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Differences of flavonoid structural genes preferentially expressed in brown and green natural colored cotton

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Abstract: We compared the expression levels of some structural genes involved in the flavonoid pathway between two brown cotton lines (brown 16 and light brown 14) and two green cotton lines (greenish 12 and light green 5). Gene expression levels of six structural genes *F3'h* (flavonoid 3'-hydroxylase), *F3'5'h* (flavonoid 3'5'-hydroxylase), *Dfr* (dihydroflavonol 4-reductase), *Lar* (leucoanthocyanidin reductase), *Ans* (anthocyanidin synthase), and *Anr* (anthocyanidin reductase) were all substantially highly expressed in both brown cotton lines than in green cotton lines. Our study also revealed differences in expression levels between the two brown cotton lines. *F3'h* and *F3'5'h* had higher expression in the brown than in the light brown fibers, suggesting that increasing expression of these genes resulted in more of the proanthocyanidin pigments that give color to the brown lines. None of the genes examined were differentially expressed in the two green lines, suggesting the color difference is not due to products of the flavonoid pathway. The results suggest that breeding efforts to introduce brown colors into white-fiber lines with high-quality fiber focus on introgressing *F3'h* and *F3'5'h* and that naturally occurring allelic variants affecting the expression levels of these genes could be used to control the intensity of brown pigmentation

Key words: Natural colored cotton (*Gossypium hirsutum* L.), gene transcription, flavonoid, pigmentation

1. Introduction

Naturally colored cotton has been studied to eliminate dyeing during processing of yarn, and to significantly reduce processing costs, environmental pollutions, and chemical residues in textile fabrics. However, it is well known that the fiber quality of naturally colored cotton is lower than that of white cotton fiber, especially with regards to fiber length, micronaire quantity, and fiber strength (Feng et al., 2011; Feng et al., 2013; Xiao et al., 2014). These undesirable qualities have limited the use of naturally colored cotton in yarn production. Breeders have attempted to cross white cotton with colored cotton to improve fiber quality of colored cotton, but the results have been unsatisfactory (Yuan et al., 2012) due to mainly a negative correlation between fiber color and fiber quality traits, presumably because of pleiotropic effects of fiber color genes (Wang et al., 2014). Additionally, distant hybridization-sterility between brown cotton cultivars and Sea-island cotton cultivar (white cotton) is an impediment not only for the improvement of fiber quality but also for map-based cloning of fiber color genes in colored cotton (Zhang et al., 1994). Finally, some products of the flavonoid pathway, along with the combined activities of the auxin inhibitor naphthylphthalamic acid receptors and PIN-FORMED proteins may affect auxin transport (Mathesius et al., 1998; Murphy et al., 2000; Peer et al., 2004), and this effect is apparently due primarily to the production of naringenin, which is negatively associated with fiber development in naturally colored cotton (Tan et al., 2013).

One potential way to get around these difficulties is to introgress specific genes that facilitate pigment production but have few deleterious pleiotropic effects into white cotton. For example, it is conceivable that increased expression of genes coding for proteins downstream of naringenin (e.g., *F3'h* and *F3'5'h*, which can directly hydroxylate naringenin (Petrussa et al., 2013)), could not only increase pigment production, but possibly reduce steady-state levels of naringenin and preserve fiber quality. In order to do this, however, information is needed on the effects of flavonoid gene expression levels on pigment production, and in particular which flavonoid genes have the greatest effect on pigment levels. To this end, this study examines the expression levels of flavonoid genes in cotton

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varieties that differ in pigmentation levels.

Recently, analyses of chemical properties have revealed that the pigment in brown fiber consists of flavonoids, specially proanthocyanidins (Li et al., 2005; Xiao et al., 2007; Feng et al., 2013; Li et al., 2013; Tan et al., 2013; Xiao et al., 2014; Feng et al., 2015). It is, therefore, expected that variation in pigment production will be affected by expression levels of some flavonoid genes. Much less is known about pigments in green-fiber cotton. Although, there is one report that the production of green pigments affected by the flavonoid biosynthetic pathway (Feng et al., 2013), the chemical structurer of pigment in green fiber is unknown.

By examining expression levels in brown and green cotton varieties with different intensities of pigmentation, we attempted to address the following specific questions: (1) Is variation in flavonoid gene expression level correlated with pigmentation intensity? (2) If so, which gene(s) is/are most likely responsible for this correlation? and (3) is green cotton pigmentation likely to be due to flavonoid production?

2. Materials and methods

2.1. Plant materials and sample collection

Advanced brown fiber F_{10} generation breeding lines 14 (light brown) and 16 (dark brown) in Figure 1 were produced by crossing white cotton (female; N84 cultivar) with brown colored cotton (male; DT cultivar) to improve fiber quality in pedigree breeding method. Advanced green fiber F_{10} generation breeding lines 5 (greenish) and 12 (light green) in Figure 1 were produced by crossing white cotton (female; N84 cultivar) with green colored cotton (male; green cultivar) to improve fiber quality. Green lines 5 and 12 (female) were subsequently backcrossed for 4 and 3 generations in an introgression program, respectively with white cotton (male, recurrent). After backcrossing, both green breeding lines were produced by selfing pollination up to F_{10} generations. Consequently, the two green lines may have slightly different genetic backgrounds.

Four replicate plants of each line were grown in pots (51L volume) in the greenhouse at Duke University, Durham NC, USA. Growth conditions were 30–32 °C during the day, and 20–22 °C at night, with a photoperiod of 16 h day, 8 h night.

Flowers were tagged on the day of anthesis (0 DPA, Day Post Anthesis). Cotton bolls were harvested at 14 DPA and immediately immersed in ice. The cotton fibers were quickly separated on ice from seeds using forceps then immediately stored at −80 °C until RNA extraction. In addition, cotton plants with white fiber (N84 cultivar) were grown as a control sample to calculate the normalized fold expression of six genes in the four naturally colored cotton cultivars.

2.2. RNA extraction, cDNA synthesis, and semiquantitative PCR

Total RNA was extracted from fibers using Sigma Aldrich Spectrum™ Plant Total RNA Kit (St. Louis, MO) and quantified spectrophotometrically using a Nano Drop-1000, (Thermo Scientific, USA). The extracts were diluted to 40 ng/μL for subsequent analyses.

Double-stranded cDNA was synthesized from total RNA using the MultiScribe™ Reverse Transcriptase Kits of Applied_Biosystems (California, USA) using random primers according to the manufacturer's instructions.

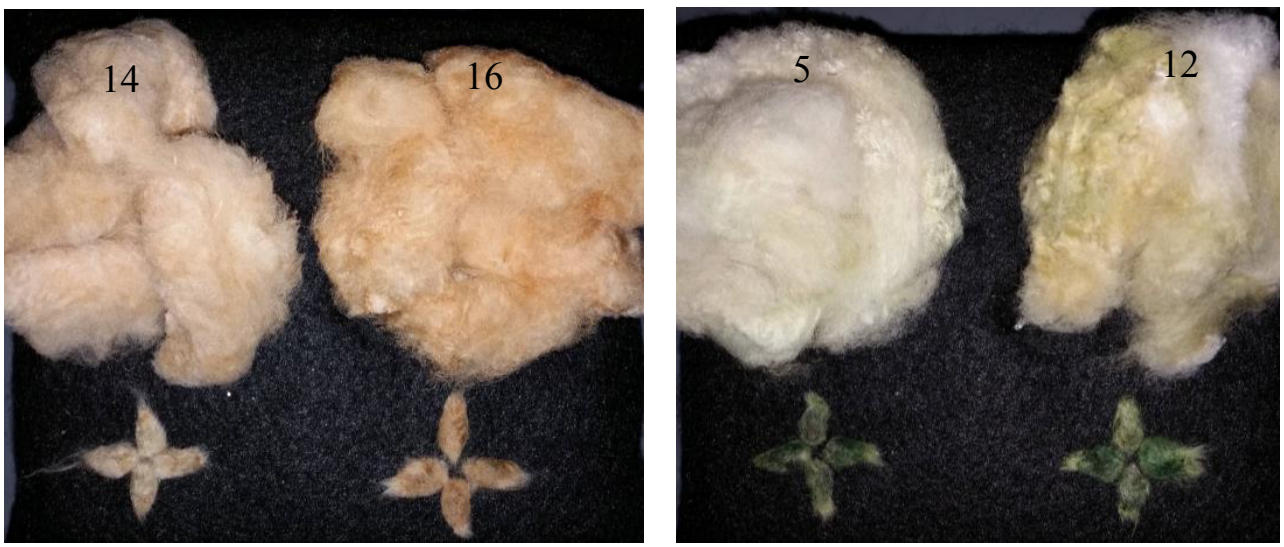

Figure 1. The image of fibers and seeds from brown colored cotton lines 14 (light brown) and 16 (dark brown) and green colored cotton lines 5 (greenish) and 12 (light green).

Specifically, a 2X master mix consisting of 10X RT buffer (2 µL), 10X RT random primers (2 µL), 25X dNTP Mix (100 mM) (0.8 µL), MultiScribe™ reverse transcriptase 50 U/µL (1 µL), and nuclease-free H_2O (4.2 µL) was prepared. Reactions (20 µL) were carried out using 10 µL of 2X RT master mix and 10 µL of RNA sample. The thermocycler (Bio-Rad, USA) program used was 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C for ¥.

Primers used to amplify individual genes *F3'5'h*, *Dfr*, *Lar*, and *Anr* were obtained from Xiao et al. (2014). The primers of *F3'h* (NM_0013227514), *Ans* (EF187442), and *UBQ7* (DQ116441) were designed using gene sequences obtained from the cotton genome by BLAST[1]. Primers (forward and reverse) were synthesized by Integrated DNA Technologies, Inc. (IDT) (USA). These primers produced PCR products of 95–155 bp for 6 flavonoid structural genes and the control gene ubiquitin (Table 1). PCR was performed using Phusion DNA polymerase (New England Biolabs) according to the manufacturer's instructions. The amplification protocol consisted of 40 cycles of 94 °C for 3.5 min, 63 °C for 30 s, and 72 °C for 2 min (Bio-Rad MyCycler Thermal Cycler PCR, CA, USA). PCR products were identified electrophoretically on a 1% agarose gel with SybrRsafe (8.0 µL; Invitrogen, USA). The gel was viewed and captured with a ChemiDoc™ MP Imaging System (170-8280) (Bio-Rad, USA).

2.3. Quantitative RT-PCR analysis

cDNA was adjusted to a concentration of 2.0 ng/µL for use in quantitative real-time PCR (qPCR) reactions. The qPCR mix consisted of 10 µL of Thermo Scientific DyNAmo HS SYBR Green qPCR Kits (USA), 1 µL of each primer (0.5 µM), 2.0 µL of template, and 6.0 µL of ddH2O 6.0 (20 µL total reaction volume). PCR was performed using a LightCycler® 96 (Roche, USA) with a program of 15 min initial denaturation step at 95 °C, followed by 45 cycles of 10 s at 94 °C and 30 s at 58 °C. All experiments involved three biological replicates for each cotton genotype, and each biological replicate was performed in duplicate.

The threshold cycle (CT) values of the triplicate qPCR runs were reported as a mean for each replicate and the fold changes of transcription levels of target genes relative to the reference genes (UBQ7). These data were then analyzed using the comparative Ct ($2^{-}\Delta\Delta Ct$) method (Balasubramanian et al., 2016). ΔCt was calculated by subtracting the Ct values of UBQ7 (control genes) from the Ct values of flavonoid synthesis gene within the same stage, whereas ΔΔCt was calculated by subtracting the ΔCt values from the colored cotton samples from that of white cotton. Normalized fold expression was calculated relative to white cotton. The possibility of contamination by gDNA was ruled out by running the PCR reactions on isolated RNA.

[1] National Center for Biotechnology Information. Basic Local Alignment Search Tool [online]. Website https://blast.ncbi.nlm.nih.gov/Blast.cgi

Table 1. Sequences of the primers used for real-time PCR analysis.

Genes	Primers	Sequences (5' to 3')	Accession number (Sequence ID)	Amplicon length (bp)
GhF3'h	*F3'h-F*	AGTGGGAGTTGGCTGATGGATT	NM_0013227514	155
	F3'h-R	CTCCTCACCCTGAAACGACAAC		
GhF3'5'h	*F3'5'h-F*	AAACATGGATGAGGCCTTTG	NM_001327621	111
	F3'5'h-R	GCAAGGGATGTGCTTAGGAA		
GhDfr	*Dfr-F*	CATGTTCGTAGGAGCTGTCG	NM_001327665	118
	Dfr-R	GGTAGGCACTCAATTGTTGAAA		
GhLar	*Lar-F*	GAATGAGCCATTCCGAACAT	XM_016880783	135
	Lar-R	GCTTCGACTACTGGCTTTGG		
GhAns	*Ans-F*	ACAATGCTAGTGGGCAGCTT	EF187442	139
	Ans-R	GCAGTTGCCTTGCATACTCA		
GhAnr	*Anr-F*	TGGGATCGAGGAAATCTACG	NM_001327416	95
	Anr-R	ACCATAATCATTGGGGAAGC		
GhUbq7	*UBQ7*	AAGCCCAAGAAGATCAAGCA	DQ116441	115
	UBQ7	CGCATTAGGGCACTCTTTTC		

Anr, anthocyanidin reductase; *Ans*, anthocyanidin synthase; *Dfr*, dihydroflavonol 4-reductase; *F3'h*, flavonoid 3'-hydroxylase; *F3'5'h*, flavonoid 3'5'-hydroxylase; *Lar*, leucoanthocyanidin reductase; *Ubq7*, ubiquitin gene.

To ensure the cDNA samples did not contain carryover DNA, we performed PCR (BIO-RAD, USA) on the RNA samples using primers for each gene. Reactions consisted of 17.6 µL of PCR buffer mix, 0.8 µL of forward primer (5mM), 0.8 µL of reverse primer (5mM), 0.8 µL of RNA.

2.4. Statistical analysis

To determine whether the four genotypes differed in gene expression levels, analysis of variance (ANOVA) using SAS software version 9 (SAS, 2018) was performed. The model consisted of a single factor (genotype). Because there are three degrees of freedom (df) associated with the main effect of genotype, three contrasts with 1 df each were performed to test the following null hypotheses: (1) the average of the brown and green genotypes did not differ; (2) The two brown genotypes did not differ; and (3) The two green genotypes did not differ. In these analyses, significance was determined after a sequential Bonferroni adjustment (Bland and Altman, 1995) to maintain an overall significance level of 0.05.

3. Results

The average expression levels of five flavonoid genes (*F3'h*, *F3'5'h*, *Dfr*, *Ans*, and *Anr*) was significantly different after corrections for multiple comparisons for the two brown lines compared to the two green lines, and that of the sixth gene (*Lar*) was marginally significantly different (Table 2). In all cases, average expression of the brown lines is higher than that of the green lines (Figure 2), indicating that the flavonoid pathway is generally upregulated more in the brown lines.

The two brown lines differed significantly in expression level for *F3'h*, *F3'5'h*, *Dfr*, and *Anr* (Table 2). The direction of the difference was not consistent, with greater expression of *F3'h* and *F3'5'h* in dark brown line 16 but greater expression of *Dfr* and *Anr* in brown line 14 (Figure 2). This pattern suggests that in brown cotton, more of the flux of the flavonoid pathway flows through *F3'h* and *F3'5'h*, producing more singly and doubly hydroxylated flavonoids in the dark brown line. By contrast, the two green lines

Table 2. The results of ANOVA for expression levels of six flavanoid genes in four cotton lines. A) Test of whether all four genotypes have equal expression. (B–D) Independent contrasts testing hypotheses: B) that the average expression for the two brown genotypes equals the average expression for the two green genotypes; C) that the two green genotypes have equal expression; and D) that the two brown genotypes have equal expression. Numerator df for all three contrasts is 1. Entries in bold indicate with nominal significance of $p < 0.05$ that remain significant after a sequential Bonferroni adjustment for an overall rejection rate of 0.05.

	F3'h	*F3'5'h*	*Dfr*	*Ans*	*Anr*	*Lar*
A.						
Genotyhpe effect MS	5331.3123	1.0655	11719.4	34.877	247.83	237.357
Genotype effect df	3	3	3	3	3	3
Error MS	19.97799	0.0121	602.18	2.594	41.766	59
Error df	8	8	8	8	8	8
Genotype effect F	266.86	88	19.46	13.45	5.93	4.02
Genotype effect P	**< 0.0001**	**<0.0001**	**0.0005**	**0.0017**	**0.0197**	0.0512
B						
green vs. brown MS	13528.7	2.3144	20295.2	103.253	342.187	702.117
green vs. brown F	677.18	191.14	33.7	39.81	8.19	11.9
green vs. brown P	**<0.0001**	**<0.0001**	**0.0004**	**0.0002**	**0.0211**	**0.0087**
C.						
green 1 vs. green 2 MS	147.3	0.0384	1388.1	0.0323	0.3314	6.427
green 1 vs. green 2 F	7.37	3.17	2.31	0.01	0.01	0.11
green 1 vs. green 2 P	0.0266	0.1128	0.1674	0.9139	0.9312	0.75
D.						
brown 1 vs. brown 2 MS	2317.9	0.8438	13474.9	1.344	400.98	3.527
brown 1 vs. brown 2 F	116.02	69.68	22.38	0.52	9.6	0.06
brown 2 vs. brown 2 P	**<0.0001**	**<0.0001**	**0.0015**	0.49	**0.0147**	0.813

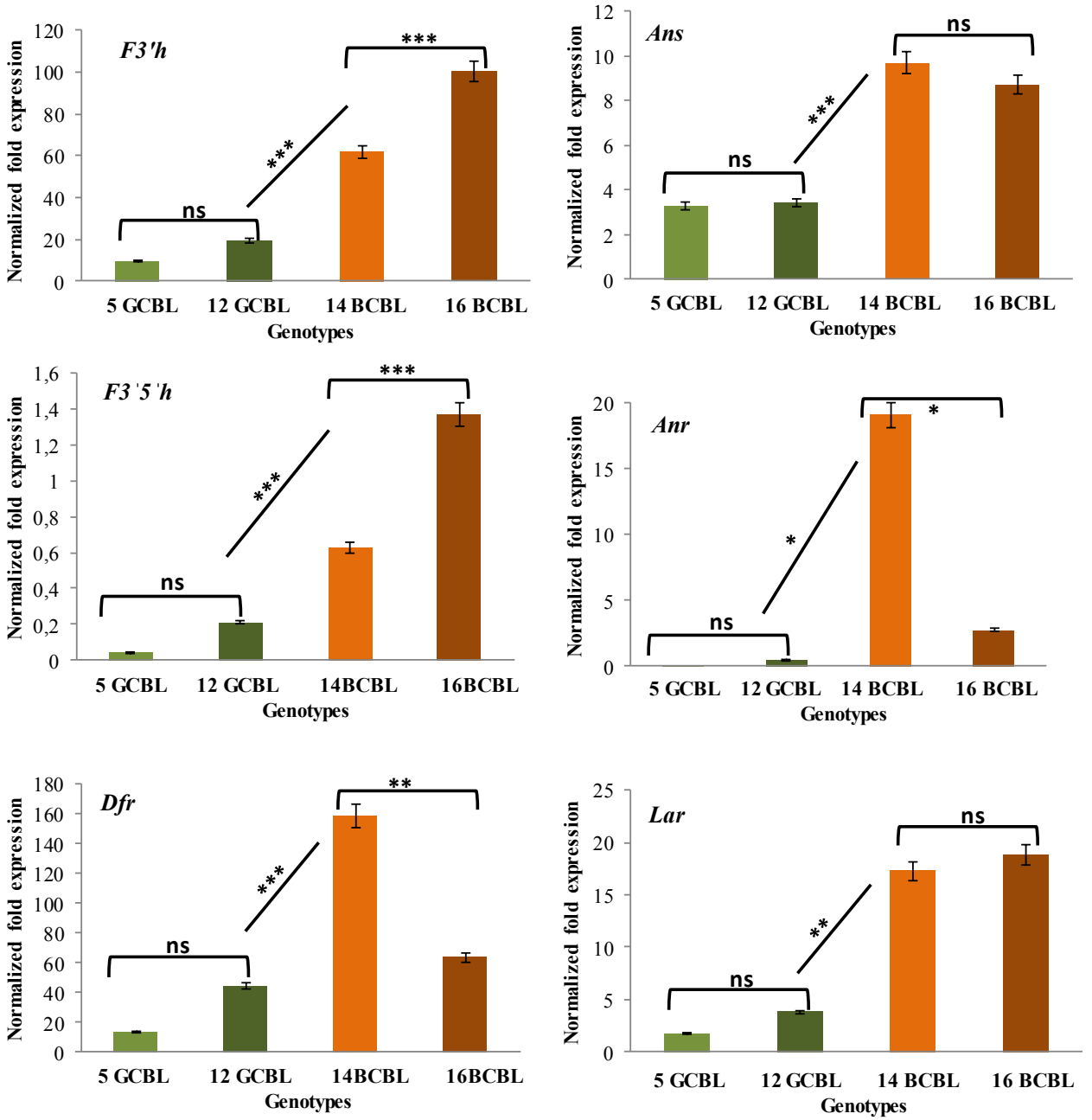

Figure 2. Relative expressions of green, white, and brown flavonoid genes during fiber development. Brackets and slanted line represent the three ANOVA contrasts performed. Bars are standard errors of three biological replicates. (GCBL: *Green Cotton Breeding Line*, BCBL: *Brown Cotton Breeding Line*). *p < 0.05, **p < 0.01, ***p < 0.001, ns; nonsignificant. All probabilities correspond to an overall probability of p = 0.05 using a sequential Bonferroni correction.

did not differ significantly in expression for any of the genes (Table 2). While difference in expression of *F3'h* was nominally significant, it did not remain so after correction for multiple comparisons. This lack of difference in gene expression between the two green lines suggests that the difference in color intensity between the lines may not be due to differences in flavonoid production, although one caveat to this inference is that we did not quantify expression levels of all genes in the flavonoid pathway.

4. Discussion

One result of this study is that the flavonoid pathway is generally downregulated in the green genotypes compared to the brown genotypes, and thus likely

produces fewer anthocyanins, anthocyanidins, flavonols, proanthocyanidins/tannins. This results is consistent with previous studies, which have demonstrated that coloration of brown cotton fibers is due to the accumulation of proanthocyanidins (Tan et al., 2013; Xiao et al., 2014; Feng et al., 2015), whereas green fibers lack these compounds (Liu et al., 2018).

A second result of this study is that there is differential expression of four flavonoid genes between the light and dark brown fiber lines. In particular, we found that *F3'h* and *F3'5'h* are expressed at higher levels, and *Dfr* and *Anr* at lower levels, in the dark brown fibers than in the light brown fibers. This result is consistent with those of Gong et al. (2014), who found that *Dfr* and *Anr* were more highly expressed in light brown fibers compared to dark brown fibers. This pattern suggests that *F3'h* and *F3'5'h* may be the rate-controlling steps in the production of proanthcyanidins in genotypes with brown pigmentation.

In the flavonoid pathway, the intermediate dihydrokaempferol (DHK) is an important branch point, where there is substrate competition between *Ans* on the one hand and *F3'h* and *F3'5'h* on the other. Because of this competition, because in dark brown cotton *F3'h* and *F3'5'h* are upregulated while *Dfr* is downregulated compared to light brown cotton, in the dark brown 16 cultivar, we expect that the dark brown cotton would produce more singly and doubly hydroxylated proanthocyanidin precursors (catechin, gallocatechin, epicatechin, epigallocatechin) than the light brown cotton, which would produce more nonhydroxylated precursors (afretichen, epiafretichen). This effect might contribute to the difference in color intensity in addition to any overall effect of a difference in the total amount of flavonoids produced.

A final result of our study is that the two green lines do not differ detectably in expression levels of any of the genes examined. While there may be subtle differences that we did not have the power to detect, this result suggests that the difference in light vs. dark green fibers is not due to differences in the production of flavonoids.

This study leaves a couple of questions unanswered. First, we do not know whether differences in expression of flavonoid genes between brown and green cotton, and between cultivars of brown cotton, are due to cis-regulatory changes in those genes or changes in the expression of transcription factors that activate those genes. This is an important question for breeders interested in introducing "color" genes into white cotton because it would be easier to introgress a small number of transcription factors into white cotton than a larger number of enzyme-coding genes. Second, we do not know whether color differences between light and dark brown cotton is actually due to the production of different proanthocyanidin precursors. This question will need to be answered in order to determine whether introgression of *F3'h* and *F3'5'h* will affect pigment intensity.

5. Conclusion

Our results indicate that in order to manipulate the intensity of brown pigmentation in cotton, a focus on two genes, *F3'h* and *F3'5'h*, is appropriate. In particular, genetic engineering approaches that upregulate these genes should result in darker fibers, while downregulation should yield lighter fibers. By contrast, our results also suggest that manipulation of flavonoid genes is unlikely to alter the intensity of pigmentation in green cotton. Remaining challenges are to determine whether differences in pigment intensity are due to cis- or transvariants, and to determine whether light and dark brown cotton have different types of pigments.

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References

Balasubramanian VK, Rai KM, Thu SW, Hii MM, Mendu V (2016). Genome-wide identification of multifunctional laccase gene family in cotton (*Gossypium* spp.); expression and biochemical analysis during fiber development. Scientific Reports. 6: 34309. doi: 10.1038/srep34309

Bland JM, Altman DG (1995). Multiple significance tests: the Bonferroni method. BMJ 310 (36986): 710. doi: 10.1136/bmj.310.6973.170

Feng HJ, Sun JL, Wang J, Jia YH, Zhang XY et al. (2011). Genetic effects and heterosis of the fiber color and quality of brown cotton (*Gossypium hirsutum*). Plant Breeding 130: 450-456. doi: 10.1111/j.1439-0523.2010.01842.x

Feng H, Tian X, Liu Y, Li Y, Zhang X et al. (2013). Analysis of flavonoids structural genes in Brown fiber of upland cotton. PLoS ONE 8: 1-10. doi: 10.1371/journal.pone.0058820

Feng H, Guo L, Wang G, Sun J, Pan Z et al. (2015). The negative correlation between fiber color and quality traits revealed by QTL analysis. PLoS ONE 10 (6): e0129490. doi: 10.1371/journal.pone.0129490

Gong W, He S, Tian J, Sun J, Pan Z et al. (2014). Comparison of the transcriptome between two cotton lines of different fiber color and quality. PLoS ONE 9 (11): e112966. doi: 10.1371/journal.pone.0112966

Hua SJ, Wang XD, Yuan SN, Shao MY, Zhao XQ et al. (2007). Characterization of pigmentation and cellulose synthesis in colored cotton fibers. Crop Science 47: 1540-1546. doi: 10.2135/cropsci2006.12.0835

Li XB, Fan XP, Wang XL, Cai L, Yang WC (2005). The cotton ACTIN1 gene is functionally expressed in fibers and participates in fiber elongation. Plant Cell 17: 859-875. doi: 10.1105/tpc.104.029629

Li Y-J, Zhang X-Y, Wang F-X, Yang C-L, Liu F et al. (2013). A comparative proteomic analysis provides insights into pigment biosynthesis in brown color fiber. Journal of Proteome 78: 374-388. doi: 10.1016/j.jprot.2012.10.005

Liu HF, Luo C, Song W, Shen, Li G, He ZG et al. (2018). Flavonoid biosynthesis controls fiber color in naturally colored cotton. PeerJ 6: e4537. doi: 10.7717/peerj.4537

Mathesius U, Schlaman HR, Spaink HP, Sautter C, Rolfe BG et al. (1998). Auxin transport inhibition precedes root nodule formation in white clover rootsand cis regulated by flavonoids and derivatives of chitin oligosaccharides. Plant Journal 14: 23-34. doi: 10.1046/j.1365-313X.1998.00090.x

Murphy A, Peer WA, Taiz L (2000). Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta 211: 315-324. doi: 10.1007/s004250000300

Peer WA, Bandyopadhyay A, Blakeslee JJ, Makam SN, Chen RJ et al. (2004). Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in Arabidopsis thaliana. Plant Cell 16: 1898-1911. doi: 10.1105/tpc.021501

Petrussa E, Braidot E, Zancani M, Peresson C, Bertolini A et al. (2013). Plant flavonoids biosynthesis, transport and involvement in stress responses. International Journal of Molecular Science 14: 14950-14973. doi: 10.3390/ijms140714950

Tan J, Tu L, Deng F, Hu H, Nie Y et al. (2013). A genetic and metabolic analysis revealed that cotton fiber cell development was retarded by flavonoid naringenin1[W][OA]. Plant Physiology 162: 86-95. doi: 10.1104/pp.112.212142

Yuan S, Hua S, Malik W, Bibi N, Wang X (2012). Physiological and biochemical dissection of fiber development in colored cotton. Euphytica 187: 215-226. doi: 10.1007/s10681-012-0653-9

Zhang J, Gong Z, Sun J, Liu J (1994). Heterosis of yield and fiber performance in interspecific crosses between *Gossypium hirsutum* and *G. barbadense*. Acta Gossypii Sinica 6: 140-145.

Wang L, Liu H, Li X, Xiao X, Ai X et al. (2014). Genetic mapping of fiber color genes on two brown cotton cultivars in Xinjiang. SpringerPlus 3: 480. doi: 10.1186/2193-1801-3-480

Xiao YH, Zhang ZS, Yin MH, Luo M, Li XB et al. (2007). Cotton flavonoid structural genes related to the pigmentation in brown fibers. Biochemical Biophysical Research Communications 358: 73-78. doi: 10.1016/j.bbrc.2007.04.084

Xiao Y-H, Yan Q, Ding H, Luo M, Hou L et al. (2014). Transcriptome and biochemical analyses revealed a detailed proanthocyanidin biosynthesis pathway in brown cotton fiber. PLoS ONE 9 (1): doi: 10.1371/journal.pone.0086344