KSÜ Tarım ve Doğa Derg 28 (1), 191-204, 2025
KSU J. Agric Nat 28 (1), 191-204, 2025
https://doi.org/10.18016/ksutarimdoga.vi.1575961

# Analysis of Flavonoids Structural Genes in Between Chalaza and Microphyll a Mutant Natural Green Cotton Fiber

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## ABSTRACT
Undesirable coloring pigments in naturally colored fibers can affect their aesthetic and commercial value in the textile industry. In our green cotton breeding program, we identified a gene mutation that causes different colored fibers to form on a single seed. We investigated the expression patterns of flavonoid biosynthesis structural genes to understand the formation of these different colors between the chalazal and microphyll parts of the seed. Significant variations in gene expression levels were observed among the examined flavonoid genes, highlighting the complexity of flavonoid biosynthesis pathways in cotton seeds. In the green fibers on the microphyll part, lower expression levels of enzymes such as *4Cl* (4-coumarate: CoA ligase), *C4h* (cinnamate 4-hydroxylase), *F3h* (flavone 3-hydroxylase), *F3′5′h* (flavonoid 3′5′-hydroxylase), *Ans* (anthocyanidin synthase), Anr (anthocyanidin reductase), and *Ufgt* (UDP-glucose: flavonoid 3-O-glucosyltransferase) were identified as potential factors influencing fiber coloration. Conversely, in the white fibers on the chalazal part, the expression levels of *Chs* (chalcone synthase) and *Chı* (chalcone isomerase) genes were lower than those in the microphyll part. This low expression is thought to be due to a mutation at the beginning of the phenylalanine pathway, preventing the formation of a green color in the white fibers on the chalazal part together with the low synthesis of the *Chı* gene. Understanding the molecular mechanisms behind these mutations is crucial for developing strategies to mitigate their effects and sustain the textile industry. The findings can inform cotton breeding programs to address unwanted coloration issues through genetic interventions, potentially enhancing the aesthetic and commercial value of naturally colored cotton fibers.

**Field Crops**

**Research Article**

**Article History**
Received : 30.10.2024
Accepted : 30.12.204

**Keywords**
Natural colored cotton (*Gossypium hirsutum* L.)
Mutation
Flavonoid
Pigmentation

# Mutant Doğal Yeşil Pamuk Lifinde Chalaza ve Microphyll Arasındaki Flavonoid Yapısal Genlerin Analizi

## ÖZET
Doğal renkli liflerdeki istenmeyen farklı renk pigmentleri, tekstil endüstrisindeki estetik ve ticari değerlerini etkileyebilir. Yeşil pamuk ıslah programımızda, tek bir tohumunda farklı renkli liflerin oluşmasına neden olan bir gen mutasyonu tespit ettik. Tohumun şalazal ve mikrofil kısımları arasındaki bu farklı renklerin oluşumunu anlamak için flavonoid biyosentez yapısal genlerinin ifade modellerini araştırdık. İncelenen flavonoid genleri arasında gen ekspresyon seviyelerinde önemli farklılıklar gözlenmiş, bu da pamuk tohumlarındaki flavonoid biyosentez yollarının karmaşıklığını vurgulamıştır. Mikrofil kısmındaki yeşil liflerde, *4Cl* (4-coumarate: CoA ligaz), *C4h* (sinamat 4-hidroksilaz), *F3h* (flavon 3-hidroksilaz), *F3′5′h* (flavonoid 3′5′-hidroksilaz), *Ans* (antosiyanidin sentaz), *Anr* (antosiyanidin redüktaz) ve *Ufgt* (UDP-glukoz: flavonoid 3-O-glukosiltransferaz) lif renklenmesini etkileyen potansiyel faktörler olarak tanımlanmıştır. Buna karşılık, şalazal kısımdaki beyaz liflerde, *Chs* (chalcone synthase) ve *Chı* (chalcone isomerase) genlerinin ifade seviyeleri mikrofil kısımdakilerden daha

**Tarla Bitkileri**

**Araştırma Makalesi**

**Makale Tarihçesi**
Geliş Tarihi : 30.10.2024
Kabul Tarihi : 30.12.2024

**Anahtar Kelimeler**
Doğal renkli pamuk (Gossypium hirsutum L.)
Mutasyon
Flavonoid
Pigmentasyon

düşüktü. Bu düşük ifadenin, şalazal kısımdaki beyaz liflerde yeşil renk oluşumunu engelleyen fenilalanin yolunun başlangıcındaki bir mutasyondan kaynaklandığı düşünülmektedir. Bu mutasyonların arkasındaki moleküler mekanizmaları anlamak, etkilerini azaltmak ve tekstil endüstrisini sürdürmek için stratejiler geliştirmek açısından çok önemlidir. Bulgular, genetik müdahaleler yoluyla istenmeyen renklenme sorunlarını ele almak için pamuk ıslah programlarını bilgilendirebilir ve potansiyel olarak doğal renkli pamuk liflerinin estetik ve ticari değerini artırabilir.

**Atıf Şekli**: Canavar, Ö., & Gören, H. K. (2025). Mutant doğal yeşil pamuk lifinde chalaza ve microphyll arasındaki flavonoid yapısal genlerin analizi. *KSÜ Tarım ve Doğa Derg*, *28* (1), 191-204. https://doi.org/10.18016/ksutarimdoga.vi.1575961.

**To Cite :** Canavar, Ö., & Gören, H. K. (2023). Analysis of flavonoid structural genes in between chalaza and microphyll a mutant natural green cotton fiber. *KSU J. Agric Nat, 28* (1), 191-204. https://doi.org/10.18016/ksutarimdoga.vi.1575961.

## INTRODUCTION

Naturally colored cotton has inherent color in the fiber. With growing consumer demand for environmentally friendly products, naturally colored cotton, which can be used with little or no processing and dyeing steps, is becoming increasingly attractive to the textile industry (Zhao and Wang 2015; Vreeland 1999). Unfortunately, only brown and green naturally colored varieties are currently available, limiting the development of the naturally colored cotton textile market. It may be possible to increase the diversity of available naturally colored fibers through conventional breeding, but this approach is limited by a lack of understanding of the process of cotton fiber color formation. In order to advance the integration of new natural colors into cotton, it is imperative to provide guidance for molecular breeding programs. The fundamental requirement for this is to explore and understand the molecular basis of pigment synthesis and deposition in cotton fibers. Additionally, the biosynthesis of flavonoids has been identified as a critical factor influencing fiber color in naturally colored cotton, with limitations in fiber quality observed in brown and green cotton fibers (Liu et al., 2018). Flavonoids and structural genes are key players in determining cotton fiber color inheritance (Gong et al., 2014). The expression profiles of key genes involved in the flavonoid biosynthetic pathway have been meticulously examined to unravel the intricate processes contributing to the formation of colored cotton fibers (Feng et al., 2013). Notably, investigations into the pigmentation of green-colored cotton fibers have provided novel insights into the underlying mechanisms, presenting potential avenues for the development of cotton varieties with consistently stable green-colored fibers (Sun et al., 2019).

Colored cotton fiber quality is a subject of significant interest due to its distinct characteristics and potential applications in the textile industry. Several studies have investigated the relationship between fiber color and quality traits in colored cotton (Xiao et al., 2014; Zhang et al., 2017). Feng et al. (2015) highlighted a negative correlation between fiber color and quality traits, underscoring the importance of quantitative trait loci (QTL) analysis in colored cotton. Breeders have attempted to cross white cotton with colored cotton to improve the 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 cultivars (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).

The coloration and quality of cotton fibers are influenced not only by genetic factors but also by environmental and developmental factors. Environmental factors include elements such as light, temperature, water availability, and nutrients. For instance, light intensity and spectrum can affect flavonoid biosynthesis, leading to changes in fiber color. High light intensity may increase flavonoid production, contributing to the formation of darker-colored fibers. Temperature is another crucial factor; lower temperatures can suppress flavonoid synthesis, resulting in lighter-colored fibers. Water stress can alter metabolic processes in the plant, changing gene expression profiles and affecting fiber quality and color. The deficiency or excess of nutrients, particularly elements like nitrogen and phosphorus, can influence flavonoid biosynthetic pathways, thereby altering fiber pigmentation.

Developmental factors encompass the intrinsic processes that affect gene expression throughout the plant's life cycle. The developmental stages of cotton fibers can cause significant changes in gene expression profiles. For example, the high expression of certain flavonoid genes during the fiber elongation stage can directly impact fiber quality and color. Additionally, changes in gene expression associated with plant aging can lead to variations in fiber pigmentation. Considering these factors together allows for a better understanding of the color and quality

characteristics of cotton fibers and enables the optimization of these traits through breeding programs.

An undesirable mutant line arose during plant breeding efforts due to the presence of mutations that some common types of undesirable mutant cotton lines encountered in our cotton breeding, which was aimed to improve the fiber quality of green cotton. Variability in fiber color distribution combined with color variations in the cotton lint can result in yarn irregularities, including thick and thin spots, slubs, and color streaks. These inconsistencies compromise yarn quality and aesthetics in the textile industry. Undesirable gene mutations in F lines through generations in plant breeding can spontaneously occur and/or inadvertently persist propagated due to several factors. During the formation of the $F_2$ and subsequent generations, the segregation of alleles according to Mendelian genetics can result in the reappearance or increased frequency of undesirable traits carried in the parent lines (Mackay et al., 2020; Khatodia et al., 2016; Ookawa et al., 2010; Xu et al., 2015; Li et al., 2015). When desirable and undesirable genes are located close to each other on the same chromosome, it can be difficult to separate them through recombinant. This linkage can cause undesirable traits to persist in breeding lines (Didelot et al., 2010). Genetic recombination during meiosis can create new allele combinations, potentially leading to the expression of undesirable traits (Mercier et al., 2015). Of course, desirable mutations can be introduced into elite lines through backcrossing. This involves crossing a hybrid with one of its parents or an individual genetically similar to its parent to maintain the majority of the elite line's genome while incorporating the new trait. Generally, crossing plants produce F1 hybrids that exhibit heterosis, where undesirable mutations are masked by the presence of dominant desirable alleles from both parents (Boerma and Walker, 2005). In our study, green cotton with low fiber quality was crossed with white cotton belonging to high fiber quality to increase its fiber quality a little more. Then, green lines in each F generation were backcrossed with white fiber (male, recurrent) cotton (4 generations, after obtaining $F_1$). After the 5th year, the backcrossed lines selected were left to self-fertilize until the $F_{10}$ generation. After selections up to the $F_{10}$ generation, it was determined that one of the 4 green lines did not resemble the green cotton that was introduced into the hybrid as the parent and that different colored fibers were produced in the seeds of that line (Figure 1). Of course, we did not examine the genes that caused the color difference in the seeds of this line throughout its generations. The reason why this mutant green cotton line was chosen, was most likely because it given a greenish color and had higher fiber quality than other lines throughout generations up to the $F_{10}$ generation.

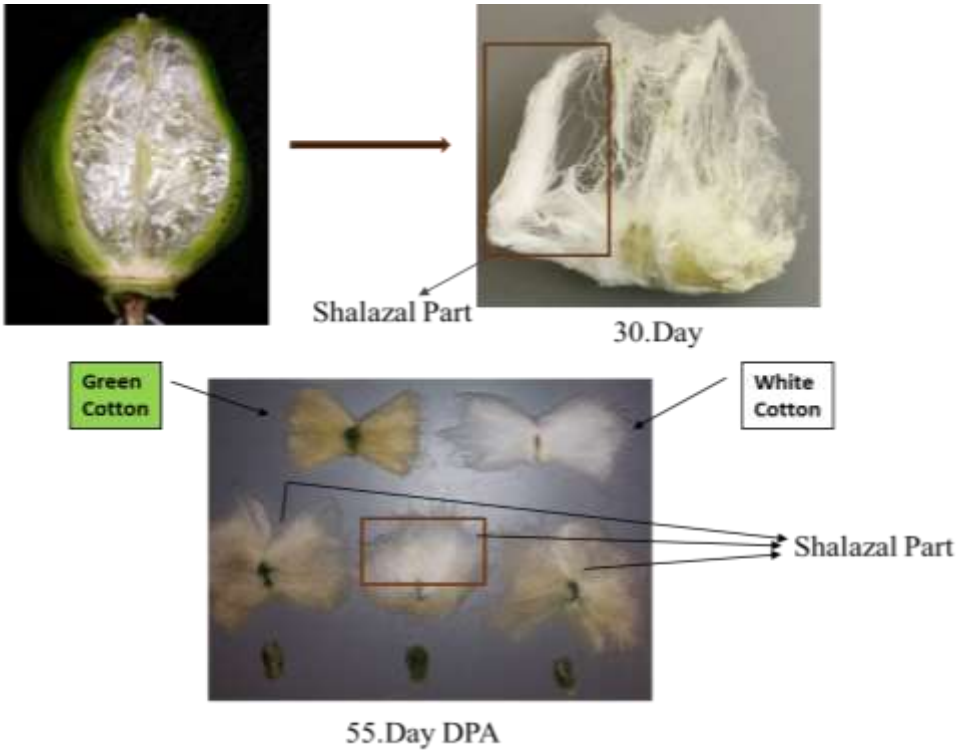


Figure 1. The mutation that occurred in fibers on a seed in the green cotton variety before/after the opening of the boll.

Şekil 1. Yeşil lif rengine sahip pamuk genotipinde koza açılmadan önce/açıldıktan sonra tohum üzerindeki liflerde meydana gelen mutasyon.

The occurrence of gene mutations in green cotton has been a subject of interest in recent research. Studies have demonstrated the feasibility of gene mutation in cotton using the CRISPR/Cas9 system, with a specific focus on genes involved in chloroplast development (Gao et al., 2017), furthermore, genome sequencing has provided novel insights into the molecular mechanisms underlying virescent mutation in cotton (Gao et al., 2021). Additionally, gene expression analyses have revealed divergent patterns between brown and green color cotton, particularly in the activation of genes encoding enzymes for the synthesis of caffeic acid derivatives, lignin, and lignan in the developing fibers of green cotton (Li et al., 2020). Moreover, the expression levels of certain structural genes related to flavonoid metabolism were found to be substantially higher in brown cotton lines compared to green cotton lines (Canavar & Rausher, 2021). These findings collectively indicate the presence of distinct genetic mechanisms underlying the pigmentation and metabolic processes in green cotton.

The aim of this research focuses on a mutation in the breeding green cotton line that was noticed during our previous study (Figure 1). In this mutation, it was observed that different color pigments were detected in the fibers of the same seed. In this context, the focus of the research is to determine the flavonoid gene expression in the fibers in the chalaza and micropyle parts of the mutation and to identify the point where the mutation starts. This scientific study is being conducted to understand the effects of this mutation at the molecular level and to develop strategies to mitigate these effects. Furthermore, identifying the specific point at which the mutation starts to develop is vital to maintain the quality of cotton fibers and ensure the sustainability of the textile industry.

## MATERIALS and METHODS

### Plant materials and sample collection

Advanced green fiber $F_{10}$ generation breeding line (Line number is 12) was produced by crossing white cotton (female; N84 cultivar) with green-colored cotton (male; green cultivar). An unregistered cultivar was used in this study: a green fiber cotton cultivar (Green) from Azerbaijan (Gürel, Akdemir, & Karadayı, 2001). After the $F_1$ generation, the Green line (female) was backcrossed by crossing with white cotton (male, recurrent) for 4 generations in an introgression program. After 4 years of backcrossing, green breeding lines were produced by selfing pollination up to $F_{10}$ generations. Seeds of the green cotton line were planted in three replicate pots (51-L volume, biological replicates), three seeds per pot, in the greenhouse of Duke University, Durham, NC, at temperatures of 30–32 °C during the day and 20–22 °C at night, and at 16 h of daily light.

Plants were thinned to one seedling per pot after germination. We ended up sampling three biological replicates (i.e., one plant from each of the three blocks) for gene expression analysis. Successive temporal samples for a biological replicate were taken from the same individual. According to Kim & Triplett (2001) and Zhang, Li, Wang, & Chee (2008), cotton fiber goes through four main developmental stages: fiber start (−3 to 5 d post-anthesis [DPA]), elongation (5–25 DPA), secondary cell wall production (15–45 DPA), and maturity or dehydration (45–70 DPA). On the day of anthesis (0 DPA), flowers and developing cotton bolls were tagged and labeled. In this study, cotton bolls and ovaries were harvested on the 30$^{th}$ day, because the beginning of light coloration can be seen at this stage after the day post-anthesis. Ovules were quickly dissected from the ovaries on ice. White fibers on chalaza and light green fibers on microphyll on a seed at the 30$^{th}$ DPA were quickly separated using forceps and also all parts of the seed such as cotyledon, epicotyl, hypocotyl, embryo, and radicle were removed to get only the seed coat, where the fiber is formed, was taken. Then immediately stored at −80 °C until RNA extraction.

### RNA extraction, cDNA synthesis, and semi-quantitative PCR

Total RNA was extracted using the Sigma Aldrich Spectrum plant total RNA kit from a part of the seed (white fiber, green fiber, and seed coat) for 30. DPA treatment. RNA was quantified spectrophotometrically using Nano Drop-1000 (Thermo Scientific). Because extraction of different tissues yielded different RNA concentrations, we diluted all extracts to the same concentration for subsequent analyses.

Double-strand complementary DNA (cDNA) was synthesized from total RNA using MultiScribe reverse transcriptase kits of Applied Biosystems using random primers according to the manufacturer's instructions. To synthesize double-strand cDNA from total RNA, the 2× Reverse Transcription Master Mix (RT) was prepared as follows: 10× RT buffer (2 µl), 10× RT random primers (2 µl), 25× dNTP mix (100 mM, 0.8 µl), MultiScribe reverse transcriptase 50 U $\mu l^{-1}$ (1 µl), and nuclease-free $H_2O$ (4.2 µl) were combined for a total reaction of 10 µl. Ten microliters of 2× RT master mix and 10 µl of the RNA sample were pipetted into each tube, pipetting up and down two times to mix on the ice box. The thermal cycler conditions (Bio-Rad) were 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C for ∞.

*4Cl*, 4-coumarate:CoA ligase; *Anr*, anthocyanidin reductase; *Ans*, anthocyanidin synthase; *C4h*, cinnamate 4-hydroxylase; *Chı*, chalcone isomerase; *Chs*, chalcone synthase; *Dfr*, dihydroflavonol 4-reductase; *F3h*, flavone 3-hydroxylase; *F3'h*, flavonoid 3'-hydroxylase; *F3'5'h*, flavonoid 3'5'-hydroxylase; *Lar*, leucoanthocyanidin

reductase; *Pal*, phenylalanine ammonia-lyase; *Ubq7*, ubiquitin gene; *Ufgt*, UDP-glucose: flavonoid 3-O-glucosyltransferase;

Table 1. Sequences of the primers used for real-time PCR analysis.
Çizelge 1. Real-time PCR analizi için kullanılan primerlerin dizileri.

| Genes | Primers | Sequences (5' to 3') | Accession number (Sequence ID) | Amplicon length (bp) |
|---|---|---|---|---|
| *GhPal* | PAL-F | AGCTTGGAACTGGGTTGTTG | XM_016878448 | 134 |
| | PAL-R | AGCACCATTCCAACCCTTTA | | |
| *GhC4h* | C4H-F | TTTGGGTCGTTTGGTACAGA | XM_016868687 | 137 |
| | C4H-R | AAAATTGCCTTGGCTTAGCA | | |
| *Gh4cl* | 4CL-F | AAGGTGCACTTTGTTCATGC | NM_001327242 | 148 |
| | 4CL-R | CGTTGCAATTTAAAAGCCAAAT | | |
| *GhChs* | CHS-F | CAGAGGAAGGACTGGAGTGG | XM_016823419 | 86 |
| | CHS-R | AGCAGCAACACTATGGAGCA | | |
| *GhChi* | CHI-F | ATGGAGTTTCTCCTCCAGCA | XM_016810061 | 85 |
| | CHI-R | GGTTTTTCACTGTCGACTCCA | | |
| *GhF3h* | F3H-F | CTGAAGAAGCTGGCCAAAGA | NM_001327494 | 99 |
| | F3H-R | TGCAAGGATTTCCTCCAATG | | |
| *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 | | |
| *GhUfgt* | UFGT-F | AAGCAGATAGCGGTGGAGAA | XM_016885447 | 118 |
| | UFGT-R | GCCTCCAACACCAAATTTTTC | | |
| *GhHiston3* | His3-F | CAGGAAATTGCCTTTCCAGA | XM_016885274 | 113 |
| | His3-R | GTATGCCTCTGCAGCTTCCT | | |
| *GhUBQ7* | UBQ7 | AAGCCCAAGAAGATCAAGCA | DQ116441 | 115 |
| | UBQ7 | CGCATTAGGGCACTCTTTTC | | |

The 13 flavonoid enzyme coding genes and two control genes examined in this study, along with their abbreviations used in this publication, are shown in Table 1. The polymerase chain reaction (PCR) primers used to amplify 85-to-154-bp fragments of the genes *Pal*, *C4h*, *4cl*, *Chs*, *Ch1* (XM_016810061), *F3h*, *F3'5'h*, *Dfr*, *Lar* and *Anr* were obtained from Xiao et al. (2014). The primers of F3'h (NM_0013227514), Ans (EF187442), His3 (XM_016885274), and UBQ7 (DQ116441) were designed using gene sequences obtained from the cotton genome by BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Canavar and Rausher 2021). Primers (forward and reverse) were synthesized by Integrated DNA Technologies. The PCR was performed using Phusion DNA polymerase (New England Biolabs) according to the manufacturer's instructions. The amplification protocol consisted of 40 cycles of

initial denaturation at 94 °C for 3.5 min followed by 63 °C for 30 s and 72 °C for 2 min (Bio-Rad MyCycler thermal cycler PCR). The PCR products were identified electrophoretically by running on a 1% agarose gel containing SybrRsafe (8.0 µl, Invitrogen). Gels were viewed with a ChemiDoc MP imaging system 170-8280 (Bio-Rad).

### Quantitative RT-PCR analysis of gene expression levels

Complementary DNA was diluted to 2.0 ng µl for use in quantitative PCR (qPCR) reactions. Quantitative reverse-transcription PCR was performed in a total volume of 20 µl with 10 µl DyNAmo HS SYBR Green (Thermo Scientific), 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), 2.0 µl cDNA template, and 6.0 µl double-distilled $H_2O$ (total volume of 20 µl). Reactions were run on a Roche LightCycler thermocycler according to the manufacturer's instructions. The PCR amplification used a 15-minute initial denaturation step at 95 °C, followed by 45 cycles with a 10-s denaturing step at 94 °C and an annealing step for 30 s at 58 °C. All experiments involved three biological replicates for each cotton genotype and stage, and each biological replicate was performed in duplicate (technical replicate).

The threshold cycle (Ct) values were reported as a mean for each replicate and the fold changes of transcription levels of target genes relative to the reference genes (UBQ7 [ubiquitin gene] and Histon 3) were analyzed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The Ct values were used to calculate ΔCt and ΔΔCt values (Balasubramanian et al., 2016). The ΔCt was calculated by subtracting the Ct values of UBQ7 and Histone 3 (two control genes) from the Ct values of the flavonoid synthesis gene within the same stage, whereas ΔΔCt was calculated by subtracting the ΔCt values of fiber stages at 30 DPA. Hence, the results were presented as relative expression of flavonoid genes in different stages normalized to that for the seed coat. Normalized fold expression among the stage of 0 DPA of the samples was calculated with seed coat as a standard. The possibility of contamination by genomic DNA was ruled out by running the PCR reactions on isolated RNA.

### Statistical Analysis

To determine whether gene expression levels were different in 2 different fiber samples taken from the chalazal and microphyll of seed, expression levels, and analysis of variance (ANOVA) were performed in the "JMP®, Version <16.0>. SAS Institute Inc., Cary, NC, 1989–2023" statistical program. The difference between the means was determined by LSD Student-t (0.05).

## RESULTS and DISCUSSION

When the results of the quantitative RT-PCR analysis of gene expression levels were examined, it was also observed that the genes were present in all three seed parts (White fiber on chalaza and green fiber on microphyll and seed coat) (Figure 2). The expression levels of 13 flavonoid genes (*Pal, C4h, 4Cl, Chs, F3'5'h, F3'h, F3h, Lar, Dfr, Ans, Anr,* and *Ufgt*) were statistically different in parts of the seeds (green part, white part and seed coat) (Table2).

When the averages were compared according to the regions where the samples were taken, it was determined that the highest expression level of the *Pal* gene was observed in green fiber, even though it was in the same statistical group as white fiber. The lowest expression of *Pal* was found in the seed coat. The *C4h* gene expression was statistically highest in white fiber and lowest in the seed coat. When *4Cl* gene expression was evaluated statistically, no difference was found between green fiber and seed coat, but white fiber had a higher expression level. It was observed that the gene was synthesized mostly in the seed coat and no difference was found in the samples taken according to fiber color. *F3'5'h* flavonoid gene expression was found to be significantly higher in white fiber samples and 46.5% more than in green fiber samples. No difference was found in *F3'h* flavonoid synthesis according to the sampled seed regions. *F3h* gene synthesis was highest in the white fiber section, while green fiber and seed coat were statistically in the same group. However, it was expressed 48.1% more in the white fiber section. When *Lar* flavonoid gene expression averages were examined, it was seen that the green fiber section came to the fore. It was 59.67% more in the green fiber section than in the white fiber section. While *Dfr* expression was found in both colored fiber regions, it was the least in the seed coat (Figure 2).

The results of our expression analyses revealed several major patterns. First, genes of the anthocyanin pathway, are expressed at detectable levels in fibers on the seed of green cotton line with green and white. All parts of the seed and color types thus should be capable of producing anthocyanins if anthocyanidins are produced. Several studies have underscored the pivotal role of flavonoid genes in cotton fiber development (Peng et al., 2020). Tan et al. (2013) revealed that the dominance of flavonoid gene expression during fiber elongation stages significantly impacts fiber development, particularly through the flavonoid naringenin's retardation effect. Canavar & Rausher (2021), further elucidated the importance of these genes by demonstrating their high expression levels during fiber elongation, suggesting their substantial involvement in this developmental phase. Gong et al. (2014) expanded, proposing a potential link between highly expressed flavonoid genes during fiber elongation and fiber quality,

highlighting their significance in cotton fiber development and quality regulation. Collectively, these findings underscore the multifaceted roles of flavonoid genes, not only in pigment formation but also in various aspects of fiber development and elongation, emphasizing their importance in regulating fiber quality and coloration across different developmental stages.

Table 2. Variance analysis of Relative expression levels taken from white part, green part, and seed coat flavonoid genes at stages of 30 DPA fiber development. *4Cl*, 4-coumarate: CoA ligase; *Anr*, anthocyanidin reductase; *Ans*, anthocyanidin synthase; *C4h*, cinnamate 4-hydroxylase; *Chı*, chalcone isomerase; *Chs*, chalcone synthase; *Dfr*, dihydroflavonol 4-reductase; *F3h*, flavone 3-hydroxylase; *F3'h*, flavonoid 3'-hydroxylase; *F3'5'h*, flavonoid 3'5'-hydroxylase; *Lar*, leucoanthocyanidin reductase; *Pal*, phenylalanine ammonia-lyase; *Ubq7*, ubiquitin gene; *Ufgt*, UDP-glucose: flavonoid 3-O-glucosyltransferase; ** P-value is less than 0.01 ($p < 0.01$).
* P-value is generally less than 0.05 ($p < 0.05$).

Çizelge 2. Beyaz lif, yeşil lif ve tohum kabuğunda, 30 DPA lif gelişim aşamasında elde edilen flavonoid genlerinin ifade seviyelerinin varyans analizi. . *4Cl*, 4-coumarate: CoA ligase; *Anr*, anthocyanidin reductase; *Ans*, anthocyanidin synthase; *C4h*, cinnamate 4-hydroxylase; *Chı*, chalcone isomerase; *Chs*, chalcone synthase; *Dfr*, dihydroflavonol 4-reductase; *F3h*, flavone 3-hydroxylase; *F3'h*, flavonoid 3'-hydroxylase; *F3'5'h*, flavonoid 3'5'-hydroxylase; *Lar*, leucoanthocyanidin reductase; *Pal*, phenylalanine ammonia-lyase; *Ubq7*, ubiquitin gene; *Ufgt*, UDP-glucose: flavonoid 3-O-glucosyltransferase; ** P-value 0.01 ($p < 0.01$).* P-value 0.05 ($p < 0.05$).

| Source | Df | Mean Square | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PAL | C4H | 4CL | CHS | CHI | F3'5'H | F3'H | F3H | LAR | DFR | ANS | ANR | UFGT |
| Part of Seed | 2 | 0.677*** | 3.08*** | 8.23*** | 0.168*** | 0.03** | 0.363*** | 0.0066 | 0.563** | 31.18*** | 64.77*** | 0.078** | 1.01*** | 0.18* |
| Replication | 2 | 0.0008 | 0.01 | 0.009 | 0.0008 | 0.002 | 0.016 | 0.0057 | 0.0096 | 0.0006 | 0.367 | 0.01 | 0.0135 | 0.588 |
| Error | 4 | 0.008 | 0.005 | 0.021 | 0.0009 | 0.003 | 0.004 | 0.0015 | 0.012 | 0.196 | 0.244 | 0.0058 | 0.0067 | 0.018 |

Table 3. qPCR results of expression levels of 13 genes related to flavonoid genes in three different regions of cotton seeds harvested on the 30th day, Lsd student-t test (0.05).

Çizelge 3. 30. günde hasat edilen pamuk tohumlarının üç farklı bölgesinde flavonoid genlerle ilgili 13 genin ifade düzeylerinin qPCR sonuçları, Lsd student-t testi (0.05).

| Genes | White fibre on chazal | Green fibre on microphyll | Seed Coat |
|---|---|---|---|
| *PAL* | 1.68 A | 1.91 A | 1.00 B |
| *C4H* | 2.96 A | 1.52 B | 1.00 C |
| *4CL* | 4.02 A | 1.32 B | 1.00 B |
| *CHS* | 0.53 C | 0.78 B | 1.00 A |
| *CHI* | 0.80 B | 0.89 AB | 1.00 A |
| *F3'5'H* | 1.29 A | 0.60 C | 1.00 B |
| *F3'H* | 0.94 ns | 1.06 ns | 1.00 ns |
| *F3H* | 1.58 A | 0.76 B | 1.00 B |
| *LAR* | 4.44 B | 7.44 A | 1.00 C |
| *DFR* | 8.58 A | 9.45 A | 1.00 B |
| *ANS* | 1.20 A | 0.88 B | 1.00 C |
| *ANR* | 2.16 A | 1.53 B | 1.00 C |
| *UFGT* | 1.49 A | 1.30 AB | 1.00 B |

In addition, to investigate the relationship between flavonoid biosynthesis levels and cotton fiber properties, Jia et al. (2022) conducted a study that identified significant correlations between differentially expressed genes related to flavonoid biosynthesis, such as *Pal, 4Cl, C4h, F3h, F3'h*, and F3'5'h, and total flavonoid content. This highlights the regulatory importance of these genes in flavonoid production, which can impact the properties of cotton fibers. Additionally, Wang et al. (2021) explored the phenylpropanoid biosynthesis pathway in bamboo, emphasizing the roles of *Pal, 4Cl*, and *C4h* genes in directing phenylpropanoid intermediates towards lignin or flavonoid biosynthesis. Understanding these mechanisms is crucial for comprehending how different genes influence the synthesis of compounds that contribute to fiber properties in cotton. By synthesizing insights from studies on flavonoid biosynthesis pathways and gene expression related to cotton fiber properties, a comprehensive understanding of how genes like *C4h* and *4Cl* influence flavonoid levels and, consequently, fiber characteristics can be achieved. These findings are essential for unraveling the intricate relationship between gene expression, flavonoid biosynthesis, and the quality of cotton fibers.

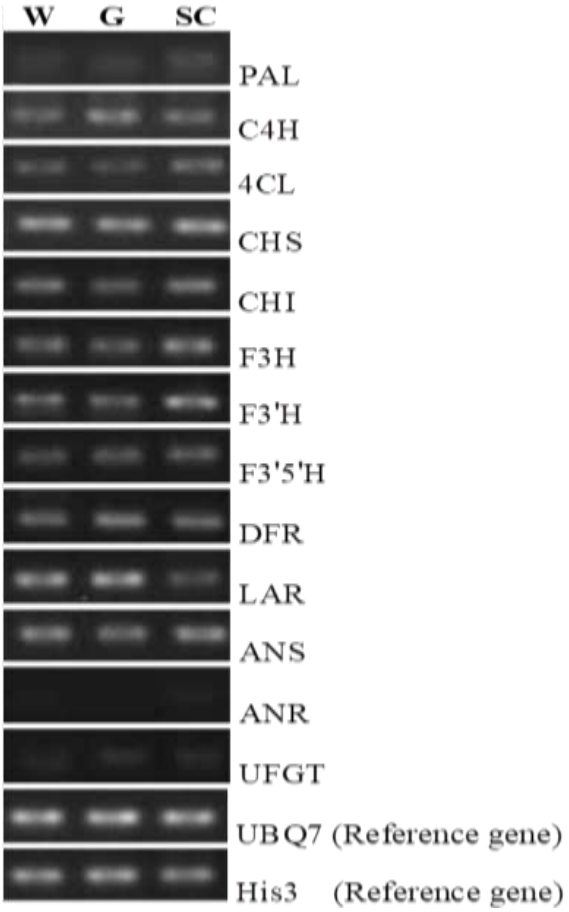


Figure 2. The results of the quantitative RT-PCR analysis of gene expression (the gel image of one replication). W: White cotton, G: Green cotton, SC: Seed coat.

Şekil 2. Gen ifadesinin kantitatif RT-PCR analizinin sonuçları (bir replikasyonun jel görüntüsü). W: Beyaz pamuk, G: Yeşil pamuk, SC: Tohum kabuğu.

The relationship between suberin and naringenin in green cotton fibers can be elucidated through a combination of studies. Suberin, a key component of the cell wall, has been identified as a monomer of glycerol (Moire et al., 1999). It is insoluble and lipophilic, contributing to the structural integrity of the cell wall (Moire et al., 1999). Studies have shown that suberin deposition can be altered by specific inhibitors, affecting the dimensions of suberin lamellae in green cotton fibers (Schmutz et al., 1996). On the other hand, naringenin, a flavonoid, has been found to impact fiber development in cotton. It can significantly retard fiber development, indicating its regulatory role in the growth processes of cotton fibers (Tan et al., 2013). Additionally, naringenin has been associated with pigment formation variations in cotton fibers, with its upregulation observed in certain cotton lines (Lv et al., 2023). The presence of suberin in green cotton fibers has been confirmed through ultrastructural and chemical analyses, highlighting its suberized nature (Yatsu et al., 1983).

The reduced expression of the *Chs* and *Chı* genes in white fibers, leading to decreased pigment formation, suggests a potential lack of suberin formation due to mutations affecting these genes. Notably, one of the *Chı* genes directly synthesizes *F3'5'H,* which is associated with suberin formation (Graça, 2015). Suberin is a lipophilic macromolecule found in specialized plant cell walls, providing insulation and protection where needed (Graça, 2015). Mutations affecting the expression of the *Chı* gene associated with *F3'5'h* synthesis may disrupt suberin formation, potentially influencing the color and quality of cotton fibers. Because Also, Few reports on the formation of the suberin layer of green cotton fiber suggest that the color of the fiber cannot be expressed when the synthesis of the suberin substances is inhibited during the fiber development (Schmutz et al 1993). The observation of lower expression levels of these genes in white fibers at 30 DPA highlights the importance of gene regulation in fiber development and coloration. The study's focus on a later developmental stage provides insights into the potential impact of mutations occurring during the peak synthesis period of these genes on their later expression levels (Fernandes et al., 2017). Understanding the molecular mechanisms underlying gene expression dynamics in colored cotton fibers is crucial for unraveling the genetic basis of fiber traits and color inheritance.

Furthermore, the link between gene expression, suberin formation, and pigment synthesis underscores the intricate regulatory networks governing fiber development. The study's findings contribute to the broader understanding of gene regulation in cotton fibers and shed light on the potential role of mutations in modulating gene expression and fiber characteristics.

Chemical analysis has further revealed the composition of suberin layers in green fibers, with a significant proportion of monomers being 22-hydroxydocosanoic acid (Sun et al., 2019). This underscores the importance of suberin in the structural composition of green cotton fibers. In the study conducted by Canavar and Rausher (2021), it was stated that *C4h* and *4Cl* flavonoid biosynthesis were at the highest, medium, and lowest levels, respectively, in cotton with brown, green, and white fiber colors. It was also observed that the synthesis of these two flavonoids was a part of the subuerin mechanism. In the same study, it was revealed that the synthesis of F3h, F3'h, and F3'5' was highest in colored cotton (due to the naringenin mechanism). However, in our study, unlike expected, an increase in the synthesis of these flavonoids in the white fiber region was observed. In Figure 3, this study, the pathway formed as a result and the synthesis rates of flavonoids in white fiber, green fiber, and seed coat are given. These results suggest that the mutation may have occurred at one or both of the two points where these genes are synthesized.

The potential effects of gene expression differences on fiber elongation in cotton, particularly concerning genes such as *4CL* (4-coumarate-CoA ligase), *C4H* (cinnamate-4-hydroxylase), *F3H* (flavanone-3-hydroxylase), *F3'H* (flavonoid 3'-hydroxylase), and *F3'5'H* (flavonoid 3',5'-hydroxylase), are significant in understanding the molecular mechanisms underlying fiber development. These genes are crucial in the flavonoid biosynthesis pathway, which has been linked to various aspects of plant growth, including fiber elongation. Research indicates that the expression of genes involved in flavonoid biosynthesis, such as C4H and F3H, is associated with the regulation of fiber elongation in cotton. For instance, (Yoo & Wendel, 2014) highlighted that several genes related to fiber elongation, including those in the flavonoid biosynthesis pathway, were over-expressed in domesticated cotton varieties compared to their wild counterparts. This suggests that enhanced expression of these genes may contribute to improved fiber characteristics, including elongation and quality (Yoo & Wendel, 2014).

Moreover, the enzymes encoded by F3H, F3'H, and F3'5'H are integral to the accumulation of flavonoids, which can influence cell wall properties and, consequently, fiber elongation. The synergistic action of these enzymes in the biosynthesis of anthocyanins and other flavonoids has been shown to affect the structural integrity of the cell wall, thereby impacting fiber growth. However, the reference provided Wang et al. (2021) does not directly support the claim regarding fiber elongation in cotton, as it focuses on anthocyanin regulatory networks in walnuts (Wang et al., 2021). Therefore, this citation has been removed. Additionally, the role of ethylene in promoting fiber elongation has been documented, with genes such as GhXB38D being implicated in this process. Ethylene signaling enhances the expression of genes involved in cytoskeleton construction and cell wall synthesis, which are essential for fiber elongation. Song (2023) discusses how ethylene treatment promotes fiber cell elongation in cotton by increasing the expression of relevant genes (Song, 2023). Furthermore, the expression of expansin genes, which are known to facilitate cell wall loosening, is also influenced by the flavonoid biosynthesis pathway. Expansins work in concert with the structural components of the cell wall to promote elongation. The identification of expansin genes that are co-expressed with flavonoid biosynthesis genes underscores the interconnectedness of these pathways in regulating fiber elongation. Lv et al. (2020) provide insights into the role of expansin genes in fiber cell growth, indicating their importance during the elongation stages of cotton fiber development (Lv et al., 2020).

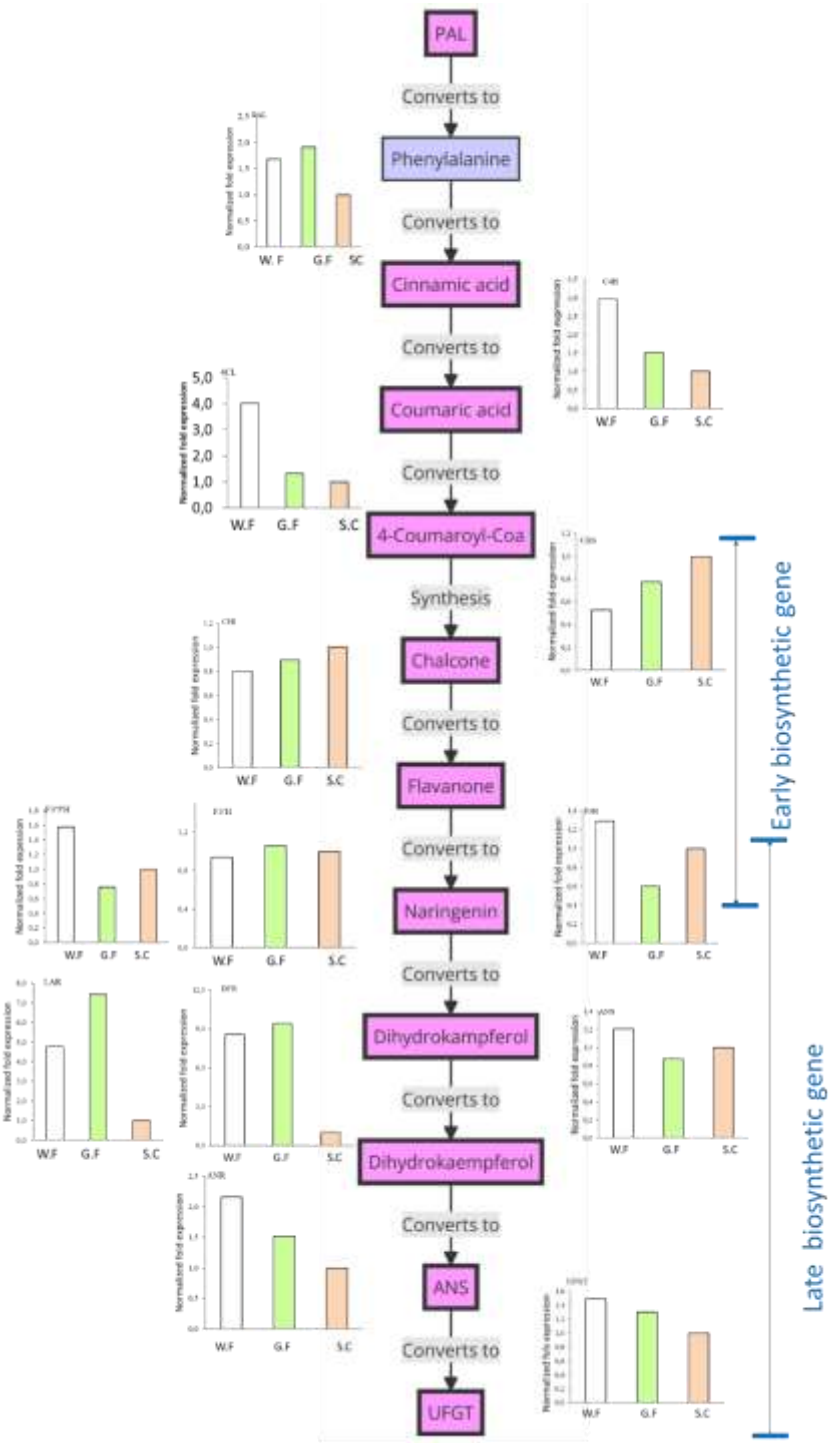


**Figure 3:** Diagram of the flavonoid pathway in green cotton fibers and expression patterns of flavonoid-related genes.Relative expression of flavonoid genes during fiber development in green cultivar. UBQ7 and His3 were used as internal controls to normalize the expression data. The fold expression of each gene was normalized to the expression of that gene in green cotton at the stage of 55 DPA (days post-anthesis). The significance of differences at a given stage of flavonoid genes is seen. Enzyme abbreviations: 4Cl, 4-coumarate:CoA ligase; Anr, anthocyanidin reductase; C4h, cinnamate 4-hydroxylase; Chi, chalcone isomerase; Chs, chalcone synthase; Dfr, dihydroflavonol 4-reductase; F3h, flavone 3-hydroxylase; F3'h, flavonoid 3'-hydroxylase; F3'5'h, flavonoid 3'5'-hydroxylase; Lar, leucoanthocyanidin reductase; Pal, phenylalanine ammonia-lyase; UBQ7, ubiquitin gene; Ufgt, UDP-glucose:flavonoid 3-glucosyltransferase.

**Figure 3:** Yeşil pamuk liflerinde flavonoid yolunun diyagramı ve flavonoid ile ilişkili genlerin ifade desenleri. Yeşil çeşitte lif gelişimi sırasında flavonoid genlerinin göreceli ifadesi. İfade verilerini normalize etmek için UBQ7 ve His3 iç kontrol genleri olarak kullanıldı. Her genin katlanma ifadesi, 55 DPA (çiçeklenme sonrası gün) aşamasındaki yeşil pamuktaki ifadesine göre normalize edildi. Enzim kısaltmaları: 4Cl, 4-coumarate:CoA ligase; Anr, anthocyanidin reductase; C4h, cinnamate 4-hydroxylase; Chi, chalcone isomerase; Chs, chalcone synthase; Dfr, dihydroflavonol 4-reductase; F3h, flavone 3-hydroxylase; F3'h, flavonoid 3'-hydroxylase; F3'5'h, flavonoid 3'5'-hydroxylase; Lar, leucoanthocyanidin reductase; Pal, phenylalanine ammonia-lyase; UBQ7, ubiquitin gene; Ufgt, UDP-glucose:flavonoid 3-glucosyltransferase.

The findings of the study regarding gene expression differences in flavonoid biosynthesis pathways in cotton have substantial implications for cotton breeding programs. Understanding the genetic mechanisms that influence fiber color and quality can facilitate targeted genetic modifications, ultimately leading to the development of cotton varieties with desirable traits. Specifically, the manipulation of flavonoid gene expression can be harnessed to enhance fiber color and quality, which are critical attributes in the textile industry (Xhing et al., 2022).

Recent research has demonstrated that specific genes involved in flavonoid biosynthesis play a pivotal role in determining fiber characteristics. For instance, the identification of key regulatory genes can inform breeding strategies aimed at improving fiber length and strength, which are essential for high-quality cotton production (Lv et al., 2023) By leveraging genetic engineering techniques such as CRISPR/Cas9, breeders can precisely edit these genes to enhance fiber properties, thereby accelerating the breeding process and increasing the efficiency of developing superior cotton varieties (Ullah et al., 2012).

Moreover, the integration of findings related to gene expression into existing breeding programs can enhance genetic diversity within cotton populations. This is particularly important given the current trend of genetic uniformity resulting from the monoculture of a few successful cultivars (Ullah et al., 2012). By introducing genetic variability through the targeted manipulation of flavonoid biosynthesis genes, breeders can create cotton varieties that not only exhibit improved fiber quality but also demonstrate greater resilience to environmental stressors such as drought and salinity (Zhu et al., 2013). This is crucial in the context of climate change, where cotton crops are increasingly exposed to abiotic stresses that can adversely affect yield and quality (Hassan et al., 2020). In conclusion, the insights gained from this study provide a robust foundation for advancing cotton breeding programs. By focusing on the genetic underpinnings of fiber color and quality through the modulation of flavonoid biosynthesis pathways, breeders can develop high-quality, diverse-colored cotton varieties that meet the evolving demands of the textile industry. The application of modern genetic tools will further enhance the ability to produce cotton that is not only aesthetically appealing but also resilient to the challenges posed by environmental stressors.

In our study, we examined gene expression levels in fibers on the 30th day post-anthesis (DPA). Literature typically indicates that these genes peak in synthesis between DPA 5 and 14, shortly after flowering. This is supported by numerous studies, including those by Lacape et al. (2012), Gilbert et al. (2013), and Canavar and Rausher (2021). However, we conducted our analysis at 30 DPA due to the visibility of colored bolls at this stage. It is possible that mutations occur much earlier, during the peak synthesis period of these genes, affecting their later expression.

## CONCLUSION

The results of this study prove that a mutation in the flavonoid biosynthesis pathway has impacted the production of key enzymes such as *4Cl/C4h* and *F3h, F3'h, and F3'5'h*. This mutation will undoubtedly lead to the formation of differently colored fibers on a single seed, presenting significant challenges for breeding programs in terms of fiber consistency and readability. The low expression of the *CHS* and *CHI* genes, which is attributed to a mutation at the beginning of the phenylalanine pathway, is a crucial finding. This mutation prevents the formation of green color in the white fibers on the chalazal part, due to the reduction in synthesis of the *CHI* gene. These insights are invaluable for breeding programs, as they reveal the genetic mutations affecting fiber color.The study emphasizes the necessity for further research to investigate the practical applications of these findings in cotton breeding. Notably, certain genes demonstrated preferential expression in specific seed regions, indicating differential regulation of flavonoid synthesis. These findings indicate that mutations in flavonoid biosynthesis pathways may contribute to variations in fiber color and quality in cotton, offering new avenues for enhancing cotton fiber diversity and quality through targeted breeding strategies.

### Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

### Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

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