods

Identification of the *CCD* gene family members in *Gossypium* spp.

Two approaches were adopted to identify cotton *CCD* genes in different *Gossypium* genomes. First, the whole genome translation protein sequences of *G. arboretum* (A2, CRIv1.0), *G. raimondii* (D5, JGI_v2.0), *G. hirsutum* (AD1, ZJUv2.1), and *G. barbadense* (AD2, H7124_ZJUv1.1) were downloaded from the CottonGen database (https://www.cottongen.org/home). Nine Arabidopsis and 13 rice CCD proteins downloaded from (https://phytozome.jgi.doe.gov/pz/portal.html) were used as a query to search for CCD proteins in four *Gossypium* species protein databases by local blast tool with default setting(e-value < 1^{e-10}). Then, the hidden Markov model (HMM) profiles with RPE65 (PF03055) domains (http://Pfam.sanger.ac.uk/), and the HMMER 3.0 software (http://hmmer.org/) (Potter et al. 2018) were used to perform local HMM searches in the four protein databases. All candidate sequences were submitted to domain analysis using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan5/) and SMART (http://smart.embl-heidelberg.de/) tools with default parameters to substantiate the existence of the two conserved domains and determine the exact location of them. Protein sequences without any one domain were rejected.

Comparative phylogenetic analysis of the cotton CCD proteins

CCD protein sequences from *G. arboretum*, *G. raimondii*, *G. hirsutum*, *G. barbadense,* Arabidopsis and rice were performed multiple alignments in MUSCLE program of Molecular Evolutionary Genetics Analysis (MEGA 7) software (Kumar et al. 2016). An unrooted phylogenetic tree was constructed based on the NJ method, with a 1000 bootstrap replicates with a Jones–Taylor–Thornton (JTT) model. The phylogenetic tree was further visualized and annotated using Evolview (https://www.evolgenius.info) (Subramanian et al. 2019).

Gene exon/intron architecture and promoter *cis*-acting element analysis of the cotton *CCD* genes

The gene exon/intron architecture of *CCD* genes from the four *Gossypium* species was determined using the Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/) based on the coding and corresponding genomic sequences (Hu et al. 2015). For determining *cis*-acting elements of *GhCCD*s promoters, 2 kb upstream sequences were analyzed in PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html) (Lescot et al. 2002) and the *cis*-acting elements were categorized based on their predicted functions.

Chromosome location and synteny analysis

Chromosomal positions of the *CCD* genes were obtained from the annotation information of cotton genome database (https://www.cottongen.org/home). The chromosomal distributions of genes were drafted on the cotton chromosomes according to the gene positions by Mapchart software (http://www.plantbreeding.wur.nl/UK/software_mapinspect.html) (Voorrips 2002). Meanwhile, the orthologous/paralogous gene pairs of the *CCD* gene in A, D genomes, At- and Dt-subgenomes were searched by InParanoid software (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) (Sonnhammer and Ostlund 2015). Additionally, the evolutionary rates Ka, Ks, and Ka/Ks ratio were estimated by KaKs_Calculator package (https://kakscalculator.herokuapp.com/) (Wang et al. 2010). On the basis of the synonymous substitutions per year (λ) of 2.6×10^{-9} for cotton, the divergent time of CCD orthologous gene pairs were estimated by $T = Ks/2\lambda \times 10^{-6}$ MYA (Zhang et al. 2015).

Transcriptome analysis and quantitative reverse-transcriptional PCR (qRT-PCR) confirmation of *GhCCD* gene expression

All raw RNA-seq data were downloaded from the NCBI Sequence Read Archive (SRA: PRJNA490626). TopHat2 (Kim et al. 2013), and cufflinks (Trapnell et al. 2010) were used to analyze transcriptome data. Gene expression was measured in fragments per kilobase million (FPKM). The TBtools program (Chen et al. 2018) was used to display the heat map of gene expressions.

Four-week-old *G. hirsutum* L. acc. Texas Marker-1 (TM-1) seedlings grown in a climate-controlled chamber (light/dark cycle: 16 h at 28 °C/8 h at 22 °C) were used for ABA and different abiotic stresses treatments. 100 µM ABA, 42 °C, 200 mM NaCl and 5% PEG6000 were used as ABA, heat, salt and PEG treatment conditions, and normal-grown TM-1 seedlings were used as a control group. Leaves collected at 3 h or 6 h after treatments onset were immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using an RNAprep Pure Plant Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The first-strand cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Dalian, China). Oligo 7.0 software was used to design the gene-specific primers for qRT-PCR (Table S5). Cotton *Histone 3* (GenBank accession no. AF094716) was used as an internal control. The qRT-PCR (Promega, Madison, WI, USA) on an ABI 7500

real-time PCR system (Applied Biosystems, USA) with three replicates. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of *GhCCD*s, and *t*-tests were used for statistical analysis.

Results

Identification and characterization of *CCD* genes in *Gossypium* spp.

CCDs were characterized as a RPE65 (retinal pigment epithelial membrane protein) domain which is key for their enzymatic oxidative carotenoid cleavage activity (Ohmiya 2009). In the present study, we employed two approaches to identify *CCD* family genes in two allotetraploid *Gossypium* species, *G. hirsutum* and *G. barbadense*, and two diploid *Gossypium* species, *G. arboreum* and *G. raimondii*, respectively. First, we used the CCD protein sequences from the dicot model plant *Arabidopsis* and the monocot model plant rice as query to perform a protein blast analysis in the four corresponding *Gossypium* genome databases, respectively. Then, we did another Pfam search and SMART programs analysis using the RPE65 domain in the same corresponding cotton genome database. By combination of the results from the two approaches and removal of the reduplicating proteins, we identified a total of 16, 15, 33 and 31 putative *CCD* members in *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*, respectively (Table S1). The cotton *CCD* genes were then named according to their chromosomal location and homologs in Arabidopsis and rice (Fig. 1 and Table S1).

We next investigated the basic features of all *CCD* genes and their corresponding encoding polypeptides from the four *Gossypium* species, e.g., the gene ID, chromosomal location, gene length, open reading frames (ORF) and deduced protein length, predicted protein molecular weights (MW) and isoelectric points (pI), predicted chloroplast transit peptide (cTP) presence and length, and predicted subcellular localization. As shown in Table S2, the predicted protein lengths of 90.5% of cotton CCDs (86 out of 95) range from 508 to 667 amino acids (aa), with molecular weight (MW) from 57.553 to 74.461 Da. There are four cotton *CCDs* (*GhCCD4b-A1*, *GhCCD4b-A2*, *GhNCED2c-D*, *GhZAS2-A*) with a predicted encoded

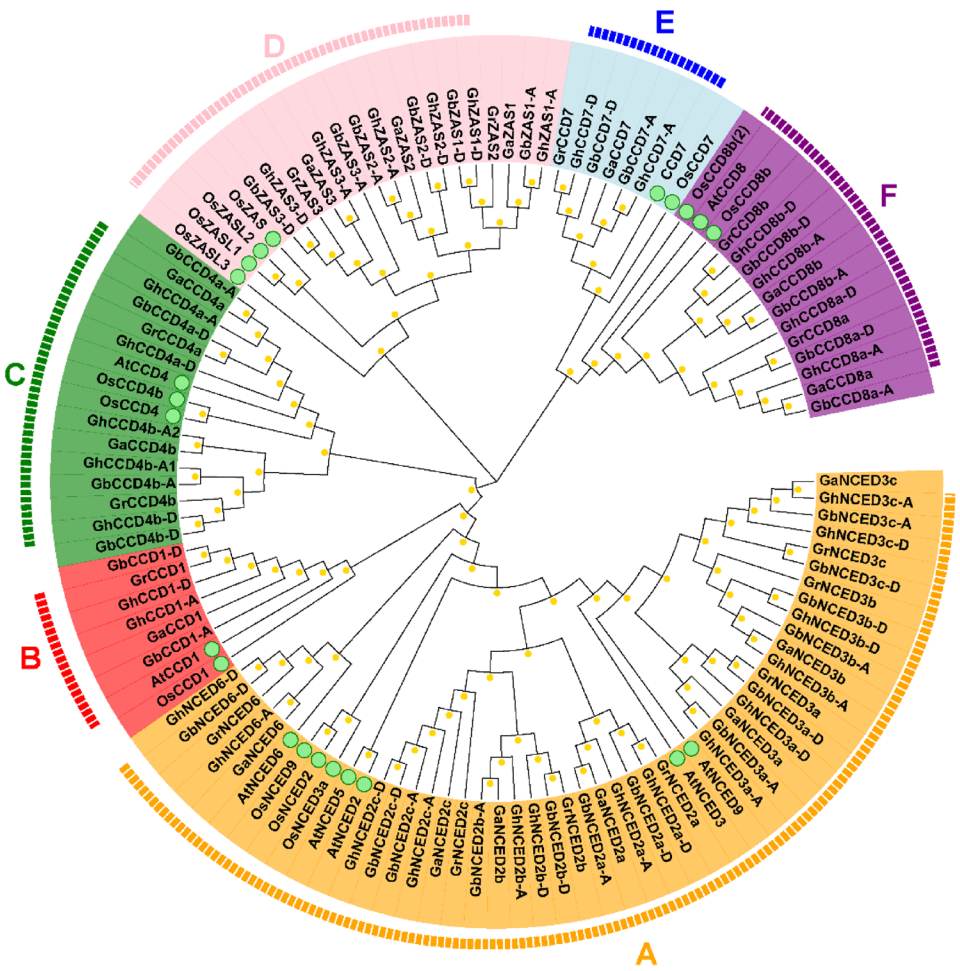


Fig. 1 Phylogenetic tree of CCD proteins from *Arabidopsis*, rice, *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii*. Full-length CCD proteins were aligned using MUSCLE program in MEGA 7.0. The phylogenetic tree was generated by the Neighbor-Joining (NJ) method with 1,000 bootstrap replicates. CCD proteins were divided into A-F sub-families distinguished by different colors. CCD proteins of Arabidopsis and rice were marked by green dots

protein length of less than 400 aa, suggesting the occurrence of deletions or truncations. The gene length of all *CCDs* ranges from 351 to 9070 bp, indicating a large diversity in the gene structure. CCD proteins usually, with the exception of CCD1s, contain an N-terminal cTP domain which targets them to the plastid, where carotenoids are synthesized and metabolized (Al-Babili and Bouwmeester 2015). We used the online cTP analysis software "ChloroP 1.1 Server" (http://www.cbs.dtu.dk/services/ChloroP/) to predict the presence of cTP in the identified CCDs. As shown in Table S2, most CCDs contain a CTP domain with the exception of CCD1s and CCD8as from all the four *Gossypium* species which were predicted without a cTP domain presence.

Classification and phylogenetic analysis of the cotton *CCD* genes

To investigate the evolutionary relationships of the 117 CCD genes from 6 different plant species including *A. thaliana*, *O. sativa*, *G. arboreum*, *G. raimondii*, *G. hirsutum* and *G. barbadense*, we generated a phylogenetic tree using the Neighbor Joint (NJ) method based on multiple sequences alignment with 1000 bootstrap replicates. As shown in Fig. 1, all CCDs from the four *Gossypium* species were clearly divided into 6 sub-families, CCD1, CCD4, CCD7, CCD8, ZAS and NCED, which is similar to the case in rice. CCD4 sub-family is divided into two sub-branches, CCD4a and CCD4b (Fig. 1). CCD4a sub-branch shows high similarity with OsCCD4s and AtCCD4; while, CCD4b branch is divergent from OsCCD4s and AtCCD4 in the phylogenetic tree. A multi-member NCED sub-family (NCED2a, NCED2b, NCED2c, NCED3a, NCED3b, NCED3c and NCED6) was identified in all the four *Gossypium* species (Fig. 1). In addition, *Gossypium* species contain a multi-member ZAS sub-family which showed high similarity with rice (Fig. 1). The phylogenetic tree indicated that *G. hirsutum* experienced significant gene family expansion, because it has more than double the number of *CCD* genes compared to *A. thaliana* and *O. sativa*. The numbers of CCD sub-family members in the two allotetraploid *Gossypium* species are almost double of those in the two diploid species, except CCD4b in *G. hirsutum* and NCED6 in *G. barbadense*. In *G. hirsutum*, we noticed that *GhCCD4b-A* was broken into two genes, *GhCCD4b-A1* and *GhCCD4b-A2*, likely due to gene truncation. In *G. barbadense*, *GbNCED6* is only present in the D-subgenome but not in the A-subgenome. These results suggest that most cotton *CCD* family genes likely retain conservative biological functions during evolution. In addition, the clusters provide evidence that *G. hirsutum* and *G. barbadense* are the result of hybridization of two diploid cotton species.

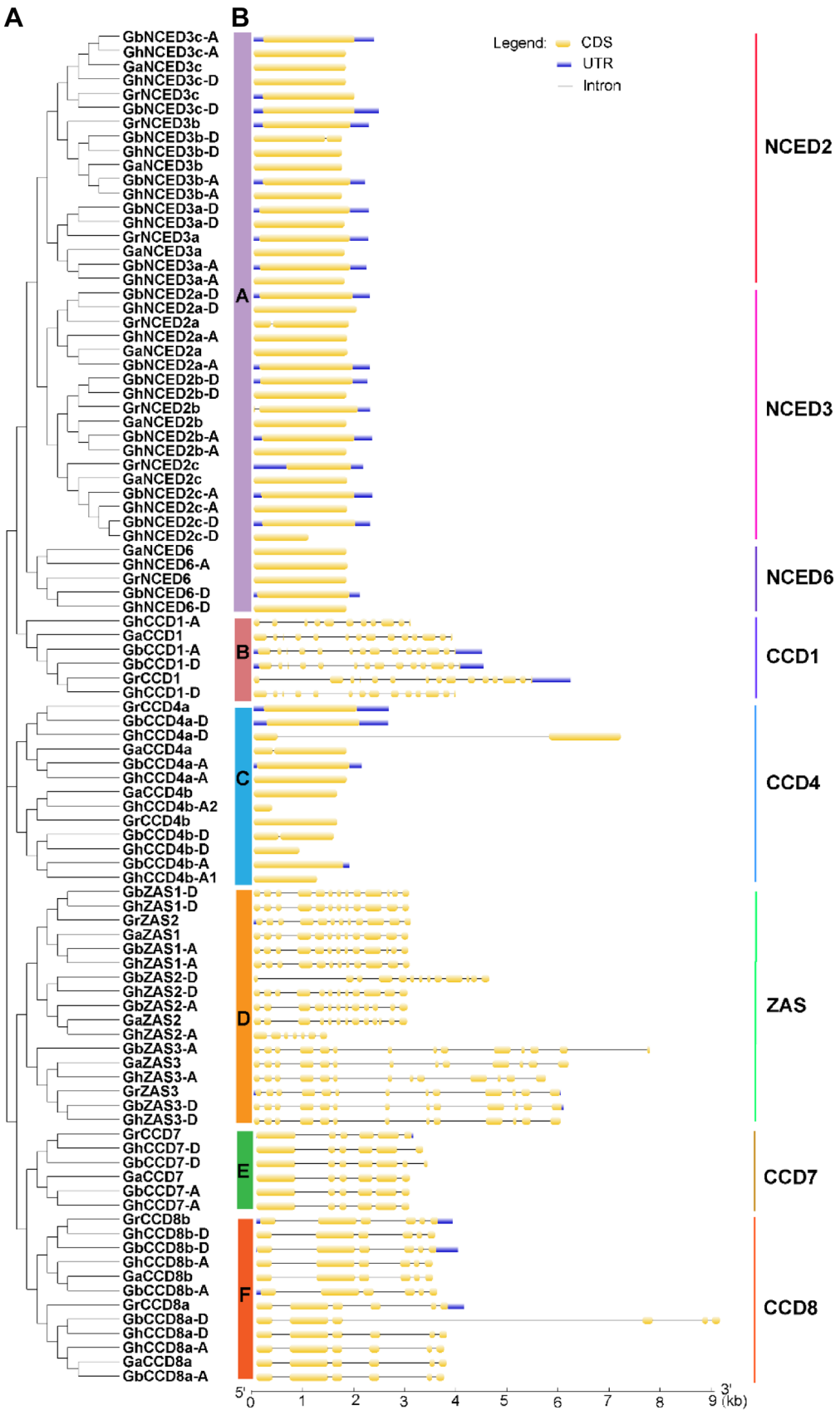
Gene structure and promoter analysis of the cotton *CCD* family genes

The gene structure, e.g., number of introns, plays an important role in the evolution of gene function (Roy and Gilbert 2006). Therefore, we compared the exon/intron number of different *CCDs* from the four *Gossypium* species using GSDS online tools (http://gsds.cbi.pku.edu.cn/). As shown in Fig. 2, the structure of the exon/intron displayed a similar pattern among the same *CCD* sub-family; while, different *CCD* sub-families displayed quite divergent exon/intron structures. Specifically, no more than one intron is present in all members of both *NCED* and *CCD4* sub-families; while all members of the *CCD1* and the *ZAS* sub-families contain no less than 10 introns except *GhZAS2-A* (Fig. 2). All *CCD7* and *CCD8* genes from the four *Gossypium* species contain five introns except *GhCCD7-D* (Fig. 2). These results suggest the common origin of the gene members from same sub-family and are in line with the functional diversity from different CCD sub-families.

The *cis*-acting elements located in gene promoters are binding sites of various transcription factors which are key to regulate gene expression (Bilas et al. 2016). To predict the gene regulation behavior of different CCDs in *G. hirsutum*, we analyzed the *cis*-acting elements in the 2 kb promoter region of *GhCCD*s and categorized them based on their functional relevance. As shown in Fig. 3a, the identified *cis*-acting elements were mainly classified into light, hormones, and stress response-related elements, suggesting complex regulation of *GhCCD*s by various environmental cues and hormonal signals. We further investigated the *cis*-acting elements that are related to hormones, which showed that most of them are targeted by ABA- and jasmonate acid-responsive genes (Fig. 3b). The other hormone related *cis*-acting elements were predicted to be targeted by salicylic acid-, gibberellins- and auxin-responsive genes (Fig. 3b). ABA, jasmonate acid and salicylic acid are important regulators of biotic and abiotic stress responses (Ku et al. 2018), suggesting the expression of *GhCCD*s are involved in these stress response processes.

Gene duplication and syntenic analysis of the *CCD* family genes in *Gossypium* spp.

Gene duplication is a common event during evolution, which is important to generate functional divergence. To study the locus relationships among the *CCD* genes in *Gossypium* spp., we identified the orthologous/paralogous gene pairs in the subgenomes of the two allotetraploid cottons, *G. hirsutum* and *G. barbadense*. 47 and 44 orthologous/


A
B
GbNCED3c-A
GhNCED3c-A
GaNCED3c
GhNCED3c-D
GrNCED3c
GbNCED3c-D
GrNCED3b
GbNCED3b-D
GhNCED3b-D
GaNCED3b
GbNCED3b-A
GhNCED3b-A
GbNCED3a-D
GhNCED3a-D
GrNCED3a
GaNCED3a
GbNCED3a-A
GhNCED3a-A
GbNCED2a-D
GhNCED2a-D
GrNCED2a
GhNCED2a-A
GaNCED2a
GbNCED2a-A
GbNCED2b-D
GhNCED2b-D
GrNCED2b
GaNCED2b
GbNCED2b-A
GhNCED2b-A
GrNCED2c
GaNCED2c
GbNCED2c-A
GhNCED2c-A
GbNCED2c-D
GhNCED2c-D
GaNCED6
GhNCED6-A
GrNCED6
GbNCED6-D
GhNCED6-D
GhCCD1-A
GaCCD1
GbCCD1-A
GbCCD1-D
GrCCD1
GhCCD1-D
GrCCD4a
GbCCD4a-D
GhCCD4a-D
GaCCD4a
GbCCD4a-A
GhCCD4a-A
GaCCD4b
GhCCD4b-A2
GrCCD4b
GbCCD4b-D
GhCCD4b-D
GbCCD4b-A
GhCCD4b-A1
GbZAS1-D
GhZAS1-D
GrZAS2
GaZAS1
GbZAS1-A
GhZAS1-A
GbZAS2-D
GhZAS2-D
GbZAS2-A
GaZAS2
GhZAS2-A
GbZAS3-A
GaZAS3
GhZAS3-A
GrZAS3
GbZAS3-D
GhZAS3-D
GrCCD7
GhCCD7-D
GbCCD7-D
GaCCD7
GbCCD7-A
GhCCD7-A
GrCCD8b
GhCCD8b-D
GbCCD8b-D
GhCCD8b-A
GaCCD8b
GbCCD8b-A
GrCCD8a
GbCCD8a-D
GhCCD8a-D
GhCCD8a-A
GaCCD8a
GbCCD8a-A
Legend:
CDS
UTR
Intron
A
B
C
D
E
F
NCED2
NCED3
NCED6
CCD1
CCD4
ZAS
CCD7
CCD8
5'
3'
0
1
2
3
4
5
6
7
8
9
(kb)

◂**Fig. 2** Gene structures of *CCD* genes from different *Gossypium* spp.. **a** The phylogenetic tree was constructed using the Neighbor-Joining (NJ) method, with 1000 bootstrap replicates. Sub-families are distinguished by different colors. **b** Exon/intron architectures of *CCD* genes from different *Gossypium* spp.. Yellow and blue boxes indicate exons and UTRs, respectively. Black lines represent introns. The size of exons and introns can be calculated according to the scale at the bottom

paralogous gene pairs were identified for *G. hirsutum* and *G. barbadense*, respectively (Fig. 4, Table S3 and S4). Most *CCDs* in the A and D subgenomes of *G. hirsutum* and *G. barbadense* have corresponding orthologous/paralogous in the A (*G. arboreum*) or D (*G. raimondii*) genomes. As shown in Fig. 4, the gene syntenic analysis indicates that the gene duplication in the two allotetraploid *Gossypium* species is mainly due to hybridization. This provides evidence that cotton *CCD* genes likely have not experienced large-scale genomic arrangements during polyploidization.

To investigate whether the duplicated orthologous/paralogous gene pairs of *G. hirsutum* and *G. barbadense* gained functional divergence during evolution, the Non-synonymous (Ka) and synonymous (Ks) divergence values were calculated. Usually, Ka/Ks ratios less than 1 indicate that the genes have undergone purifying selection, while ratios above 1 point to positive selection. For *G. hirsutum*, out of the Ka/Ks ratios of the 49 duplicated gene pairs, 36 were < 0.5, nine were between 0.5 and 1.0, and five gene pairs were > 1.0 (Table S3). For *G. barbadense*, out of the Ka/Ks ratios of the 44 duplicated gene pairs, 30 were < 0.5, 11 were between 0.5 and 1.0, and three gene pairs were > 1.0 (Table S4). These results suggest that most *CCDs* of both *G. hirsutum* and *G. barbadense* have been subjected to a strong purifying selection, with exception of five *CCDs* (*GhCCD4b*, *GhNCED2b-A, GhNCED3c-A*, *GhNCED6-A* and *GhZAS1-A*) in *G. hirsutum* and three *CCDs* (*GbNCED2b-A*, *GbZAS1-A*, *GbCCD4b-D*) in *G. barbadense* that likely experienced positive selection (Table S3 and S4).

Expression patterns of the *CCD* genes in *G. hirsutum*

Spatial expression pattern of genes is important for the study of their biological functions. Therefore, we analyzed the tissue expression pattern of different *CCD*s in *G. hirsutum*, using the published transcriptome data downloaded from NCBI (SRA: PRJNA490626) for 10 different tissues mirroring key developmental stages in cotton growth and development. These tissues include roots, stems, leaves, petals, torus, stamens, and fibers sampled at 5, 10, 20 and 25 days post anthesis (DPA). As shown in the Heat Map in Fig. 5, *CCD1* sub-family showed ubiquitous high expression in all selected tissues. Both *CCD7* and *CCD8* sub-families exhibited high expression in stems and leaves (Fig. 5). All members of the *ZAS* sub-family were expressed at low levels in all tissues, with exception of *GhZAS1-A*, *GhZAS3-A* and *GhZAS3-D*, which showed a stem-specific expression pattern (Fig. 5). For *CCD4* sub-family, *GhCCD4a-A* exhibited a ubiquitous expression with high expression rates in torus and stamens, while all the other members showed a general low expression in all tissues (Fig. 5). Members of the *NCED* sub-family were characterized by different expression patterns, with four and eight members showing ubiquitous and tissue specific expression pattern, respectively (Fig. 5).

CCD genes in *G. hirsutum* are induced by different abiotic stressors

To get insight into the responses of *Gossypium CCD*s to abiotic stressors, we analyzed the related transcriptome data from NCBI (SRA: PRJNA490626). The analyzed data include four different abiotic stress factors, i.e., cold, heat, salt and PEG treatment at time points 1, 3, 6, and 12 h. As shown in Fig. 6, *GhCCD4a-A/D* and *GhNCED3*s were induced under most abiotic stress conditions. Most members of the *CCD7*, *CCD8*, *GhNCED2*s and several *ZAS* sub-family members showed specific induction by heat stress (Fig. 6). These results suggest that *GhCCD*s likely contribute to abiotic stress responses.

To confirm the published transcriptome data and to investigate the effect of ABA application on the expression of *CCD* genes in *G. hirsutum*, we performed a qRT-PCR analysis from seedling exposed to heat-, salt-, PEG- and ABA-treatment. Four *NCED*s (*GhNCED3a-A/D*, *GhNCED3c-A/D*), two *CCD4a* (*GhCCD4a-A/D*) genes and four *ZAS* genes (*GhZAS1-A/D*, *GhZAS3-A/D*), which were obviously induced by different abiotic stress factors in the transcriptome data, were selected for the qRT-PCR investigation. As shown in Fig. 7, the four *NCED*s, *GhCCD4a-D* and *GhZAS1-A* were clearly induced by ABA application. The transcript levels of *GhNCED3a-A*, *GhNCED3c-D*, *GhCCD4a-A*, and *GhZAS1-A/D* increased significantly upon heat treatment, while salt treatment for 6 h enhanced the expression of *GhNCED3a-D*, *GhNCED3c-A*, and *GhCCD4a-D*. In addition, *NCEDs* and *CCD4a*s, especially *GhNCED3c-D* and *GhCCD4a-A*, were strongly induced by PEG treatment. Most of these results are consistent with the transcriptomic data analysis (Fig. 6) and support the importance of *GhCCD*s in the reaction to abiotic stresses.

Discussion

CCDs are key enzymes involved in carotenoid metabolism and the production of phytohormones, plant bioactive molecules, as well as plant pigmentations (Ohmiya 2009; Felemban et al. 2019; Wang et al. 2021). In this study, we comprehensively characterized the *CCD* gene family in four

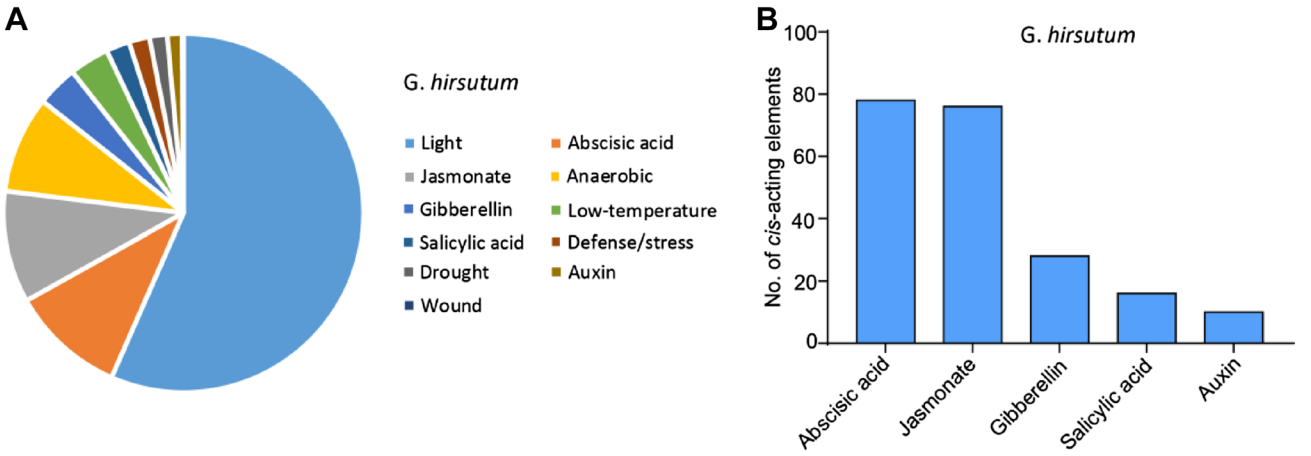


Fig. 3 Classification of *cis*-acting elements in *GhCCD* gene promoters. **a** The percentage of different *cis*-acting elements in the *GhCCD* promoters. **b** Numbers of *cis*-acting elements related to hormone response. The 2 kb promoter region of the *GhCCD* genes was used for *cis*-acting elements analysis

Gossypium species, including two allotetraploid cotton, *G. hirsutum* and *G. barbadense*, and two diploid cotton, *G. arboreum* and *G. raimondii*. *CCDs* from all four *Gossypium* species are classified into six sub-families corresponding to the *CCD1*, *CCD4*, *CCD7*, *CCD8*, *ZAS* and *NCED* clades, similar to rice and other mycorrhizal plants; in contrast, *Arabidopsis* lacks ZAS sub-family (Fig. 1) (Wang et al. 2019). In addition, the gene structural analysis showed that the pattern of exons/introns distribution was highly conservative among the same sub-family and quite diverse in different sub-families (Fig. 2). Introns number is an important factor for the evolution of gene function and regulation, and exon/intron structure differences usually result from insertion/deletion events in genes and are useful for investigating gene evolution (Lecharny et al. 2003). Furthermore, introns are considered to be under weak selection pressure, and gene families with fewer or no introns are usually thought to be evolutionarily advanced (Roy and Gilbert 2005; Roy and Penny 2007). Thus, it seems that the *CCD4* and *NCED* sub-family likely evolved faster than the other *CCD* sub-families (Fig. 2). This is consistent with reported biological functions of *CCD4s* and *NCEDs*, which are involved in secondary metabolism and abiotic stresses, respectively, and are highly adapted the environmental cues (Ohmiya 2009). Moreover, it is supposed that the intron-free structure of *NCED* genes is related with the rapid induction of ABA production and ABA-mediated stress response in plants (Fig. 2) (Wang et al. 2017).

It is assumed that allotetraploid cotton arose due to one hybridization event between A-genome of *G. arboreum* (A2) and D-genome of *G. raimondii Ulbrich* (D5) about 1–1.5 million years ago (MYA) (Endrizzi et al. 1985; Wendel 1989). The genome-wide syntenic analysis for *CCD* genes among allopolyploid and diploid *Gossypium* species well supported the above assumption (Fig. 4). Gene duplication generates functional divergence, which is essential for environmental adaptability and speciation (Conant and Wolfe 2008). The similar distribution pattern of *CCD*s on the chromosomes of A- and D-subgenome of *G. hirsutum* and *G. barbadense* indicates that *CCD*s likely have not experienced large-scale genomic arrangements during polyploidization (Fig. 4). However, the unequal *CCD* gene number between *G. hirsutum* and *G. barbadense* suggests that gene loss or addition through segmental events may have occurred. Nevertheless, an incomplete genome assembly or inaccurate gene annotation could also be a reason for this observation. We speculate that the *CCD* gene family underwent a quite conservative evolution after polyploidy. Consistently, evolutionary analysis showed that purifying selection dominated the evolution of these genes (Table S3 and Table S4). However, four *CCD*s (*GhCCD4b*, *GhNCED2b-A, GhNCED3c-A*, *GhNCED6-A* and *GhZAS1-A*) in *G. hirsutum* and three *CCDs* (*GbNCED2b-A*, *GbZAS1-A*, *GbCCD4b-D*) in *G. barbadense*, which likely experienced positive selection during evolution, might acquire new biological functions (Table S3 and Table S4).

Cotton *CCD*s are classified into 6 sub-families according to the phylogenetic analysis. *CCD1* sub-family is highly conservative among different species. CCD1 enzymes are characterized by a wide substrate and low double-bond specificity by cleaving various cyclic and acyclic all-*trans*-carotenoids as well as apocarotenoids (Ilg et al. 2009, 2014). Cotton CCD1s are predicted to be localized in cytoplasm and are ubiquitously expressed in all tissues (Figs. 1 and 5), suggesting *Gossypium CCD1*s likely have conservative biological functions, e.g., to scavenger unnecessary carotenoids and apocarotenoids similar as *AtCCD1* (Ilg et al. 2014). *CCD4* sub-family members were showed to affect

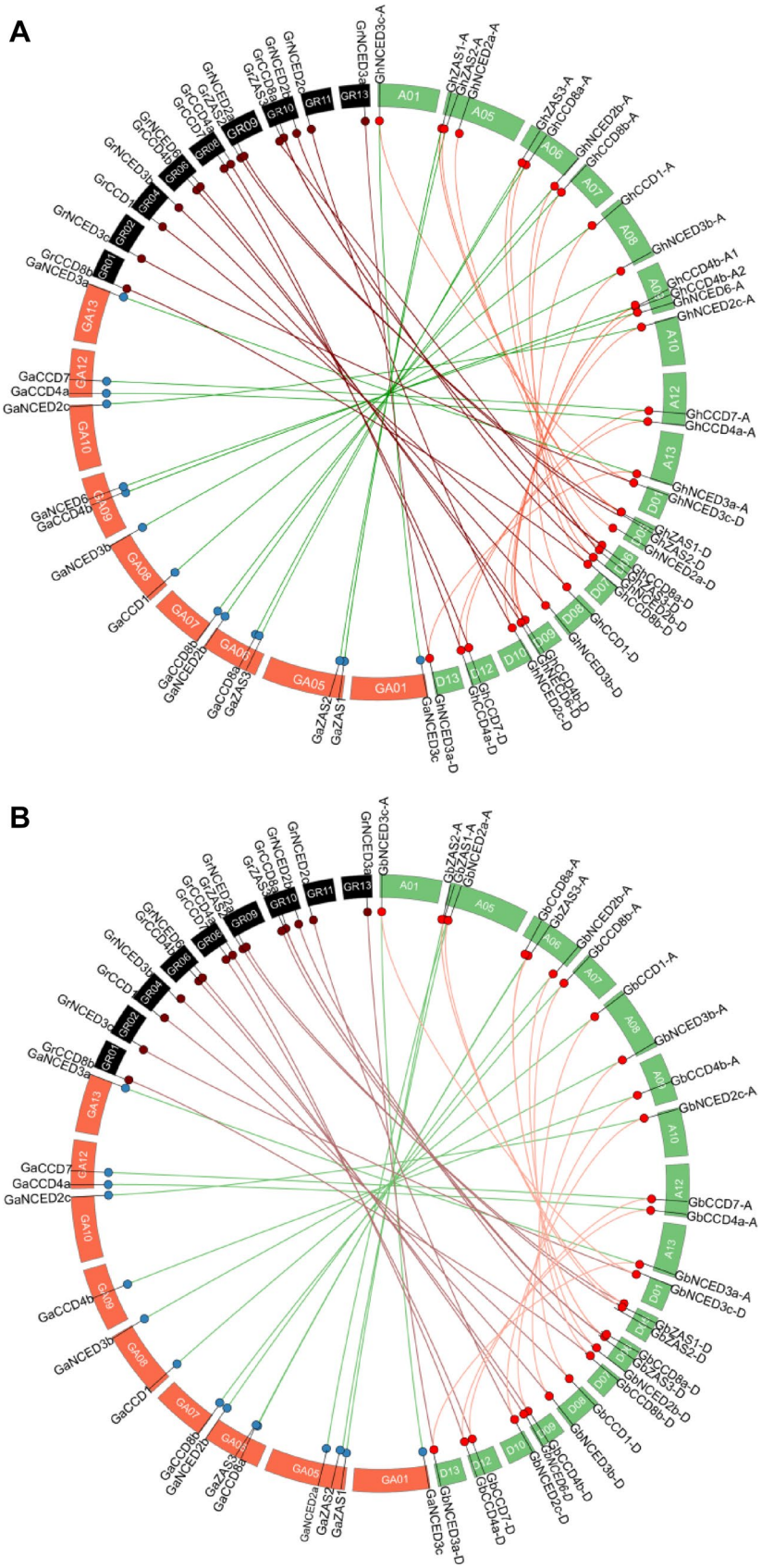


Fig. 4 Genome-wide synteny analysis for *CCD* genes among allopolyploid and diploid *Gossypium* species. **a** Synteny analysis between *G. hirsutum* and two diploid species, *G. arboreum* and *G. raimondii*. **b** Synteny analysis between *G. barbadense* and two diploid species, *G. arboreum*and *G. raimondii*. Green lines link gene pairs between *G. arboreum* and *G. hirsut*um or *G. barbadense*; dark red lines connect gene pairs between *G. raimondii* and *G. hirsutum or G. barbadense*; light red lines bridge gene pairs between At- and Dt-subgenome in allopolyploid cotton. Red boxes indicate chromosomes of *G. arboreum*, dark boxes indicate chromosomes of *G. raimondii*, and green boxes indicate chromosomes of *G. hirsut*um or *G. barbadense*

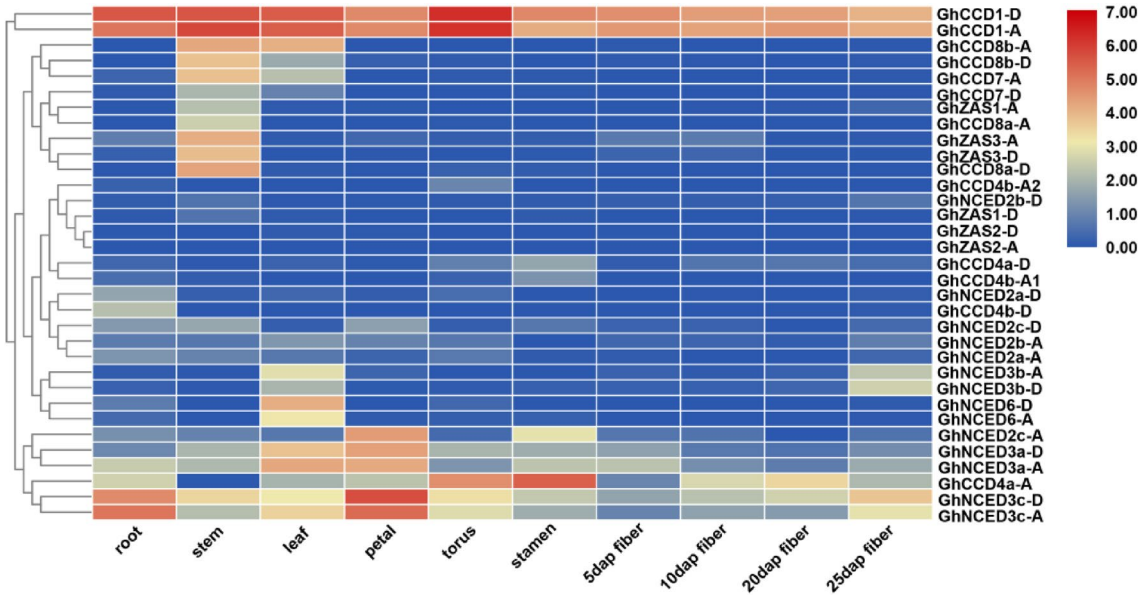
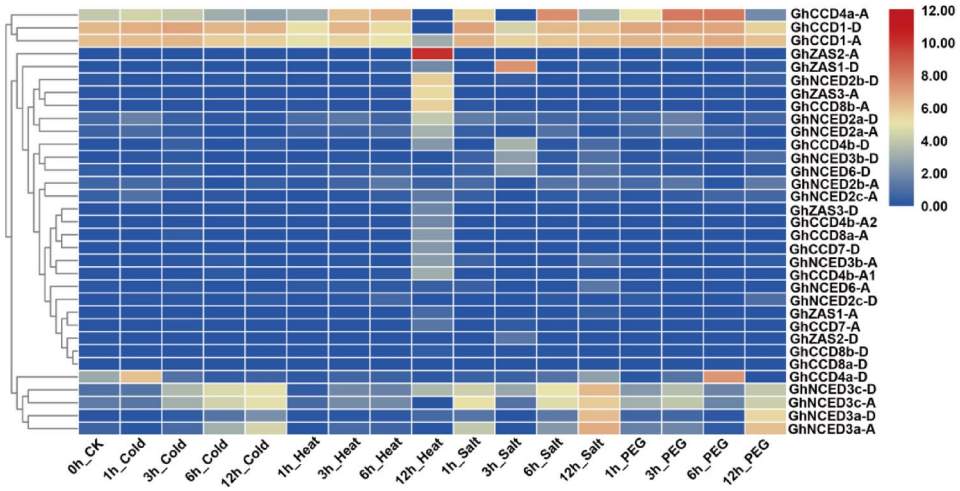


Fig. 5 Tissue expression pattern of *GhCCD* genes. Heat map of the tissue expression pattern of 33 *GhCCD* genes in 10 different tissues, including roots, stems, leaves, petals, torus, stamens, and fibers sampled at 5, 10, 20 and 25 DPA. The expression data were analyzed using the published transcriptome data downloaded from NCBI (SRA: PRJNA490626)

Fig. 6 Heat map of the expression of *GhCCDs* under different abiotic stressors. The abiotic stressors include cold, heat, salt and PEG treatment for 1, 3, 6, 12 h. The expression data were analyzed using the published transcriptome data downloaded from NCBI (SRA: PRJNA490626)



carotenoid profile in a tissue-dependent manner in different species (Gonzalez-Jorge et al. 2013; Ohmiya et al. 2006; Zheng et al. 2019). Cotton *CCD4* sub-family seems have two sub-branches, *CCD4a* and *CCD4b* based on the phylogenetic analysis (Fig. 1). The *CCD4a* sub-branch is highly similar to *OsCCD4*s rice and *AtCCD4*, but *CCD4b* seems represent a different sub-branch (Fig. 1). In *G. hirsutum*, the *GhCCD4a-A* is ubiquitously expressed with high levels in stamen, and *GhCCD4a-D* showed a specific expression in torus and stamen, suggesting *GhCCD4a* might have important roles in stamen (Fig. 5). *GhCCD4b-A1* and *GhCCD4b-A2* supposedly originate from the truncation of *GhCCD4b-A*, which was likely accompanied by the loss of function of both genes, as supported by the fact that both genes are almost not expressed in all tissues (Fig. 5). In addition, *GhCCD4b-D* is predicted to be cytoplasm localized different with the plastid localization of *GhCCD4a*s, suggesting this gene might evolve new biological functions (Fig. 5).

CCD7 and *CCD8* are mainly involved in the biosynthesis of strigolactones (Alder et al.2012). Strigolactones are quite conservative plant hormones which are synthesized even in moss (Decker et al. 2017). Members of cotton CCD8 and CCD7 sub-families showed high similarity to CCD8 and CCD7 from both rice and Arabidopsis based on the phylogenetic analysis, indicating conservative biological functions of them (Fig. 1). As we know, CCD7 and CCD8 catalyzed

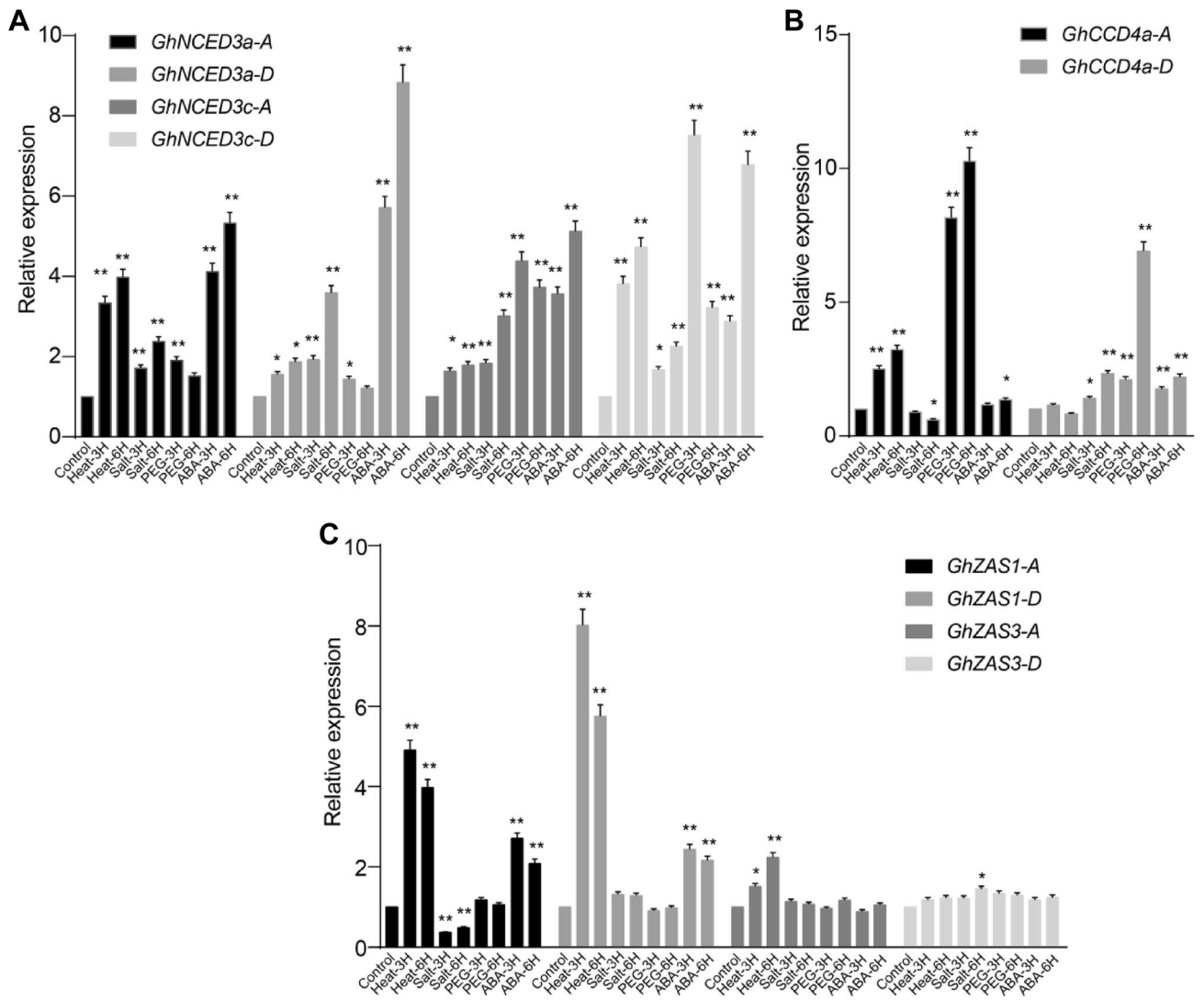


Fig. 7 qRT-PCR expression analysis of selected *GhCCD* genes under different abiotic stresses treatments or upon exposure to exogenous ABA for 3 h and 6 h. Relative expression level of selected *GhNCED*s (**a**), *GhCCD4s* (**b**) and *GhZAS*s (**c**) under different conditions. Relative gene expression levels were normalized to the reference gene (*GhHistone 3*) and were re-adjusted to the expression levels in the control, which were set as 1. Average fold change ± SE ($n = 3$). Two-tailed Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$

strigolactone biosynthesis steps that occur in plastids; however, CCD8as from all the four *Gossypium* species were predicted as cytoplasm localized, suggesting CCD8as might acquire unknown biological functions rather than involvement in strigolactone biosynthesis. In the other side, CCD7s and CCD8bs from all the four *Gossypium* species were predicted as plastid localized and have similar tissue expression patterns, suggesting they are likely involved in strigolactone biosynthesis (Figs. 1 and 5).

NCEDs catalyze the stereospecific cleavage of 9-*cis*-epoxycarotenoids into the ABA precursor xanthoxin (C15) (Schwartz et al. 1997; Giuliano et al. 2003). The gene numbers of *NCED* sub-family dominated the *CCD* gene family In Arabidopsis and the four *Gossypium* species, suggesting the gene expansion and important roles of this sub-family (Fig. 1). The transcriptome analysis of *NCEDs* in *G. hirsutum* shows that *GhNCED3c-A/D* and *GhNCED3a-A/D* were generally high expressed in most tissues (Fig. 6). Furthermore, *GhNCED3c-A/D* and *GhNCED3a-A/D* were all strongly induced by ABA application (Fig. 7a), suggesting their involvement in ABA biosynthesis and abiotic stresses (Wan and Li 2006).

*ZAS*s belong to a *CCD* sub-family which is not present in *Brassicacea* species. In rice, OsZAS catalyzes the cleavage of apo-10′-zeaxanthinal to produce zaxinone (Wang et al. 2019). Although Arabidopsis doesn't have ZAS homologs, zaxinone was detected in this specie and exogenously applied zaxinone was shown to promote

strigolactone and ABA biosynthesis (Ablazov et al. 2020). Cotton ZASs showed high similarity to OsZAS based on phylogenetic analysis, suggesting a similar enzymatic activity (Fig. 1). However, *Gossypium* spp. are dicotyledonous plants, different from *O. sativa*. Thus, the biological functions of ZASs in *Gossypium* species are still open and interesting questions.

Abiotic stress is becoming a major constraint for cotton fiber yield, due to insufficient farm land and climate change. *CCD* genes have been reported to play key roles in regulating biotic and abiotic stress responses in different species, (Wang et al. 2013; Zhou et al. 2019). The *cis*-acting elements analysis of the promoter regions in *GhCCD*s showed that most elements are related to light, hormones, and response to different abiotic stresses, such as drought, wound, and low temperature (Fig. 3). Most identified hormone-regulated *cis*-acting elements in the promoters of *GhCCD*s are related to ABA and jasmonate acid, the major two hormones regulating stress responses, suggesting *GhCCDs* are likely involved in various biotic and abiotic stresses (Fig. 3). This assumption was substantiated by transcriptome data of *GhCCD*s under various abiotic stress conditions, including cold, heat, salt and PEG treatments (Fig. 6) and by qRT-PCR analysis performed in this study (Fig. 7). Consistently, it was previously reported that the expression of *NCEDs* in *Gossypium* is induced by drought stress and H_2O_2 application (Kong et al. 2016). *CCD4* genes were also shown to be enhanced by various abiotic stress factors in different species (Wang et al. 2013; Rubio-Moraga et al. 2014). In the present study, we show that members of *GhNCED*, *GhZAS*, *GhCCD7* and *GhCCD8* sub-families are specifically promoted by heat stress (Figs. 6 and 7). *GhNCED*s and *GhCCD4a*s were strongly induced by PEG treatment, suggesting possible roles of these genes in drought stress response (Fig. 7). Taken together, our study identifies the *CCD* gene family in four *Gossypium* species and suggests key roles of *GhCCDs* in regulating abiotic stress response, which paves the way for investigating their biological functions in the future.

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Author contributions KJ, YM and SZ designed the research. KJ and YG did the qRT-PCR experiments. KJ, YM, SZ, YG, YZ, JG, KL, WF, ZJ and WL analyzed the data with the input of L-SPT. KJ and SZ wrote the manuscript. YM, L-SPT and WL revised the manuscript.

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Availability of data and materials Nine Arabidopsis and 13 rice CCD proteins downloaded from (https://phytozome.jgi.doe.gov/pz/portal.html). All raw RNA-seq data were downloaded from the NCBI Sequence Read Archive (SRA: PRJNA490626).

Declarations

Conflicts of interest The authors declare that they have no conflict of interest in the publication.

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