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Cotton flavonoid structural genes related to the pigmentation in brown fibers

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Abstract

Five flavonoid structural genes, encoding chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, anthocyanidin synthase, and anthocyanidin reductase, were cloned from a brown-fiber cotton line (T586). The predicted proteins of these genes exhibit high sequence similarity with corresponding enzymes from various plants. RT-PCR analysis showed these genes are developmentally co-regulated and preferentially expressed in developing fibers of T586. Expression analyses and dimethylaminocinnaldehyde staining demonstrated that high transcript levels of these genes in developing fibers and presence of proanthocyanidins in mature fibers co-segregated with brown fiber in a recombination inbred line population. Our results indicated that the cloned flavonoid structural genes and proanthocyanidins were involved in the pigmentation in brown cotton fibers.

Keywords: Cotton (*Gossypium hirsutum* L.); Naturally colored fiber; Brown fiber; Proanthocyanidin; Flavonoid biosynthesis; Flavonoid structural gene

Processing and dying procedures are the major pollution source in the cotton industry, and also lead to trace chemical contaminants in textiles harmful to human health [1,2]. Compared to commonly used white cotton, naturally colored cotton is much friendlier to environment, for it requires no or less processing and dying steps [1–3]. With increased concern to environment and health in the modern society, naturally colored cotton becomes more and more attractive to textile industry and cotton production. However, naturally colored cotton is generally inferior to white cotton in one or more aspects, which may be partially attributed to the pleiotropic effects of fiber color genes. For example, brown fiber gene inhabits fiber development and suppresses fiber quality [1,2].

Brown and green are the most common fiber colors in the naturally colored cottons. Traditional genetic analyses revealed that the brown fiber was controlled by 6 loci (L_{c1-6}) in cotton, while the green fiber was conditioned by 1 locus (L_g) [4]. Recently, extraction [1,3] and washing [5] experiments demonstrated that pigments in naturally colored fibers might belong to flavonoids. But exact structures and chemical properties of the pigments in naturally colored fibers and the molecular basis of pigment synthesis and deposition are essentially unknown.

To analyze the molecular basis of pigmentation in cotton fibers, we performed cDNA amplified fragment length polymorphism (cDNA-AFLP) analysis to compare expression profiles between the brown and white fibers from a recombination interbred line (RIL) population, which was derived from the cross of a white-fiber cultivar (Yumian No. 1) and a brown-fiber line (T586) [6]. On the basis of cDNA-AFLP analysis, we cloned five flavonoid structural genes from T586 fiber and further determined their transcript levels in various tissues of T586 and in the developing

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fibers from different-colored lines in the RIL population. Our results demonstrated that the cloned flavonoid structural genes and the flavonoid pathway were related to the pigmentation in brown cotton fibers.

Materials and methods

Population construction and cDNA-AFLP analysis. The F_2 population was constructed as previously described [6]. Two hundreds and seventy F_2 individuals were selected randomly and planted in the field to obtain $F_{2:3}$ family lines. One individual of each family line was randomly selected to produce next generation. This procedure was continued in the following generations until a $F_{2:7}$ RIL population was obtained. Due to the segregation of L_{c1} and L_g genes, there exist three fiber colors (white, brown, and green) in the RIL population.

The total RNAs were extracted from fibers of 8–10 days post anthesis (DPA) of brown- or white-fiber lines of the RIL population using a modified CTAB method [7]. The RNAs from 6 brown-fiber lines and 6 white-fiber lines were equally mixed to construct two RNA bulks (BF, brown fiber and WF, white fiber, respectively). The double-stranded cDNAs were synthesized from the bulked RNAs using a cDNA synthesis kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Then the cDNA was purified and subjected to cDNA-AFLP analysis as previously described [8].

Cloning of flavonoid structural genes. To clone corresponding genes from cotton fiber, an *Arabidopsis* chalcone isomerase (CHI), a *Malus* flavanone 3-hydroxylase (F3H), a *Daucus carota* anthocyanidin synthase (ANS) and a cDNA-AFLP differential fragment (Table 1) were used as probe sequences to query cotton ESTs in GenBank with the tBLASTn program (http://www.ncbi.nlm.nih.gov/blast). The homologous ESTs were assembled into contigs using SeqMan program of DNAStar software (DNAStar, WI, USA), and the contigs were subjected to BLASTX analysis (http://www.ncbi.nlm.nih.gov/blast) to find potential full-length ORFs. Subsequently, primers encompassing the putative ORFs were synthesized to amplify the cotton flavonoid structural genes using the cDNA derived from T586 fibers of 8–10 DPA as template (Table 1). To clone the anthocyanidin reductase (ANR) gene from cotton, the primers were designed according to the *Gossypium arboretum* BAN gene [9]. The amplified cDNAs were cloned and sequenced. The predicted proteins were further used to perform homology search in GenBank using BLASTP program (http://www.ncbi.nlm.nih.gov/blast). To further characterize these cotton flavonoid structural genes, we performed multiple alignment and phylogenetic tree analyses using the predicted proteins and their homologs. The alignments were generated using CLUSTALW [10] in DNAStar software (DNAStar, WI, USA), and the phylogenetic trees were viewed by TREEVIEW [11] programs.

Analysis of mRNA expression of the flavonoid structural genes by reverse transcription-PCR. The RNAs are extracted from various tissues and organs as described above. The cDNAs were synthesized with a First-Strand cDNA Synthesis Kit (MBI, ON, Canada) according to the manufacturer's instructions. The primers to specifically amplify the five flavonoid structural genes were as listed in Table 1. Ubiquitin gene (UBI) was amplified as RNA standard with the primers, 5′-CAG ATC TTC GTC AAA ACC CT-3′ and 5′-GAC TCC TTC TGG ATG TTG TA-3′, which were designed according to a cotton EST homologous to the polygene of plant ubiquitin (GenBank Accession No. AI727463). PCRs were amplified for 22–28 cycles.

Dimethylaminocinnamaldehyde staining of cotton fiber. The presence of proanthocyanidins in cotton fiber was monitored by dimethylaminocinnamaldehyde (DMACA) staining [12]. Mature seeds with different-colored fibers were immersed in 0.1% DMACA (Fluka, NY, USA) in 6 N HCl: 95% ethanol (1:1). After staining for 10 min, the seeds and fibers were washed for three times with distilled water. As control, seeds (with fibers) were treated in 6 N HCl: 95% ethanol (1:1). To determine the segregation of presence of fiber proanthocyanidins, ten white, green, and brown-fiber lines from the RIL population were detected.

Results and discussion

Cloning and sequence analysis of the cotton flavonoid structural genes

A total of 34 differential bands between the BF and WF bulks were obtained from 13 primer combinations. After sequencing and BLASTX analysis, a fragment of 164 bp (designated as 02021) amplified from the BF bulk with the primer recombination E-AAG/M-CAA was found to be highly homologous (63–78% identical at the amino acid level) to plant dihydroflavonol 4-reductase (DFR), an important enzyme in the flavonoid pathway (Fig. S.1, [13,14]). Based on the differential expression of DFR-homologous sequence in brown and white fibers, we envisioned that the flavonoid pathway may participate in the pigmentation in brown fibers. To test this hypothesis, we cloned five flavonoid structural genes (*GhCHI*, *GhF3H*, *GhDFR*, *GhANS*, and *GhANR*; GenBank Accession Nos. EF187439–EF187443) from T586 fibers. As shown in Table 2, all the cloned genes encoded proteins with high sequence similarity to corresponding proteins (including catalytically verified proteins) in GenBank.

Plant CHIs are classified into two types according to their different substrate specificity [15]. The type I CHIs generally found in nonlegumes can only isomerize 6′-hydroxychalcones into 5-hydroxyflavanones, while the type

Table 1
The probe sequences, ESTs and primers used in this study

Genes	Probe sequences	ESTs	Primer sequences (5′ → 3′)
GhCHI	*Arabidopsis* CHI (AAA32766)	BQ403301, BE054382	CHI-1, GGGAAAATGTCTACGTCACC CHI-2, CTGGTCTGAAACAGACACTG
GhF3H	*Malus* F3H (AF117270)	BQ410345, BG445449	F3H-1, TCAATGGCTCCTTCAACTCT F3H-2, CCAGCAGTAGGGTAAAAGTT
GhDFR	02021	BQ407603, BG441546, BQ403753, BQ416263, BG445812, AI726674, AI727078	DFR-1, GGTCTTTCTTTATGCCAACT DFR-2, AGACATGGGTAGGCACTCAA
GhANS	*Daucus carota* ANS (AAD56581)	BG446535, BG441402, BE054710	ANS-1, AAATGGTGACCTCAGTGCTA ANS-2, TCAGTTGGACAGATTATCCTG
GhANR	*Gossypium arboretum* BAN (BN000165)		BAN-1, AAGCATGGCCAGCCAGATC BAN-2, CAGAGCGCTTCACTTGAGC

Table 2
The sequence similarity of cotton flavonoid synthases to their homologs

Predicted proteins	Amino acids	Homologous proteins			
		Classes	Species	Accession Nos.	Identity (%) and aligned regions
GhCHI	227	Chalcone isomerase	*Citrus sinensis*	BAA36552	77, 1–216
			Vitis vinifera	CAA53577	74, 1–215
			Arabidopsis thaliana	AAA32766	69, 6–214
GhF3H	368	Flavanone 3-hydroxylase	*Citrus sinensis*	BAA36553	86, 1–368
			Arabidopsis thaliana	AAM65101	83, 1–368
			Malus × domestica	AF117270	82, 1–368
GhDFR	355	Dihydroflavonol 4-reductase	*Vitis vinifera*	CAA72420	80, 16–351
			Citrus sinensis	AAS00611	77, 23–354
			Arabidopsis thaliana	BAD95233	72, 20–353
GhANS	354	Anthocyanidin synthase	*Citrus sinensis*	AAT02642	82, 1–352
			Malus × domestica	AAZ79374	79, 4–352
			Arabidopsis thaliana	CAD91994	78, 8–352
GhANR	336	Anthocyanidin reductase	*Vitis vinifera*	BAD89742	83, 1–336
			Camellia sinensis	AAT68773	82, 1–336
			Medicago truncatula	AAN77735	74, 9–334

II CHIs from leguminous plants can convert both 6′-deoxychalcones and 6′-hydroxychalcones into 5-deoxyflavanones and 5-hydroxyflavanones, respectively [15]. Alignment of the predicted GhCHI protein with its homologous proteins revealed that all residues forming the CHI active site [16] were conserved in GhCHI (Fig. S.2). Among the residues proposed to determine the substrate preference (Thr190 and Met191 in *Medicago sativa* CHI, [16]), Thr190 was conserved in the two legume CHIs, but replaced by Ser in all nonlegume CHIs (including GhCHI) (Fig. S.2), indicating that the *GhCHI* gene encoded a type I CHI related to the biosynthesis of general flavonoids [15].

Both F3H and ANS belong to the 2-oxoglutarate-dependent dioxygenases superfamily, which also include other flavonoid synthesases (flavonol synthases, flavone synthases I) and enzymes involved in other pathways such as GA 3-oxidases, GA 2-oxidases, and aminocyclopropane carboxylic acid oxidases [17,18]. Multiple sequence alignment of GhANS and GhF3H with their homologous proteins (Fig. S.3) revealed that both proteins contained the conserved amino acids typical of 2-oxoglutarate-dependent dioxygenases, including the Fe-binding sites (His217, Asp219, and His275 in GhF3H; His234, Asp236, and His290 in GhANS), and the amino acids (Arg285 and Ser287 in GhF3H; Arg300 and Ser302 in GhANS) interacting with 2-oxoglutarate [17,19]. In a phylogenetic tree constructed using 8 families of 2-oxoglutarate-dependent dioxygenases (Fig. 1A), each protein family formed a unique clade, and GhF3H and GhANS were both clustered in the corresponding clades including all the investigated F3Hs and ANSs, respectively.

DFRs usually catalyze the reduction of dihydroflavonols to leucoanthocyanidins, and also use flavanones as substrates to synthesize flavan 4-ols in certain species (Fig. S.1, [13,14]). Several studies demonstrated that an amino acid (Asp or Asn) at the position corresponding to the 134th residue of *Gerbera* DFR affected substrate preference towards dihydroflavonols with different hydroxylation patterns on B ring [21,21]. An alignment of the deduced GhDFR sequence with its homologs (Fig. S.4) showed that GhDFR contained an Asp residue at that position, suggesting that GhDFR was an Asp-type DFR which preferred dihydroflavonols with two or three hydroxyls on B ring as substrate [20].

Both DFRs and ANRs belong to the reductase–epimerase–hydrogenase (RED) protein superfamily [9,22]. BLAST analysis demonstrated that DFRs and ANRs were closely related, and were also distantly related to other protein families of the RED superfamily, such as cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductases (CCR), and bacterium nucleoside-diphosphate-sugar epimerases (NDSE) (data not shown). In a phylogenetic tree constructed using six ANRs, six DFRs, three CADs, three CCRs, and two NDSEs (Fig. 1B), GhANR and GhDFR were clustered into two closely related but unique clades including all the ANSs and DFRs, respectively. Taken together, sequence comparisons indicated that all the five cotton flavonoid structural genes (*GhCHI*, *GhF3H*, *GhDFR*, *GhANS*, and *GhANR*) were orthologous to the corresponding genes from other species and might be biologically functional in cotton.

Expression profiles of cotton flavonoid structural genes

To determine whether the cloned cotton flavonoid structural genes were related to the pigmentation in cotton fibers, we detected their transcript levels in various tissues of T586. As shown in Fig. 2, transcripts of all the five genes were not detected in red-pigmented leaves, while in red-spotted petals and ovules, only medium transcript levels were detected except the *GhANS* gene in petals. All these genes exhibited high transcript levels in developing fibers, which reached to maximum at 16 DPA, and decline at 20–30 DPA. This observation indicated that these flavonoid structural genes expressed preferentially in developing fibers, and their temporal expression profiles were

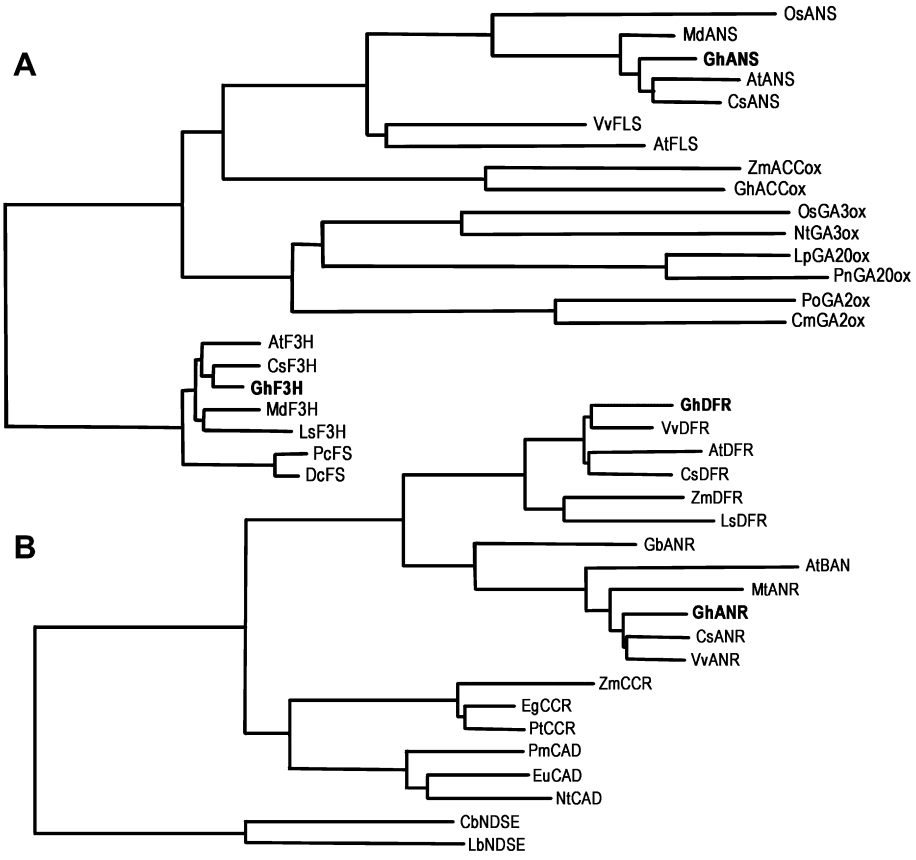

Fig. 1. Phylogenetic analyses of 2-oxoglutarate dependent dioxigenases (A) and reductase-epimerase-hydrogenase (RED) proteins (B) from various species. Proteins of the 2-oxoglutarate dependent dioxigenase superfamily include 5 ANSs from *G. hirsutum* (GhANS, EF187442) *O. sativa* (OsANS, CAA69252), *Malus* × *domestica* (MdANS, AAZ79374), *A. thaliana* (AtANS, CAD91994), and *C. sinensis* (CsANS, AAT02642), 5 F3Hs from *G. hirsutum* (GhF3H, EF187440), *Malus* × *domestica* (MdF3H, AAD26206), *A. thaliana* (AtF3H, AAM65101), *C. sinensis* (CsF3H, BAA36553) and *Lilium speciosum* (LpF3H, BAE79203), 2 flavonol synthases from *A. thaliana* (AtFLS, NP_196481), and *V. vinifera* (VvFLS, BAE75810), two flavone synthases I from *Daucus carota* (DcFS, AAX21536) and *Petroselinum crispum* (PcFS, AAP57393), 2 gibberellin 2-oxidases from *Cucurbita maxima* (CmGA2ox, CAC83090) and *Populus alba* × *Populus tremuloides* (PoGA20ox, AAQ93035), 2 gibberellin 3-oxidases from *Nicotiana tabacum* (NtGA3ox, BAA89316) and *O. sativa* (OsGA3ox, BAB62072), 2 gibberellin 20-oxidases from *Lolium perenne* (LpGA20ox, AAG43042) and *Populus nigra* (PnGA20ox, BAC56963) and 2 aminocyclopropane carboxylic acid oxidases from *G. hirsutum* (GhACCox, AAZ83343) and *Zea mays* (ZmACCox, AAR25561). The aligned RED proteins are 6 dihydroflavonol 4-reductases from *G. hirsutum* (GhDFR, EF187441), *A. thaliana* (AtDFR, BAD95233), *V. vinifera* (VvDFR, CAA72420) *C. sinensis* (CsDFR, AAS00611), *Z. mays* (ZmDFR, CAA75997) and *L. speciosum* (LsDFR, BAE79202), 6 anthocyanidin reductases from *G. hirsutum* (GhANR, EF187443), *A. thaliana* (AtBAN, NP_176365), *C. sinensis* (CsANR, AAT68773), *Medicago truncatula* (MtANR, AAN77735), *V. vinifera* (VvANR, BAD89742) and *Ginkgo biloba* (GbANR, AAU95082), 3 cinnamyl alcohol dehydrogenases from *Plantago major* (PmCAD, CAJ43716), *Eucommia ulmoides* (EuCAD, AAY26021) and *N. tabacum* (NtCAD, AAX15955), 3 cinnamoyl-CoA reductases from *Eucalyptus gunnii* (EgCCR, CAA56103), *Populus trichocarpa* (PtCCR, CAA12276) and *Z. mays* (ZmDFR, CAA66707) and nucleoside-diphosphate-sugar epimerases from 2 bacteria *Clostridium beijerincki* (CbNDSE, ZP_00909563) and *Lactobacillus brevis* (LbNDSE, YP_794413).

consistent with the time course of pigment deposition in colored cotton fibers, which peaked at 20 DPA [23]. Compared to T586 fibers, transcript levels of the five flavonoid structural genes in the fibers from white fiber cultivar Yumian No. 1 at 8-, 16- and 20-DPA were very low or undetectable (Fig. 2), implying that these genes and the flavonoid pathway might be involved in the pigmentation in cotton fibers.

With two fiber color genes (L_{c1} and L_g) [6], T586 may deposit both brown and green pigments in fiber. To further clarify the relationship of flavonoid structural genes and fiber pigmentation, we detected transcript levels of these genes in the developing fibers from seven brown-fiber, six green-fiber, and six white-fiber lines in the RIL population. As shown in Fig. 3, all the five flavonoid structural genes had high transcript levels in brown fibers, while their expressions in green fibers and white fibers were very low or undetectable, suggesting that high-level expressions of these flavonoid structural genes co-segregated with brown fiber in the RIL population. This result suggested that the cloned flavonoid structural genes were related to brown pigmentation in cotton fibers, but unrelated to green pigmentation.

A striking characteristic of flavonoid structural genes is their co-regulations [13,14,24]. Except in petals, the five flavonoid structural genes were expressed in parallel in various tissues and in different varieties (lines) (Figs. 2 and 3), suggesting that these genes were co-regulated in cotton, especially in developing fibers. This result implied that the brown fiber gene in T586 (L_{c1}) might be a flavonoid regulatory gene controlling the expressions of flavonoid structural genes in cotton fiber.

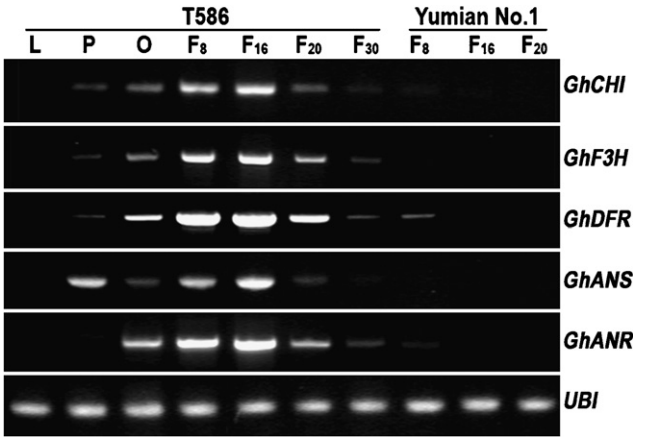

Fig. 2. Reverse transcription-PCR analysis of the transcriptional levels of cotton flavonoid structural genes. The RNAs are from leaves (L), petals (P), 12-DPA ovules (O), and fibers of 8, 16, 20, and 30 DPA (F_8, F_{16}, F_{20}, and F_{30}, respectively). The primers to specifically amplify the five flavonoid structural genes (*GhCHI*, *GhF3H*, *GhDFR*, *GhANS,* and *GhANR*) are listed in Table 1. Ubiquitin (*UBI*) gene is amplified as RNA standard. All the genes are amplified for 26 cycles.

Detection of proathocyanidins in cotton fibers

The relatedness of flavonoid structural genes to brown fiber indicated the brown pigments in cotton finer belonged to flavonoids, which are well-known as plant pigments coloring most flowers, seeds and fruits [13,14]. Flavonoids include a large group of phenolic secondary metabolites synthesized *via* a complex pathway which comprises of several branches leading to different classes of flavonoids, including pigments (aurones, anthocyanins, phlobaphenes, and proanthocyanidins) and co-pigments (flavones and flavonols) (Fig. S.1). In the flavonoid pathway, anthocyanins and proanthocyanidins share common steps from 4-coumaroyl CoA to anthocyanidins (Fig. S.1, [13,14]). Anthocyanidins can be converted to various anthocyanin pigments via different types of glycosylation and methylation [14]. For the biosynthesis of polymeric proanthocyanidins, the initiating units (flavan-3-ols) are first synthesized from leucoanthocyanidins and anthocyanidins, catalyzed by leucoanthocyanidin reductase and ANR, respectively [9,22]. As elongating units, leucoanthocyanins are then added to flavon-3-ols to form polymeric proanthocyanidins in vacuole [14]. Expression analyses demonstrated that the *GhANR* gene participated in the pigmentation in brown fiber, implying that the flavonoid pathway in brown fibers might direct to polymeric proanthocyanidins.

To further clarify the relationship of proantocyanidins with the pigmentation in brown fiber, we employed DMACA staining method to detect proanthocyanidins in mature fibers with different colors from the RIL population. As shown in Fig. 4, after DMACA staining, all the brown fibers (from 10 RILs) turned blue, while no significant color change was observed in the white and green fibers (from each 10 RILs), suggesting that proanthocyanidins deposited in brown fibers, but not in white and green fibers. Along with the expression analyses of flavonoid structural genes, this observation indicated that the pigments in brown fibers belonged to proanthocyanidins.

In conclusion, by expression analyses and DMACA staining, we demonstrated that flavonoid structural genes and proanthocyanidins were involved in the pigmentation in brown cotton fiber. To our knowledge, this is the first report on the molecular basis of the pigmentation in colored cotton fibers (at least in brown fibers). The cloning of flavonoid structural genes related to the pigmentation in naturally colored fiber may pave the way to dissect the relationship between fiber pigmentation and development, and can also facilitate to develop different-colored cottons by manipulating flavonoid structural and regulatory genes in cotton fiber *via* biotechnology techniques [12,24,25].

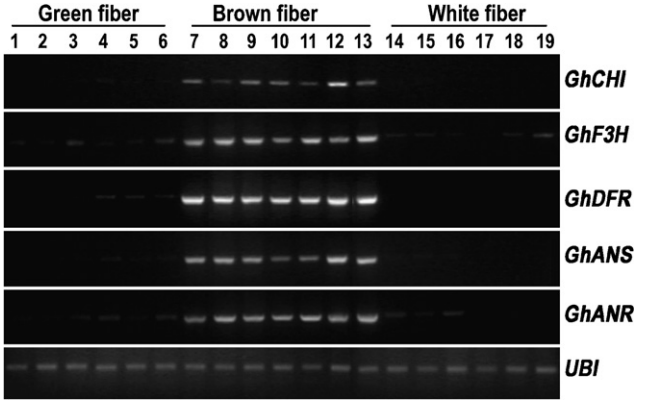

Fig. 3. RT-PCR analysis of the transcriptional levels of cotton flavonoid structural genes in different-colored fibers in the RIL population. The RNAs are from 8- to 10-DPA fibers of different lines with green (lanes 1–6), brown (lanes 7–13) and white (lanes 14–19) fibers in the RIL population. The cDNA synthesis and amplifications are performed as in Fig. 2, except that the flavonoid structural genes are amplified for 25 cycles, and the ubiquitin gene for 24 cycles.

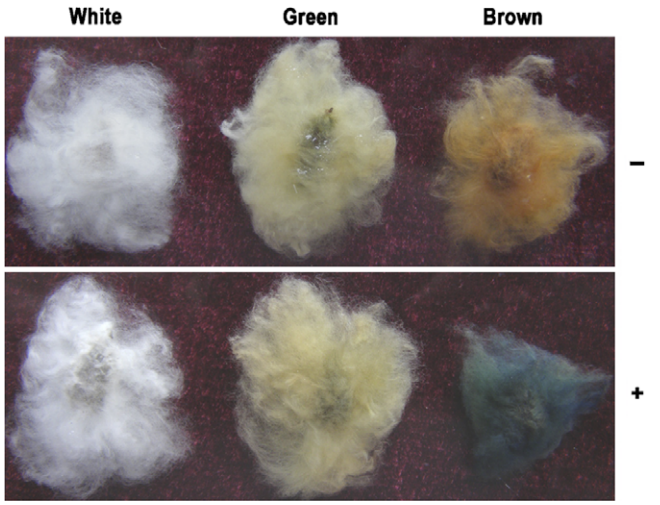

Fig. 4. Detection of proanthocyanidins in different-colored fibers. The mature seeds with different-colored fibers were treated with 6 N HCl: 95% ethanol (−) or by 0.1% DMACA in the same solution (+).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.084.

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