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## ORIGINAL RESEARCH PAPER

# Effects of light on in vitro fiber development and flavonoid biosynthesis in green cotton (*Gossypium hirsutum*)

Senhe Qian<sup>1,2</sup>, Liang Hong<sup>1</sup>, Yongping Cai<sup>1</sup>, Junshan Gao<sup>1</sup>, Yi Lin<sup>1\*</sup><sup>1</sup> College of Life Science, Anhui Agricultural University, 130 West Chang jiang Road, HeFei, Anhui 230036, China<sup>2</sup> Department of Biochemistry, Anhui Polytechnic University, Beijing Road, Wuhu, Anhui 241000, China\* Corresponding author. Email: [linyi3000@163.com](mailto:linyi3000@163.com)**Abstract**

As an environmental factor, light influences the physiological functions and secondary metabolism of plants. However, the role of light in cotton fiber development and pigment biosynthesis has not yet been thoroughly explored. In this study, ovules of green cotton were cultured in vitro under dark and light conditions, and fiber and ovule growth parameters as well as fiber carbohydrate and cellulose contents and the expression of genes related to fiber development were investigated to elucidate the effect of light on fiber development. In addition, to investigate the effect of light on fiber pigment biosynthesis, the fiber flavonoid content and related gene expression were determined. The results demonstrated that the fiber length and the expression levels of fiber elongation genes under light culture were significantly lower than under dark culture, however, the ovule and fiber weight were significantly higher than under dark culture. The fiber developed under light culture had higher carbohydrate concentrations and carbohydrate transformation rate than under dark culture. Additionally, light culture exhibited higher cellulose contents and expression levels of cellulose biosynthesis genes compared with dark culture. In contrast, the pattern of the effect of light on flavonoid biosynthesis differed from that for cellulose biosynthesis. At 10 DAC (days after culture) and 20 DAC, the flavonoid contents and the expression levels of genes related to flavonoid biosynthesis were lower than under dark culture. However, the flavonoid contents and gene expression levels observed at 30 DAC and 40 DAC were higher in the light culture than in the dark culture. These results suggested that light hindered fiber elongation, but promoted carbohydrate accumulation and carbohydrate transformation, which resulted in fiber weight gain and increased cellulose accumulation in fibers. In addition, light inhibited flavonoid biosynthesis at early stage of fiber development, but promoted it at later stages. These findings provide the basis for intensive study of fiber development and flavonoids biosynthesis in green cotton.

**Keywords**

light; in vitro culture; green cotton; fiber development; flavonoid biosynthesis; gene expression

**Introduction**

Colored cotton contains naturally pigmented fiber that grows in shades of green and brown. Although there are many drawbacks to colored cotton, such as its short fiber length, poor fiber strength, low cellulose contents, and uneven color [1], colored cotton remains widely accepted by people because it is environmentally friendly and

harmless to human health [2]. Green cotton is a kind of naturally colored cotton that accumulates green pigment in its fiber, and its fiber quality is worse than that of brown cotton.

Fiber development in green cotton is similar to that in white cotton, which is influenced by the expression of fiber elongation genes and cellulose biosynthesis genes [3,4]. Fiber differentiation is related to the expression of the *Expansin* gene (*GhEXP*) and the sucrose synthase gene (*GhSuSy*) [5]. The expansin protein can regulate the expansion of the cell wall, which can break the hydrogen bonds between cellulose microfibrils and hemicelluloses, promote polymer sliding, and cause cell wall stretching [6]. *GhEXP1*, which is related to fiber elongation and specific expression during fiber elongation, is a member of the *Expansin* gene family [7]. Sucrose synthase can catalyze the transformation of sucrose into fructose and uridine diphosphate glucose (UDPG), which can increase intracellular osmotic stress and promote the initiation of ovule epidermal cells [8]. Furthermore, UDPG can be transported to the cellulose synthase catalytic subunit and is mainly used for the biosynthesis of cellulose [9].  $\beta$ -1,3-glucanase can degrade  $\beta$ -1,3-dextran and transport sugar molecules to the non-reducing end of cellulose to synthesize fibrilla [10]. In addition, cellulose synthase can catalyze UDPG to form  $\beta$ -1,4-glucose polymers (cellulose); *GhCelA1* and *GhCelA2* are two key genes involved in the process of cellulose biosynthesis [11].

The fiber pigment of green cotton is one of the important features that differentiates green cotton from white cotton. The composition of fiber pigment in green is complicated [12–14], but recent research has demonstrated that flavonoids are the main components [15]. Flavonoids are synthesized via the pathway of phenyl propanoic acid metabolism, in which phenylalanine ammonia lyase (*PAL*) is the first enzyme to catalyze a reaction. Chalcone synthase (*CHS*), flavanone-3-hydroxylase (*F3H*), dihydroflavonol reductase (*DFR*), anthocyanidin synthase (*ANS*), and anthocyanidin reductase (*ANR*) are the major intermediate enzymes in flavonoid biosynthesis [16]. Therefore, the expression levels of these genes will influence the flavonoid contents of green cotton fiber directly.

Light is a predominant energy source for plant photosynthesis and also an important signal for plant growth and development [17]. It is now known that plants contain photoreceptors, such as phototropins, cryptochromes, phytochromes, among others. Absorption of light by these photoreceptors stimulates signaling pathway that ultimately influences the physiology of a plant [18]. Previous studies showed that light affects fiber elongation, cellulose accumulation, fiber development genes expression, fiber quality formation, and fiber color in field planted cotton [5,19,20]. The effects of light on plant growth and development are mainly because light can affect the expression of photosynthetic genes, which affect the biosynthesis of carbohydrate and secondary metabolites in plant [21,22]. At present, the reasons for poor fiber quality in green cotton are being unveiled, and most research on the light influencing fiber development focuses on field plantings of cotton (such as boll shading) [5,20,23]. However, the fiber of green cotton grows in the dark environment because of the boll shell, whether the light has an effect on its development and pigment biosynthesis is still unknown. In this study, a green fiber cultivar (Lvzumian No. 1) was used to measure the effects of light on fiber growth and development in vitro and on the gene expression of key enzymes involved in cellulose and flavonoid biosynthesis.

## Material and methods

### Plant material

The green cotton genotype Lvzumian No. 1 (*Gossypium hirsutum*) was cultivated at the experimental farm of Anhui Agricultural University, in Hefei, China. Conventional practices of cultivation were used throughout the growth period. The cotton flowers on the upper branches of the plants were tagged on the day of anthesis, and the flowers on the 1st day post-anthesis (DPA) were harvested between 8 a.m. and 10 a.m.

## Ovule culture

The harvested ovaries were sterilized in 75% ethanol and 0.1% mercuric chloride for 2 min and were then rinsed with sterile distilled water 5 times. The ovules were subsequently carefully dissected from these ovaries under sterile conditions and immediately floated on the BT medium in a 100-mL culture bottle [24]. The BT was modified from the Murashige and Skoog medium [25]. Nitrogen was in the form of  $\text{KNO}_3$  instead of  $\text{NH}_4\text{NO}_3$ , and a mixture of glucose and fructose was substituted for sucrose. Plant growth regulators (IAA  $5.0 \mu\text{mol L}^{-1}$  and  $\text{GA}_3$   $1.0 \mu\text{mol L}^{-1}$ ) were filter-sterilized and added to the medium after autoclaving. Each culture bottle contained 30 mL of liquid medium and approximately 20 ovules. The cultures were divided into two parts. One part was cultured in the light at  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  (which was found optimal in the preliminary experiments) at a constant  $30^\circ\text{C}$  under a 12-h photoperiod, and the other was cultured in the dark at the same temperature. The ovules were collected at 10, 20, 30, and 40 days after culture (DAC). About 40 ovules were used to determine ovule and fiber growth parameters, and the remainder were stored at  $-70^\circ\text{C}$  prior to other analyses.

## Determination of fiber and ovule growth parameters

Fiber length was measured using the water washing method [26]. The ovules were boiled in water for 2 min to detach fibers, and after cooling, the ovules were placed on a slide and rinsed with purified water. The fibers were straightened, and fiber length was measured using a ruler. To determine the fresh weight of the ovules and fibers, the total weight of the ovules and fibers was estimated, and after removal of the fibers, the remaining sample was weighed to obtain the ovule fresh weight. Then, the total weight minus the ovule fresh weight was considered to be the fiber fresh weight. Three replicates were included in each assay, and each replicate was composed of six ovules.

## Determination of carbohydrate and cellulose contents

Fiber samples (0.25 g, dry weight) were placed in test tubes, and 5 mL of distilled water was added. The fibers were distilled in boiling water 4 times, the first time for 30 min and then for 15 min. The fiber solvent was then filtered, and distilled water was added to a final volume of 25 mL. Carbohydrate contents were determined using the anthrone method [27].

Cellulose contents were measured in three biological replicates using the method of Viles and Silverman (1949) [28]. The residue obtained from extracting the soluble carbohydrates was dried in the oven to a constant fiber weight. The residue was hydrolyzed with 80% (v/v)  $\text{H}_2\text{SO}_4$  for 30 min in cold water and then diluted with distilled water. After filtering, distilled water was added to a final volume of 100 mL. The post-hydrolysis sugar content was determined in accordance with the soluble carbohydrate method.

## Determination of total flavonoid contents

Methanol (20 mL) was added to a 0.25 g (dry weight) of fiber, and the fibers were extracted for 1.5 h via the reflux method in a  $75^\circ\text{C}$  water bath. The fiber solvent was filtered using Whatman #1 filter paper, and methanol solution was added to a volume of 25 mL. Flavonoid contents were measured using the method of Wang et al. [29]. The sample solvent (1 mL) was placed in a flask, and 30% (v/v) ethanol was added to a volume of 5 mL. Then, 0.3 mL of  $\text{NaNO}_2$  5% (w/v) was added, and the mixture was mixed thoroughly and left on the bench for 5 min before 0.3 mL of 10% (w/v)  $\text{Al}(\text{NO}_3)_3$  was added. Six minutes later, 2 mL of 1 N NaOH was added, followed by 30% (v/v) ethanol to a final volume of 10 mL. The absorbance was measured at 510

nm after the sample had been incubated on the bench for 10 min. Three biological replicates were performed.

### RNA extraction and qPCR assays

Total RNA was isolated according to the instructions of the RNAPrep Pure Plant Kit (TIANGEN, DP441, China). When total RNA was isolated, the  $A_{260}/A_{280}$  ratio was measured and RNA gel electrophoresis was conducted to assess RNA quality (Fig. 1). The reaction system for removing genomic DNA contained 2.0  $\mu\text{L}$  of 5 $\times$  gDNA eraser buffer, 1.0  $\mu\text{L}$  of gDNA eraser, 1.0  $\mu\text{L}$  of total RNA and 6.0  $\mu\text{L}$  of RNase-free  $\text{dH}_2\text{O}$ , and the reaction was carried out at 42°C for 2 min. The obtained RNA was reverse-transcribed into cDNA using the M-MLV RTase cDNA Synthesis Kit (Takara, China). The total RNA was quantified to 1000 ng when conducting a reverse-transcription for 20  $\mu\text{L}$  reaction system. qPCR was performed using the ABI 7500 Real-Time PCR System. The 20- $\mu\text{L}$  reaction system contained 10  $\mu\text{L}$  of SYBR Green, 0.8  $\mu\text{L}$  of forward primers, 0.8  $\mu\text{L}$  of reverse primers, 0.4  $\mu\text{L}$  of ROX reference dye, 2  $\mu\text{L}$  of cDNA, and 6  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . The amplification program was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The  $2^{-\Delta\Delta\text{CT}}$  method was used to calculate the difference of gene expression. The  $\Delta\Delta\text{C}_\text{T}$  was calculated in the following equation:  $\Delta\Delta\text{C}_\text{T} = [(C_\text{T target gene} - C_\text{T internal control}) \text{ sample A} - (C_\text{T target gene} - C_\text{T internal control}) \text{ sample B}]$ , in which *sample A* was the fiber under dark and light culture from 10 DAC to 40 DAC and *sample B*, the fiber under dark culture at 10 DAC as standard. PCR amplification efficiency was determined by means of calibration curves. For calibration curves, five template concentrations were plotted on the  $x$  axis and the corresponding  $\text{C}_\text{T}$ s were plotted on the  $y$  axis. The annealing temperature and the primers were modified to ensure that the  $R^2$  values of the calibration curve were about 0.99, and the amplification efficiency in the range of 90–105%.

The primers for the fiber development genes and flavonoid structural genes were designed according to the conserved regions of the sequences based on the NCBI nucleotide database. Meanwhile, primers designed for qPCR were positioned in the junctions of two exons that included intronic spacers (Tab. 1). Ubiquitin 7 (*GhUBQ7*) was amplified as an internal control, for which the primers were synthesized according to Tan et al. [16]. Three biological repeats and at least two technical repeats for each assay were performed.

### Data analysis

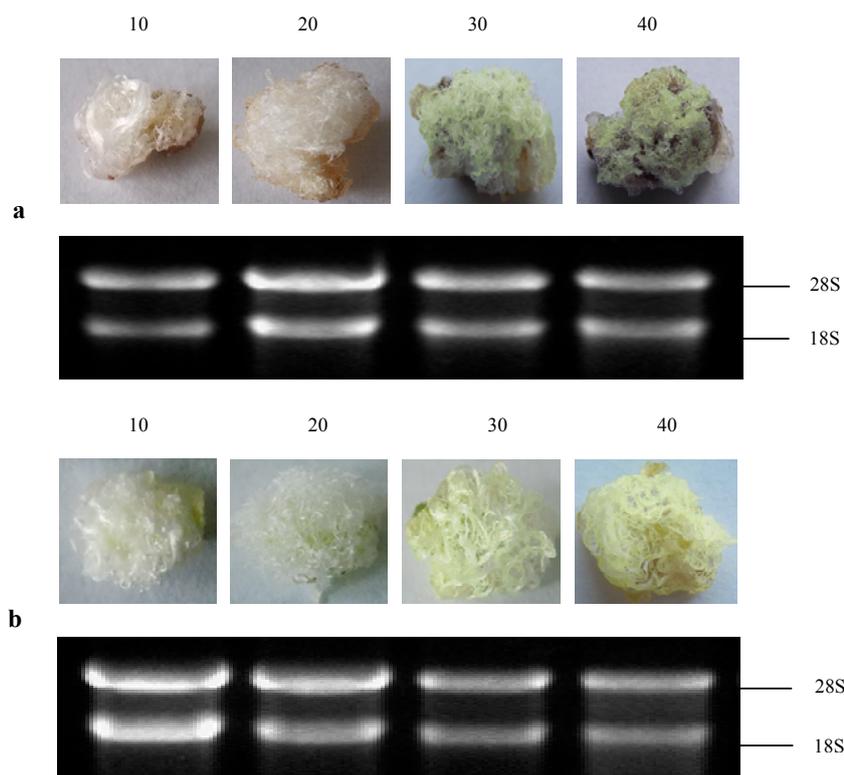
Graphs were generated using Excel software (Microsoft Corp., Redmond, WA, USA).  $T$ -tests for significant differences between means were performed using SAS 9.1 software.

## Results

### Growth of ovules and fibers

The growth of the green cotton ovules and fiber under light and dark cultures from 10 DAC to 40 DAC is shown in Fig. 1. The color of the fiber was white at 10 DAC and 20 DAC and became green after 20 DAC under both dark and light culture.

The fiber length, ovule fresh weight, and fiber fresh weight increased gradually during fiber development under both dark and light culture (Fig. 2a–c). At 10 DAC, the differences in fiber length, ovule fresh weight, and fiber fresh weight were insignificant between the light and dark cultures. From 20 DAC to 40 DAC, the fiber lengths measured under light culture were 11, 13.2, and 13.33 mm, which were significantly shorter than those measured under dark culture. At 30 DAC and 40 DAC, the ovule fresh weights obtained under light culture were 117.48 and 172.3 mg, respectively, and the fiber fresh weights were 80.96 mg and 136.05 mg, respectively, which



**Fig. 1** Ovules and fiber RNA of green cotton under different culture conditions. **a** Ovules and fiber RNA under dark culture. **b** ovules and fiber RNA under light culture. The numbers 10, 20, 30, and 40 indicate the stages of fiber development (DAC).

were significantly greater than the values measured in the dark culture. In addition, from 20 DAC to 40 DAC, the ratios of fiber fresh weight to fiber length recorded in the light culture were significantly higher than in the dark culture (Fig. 2d).

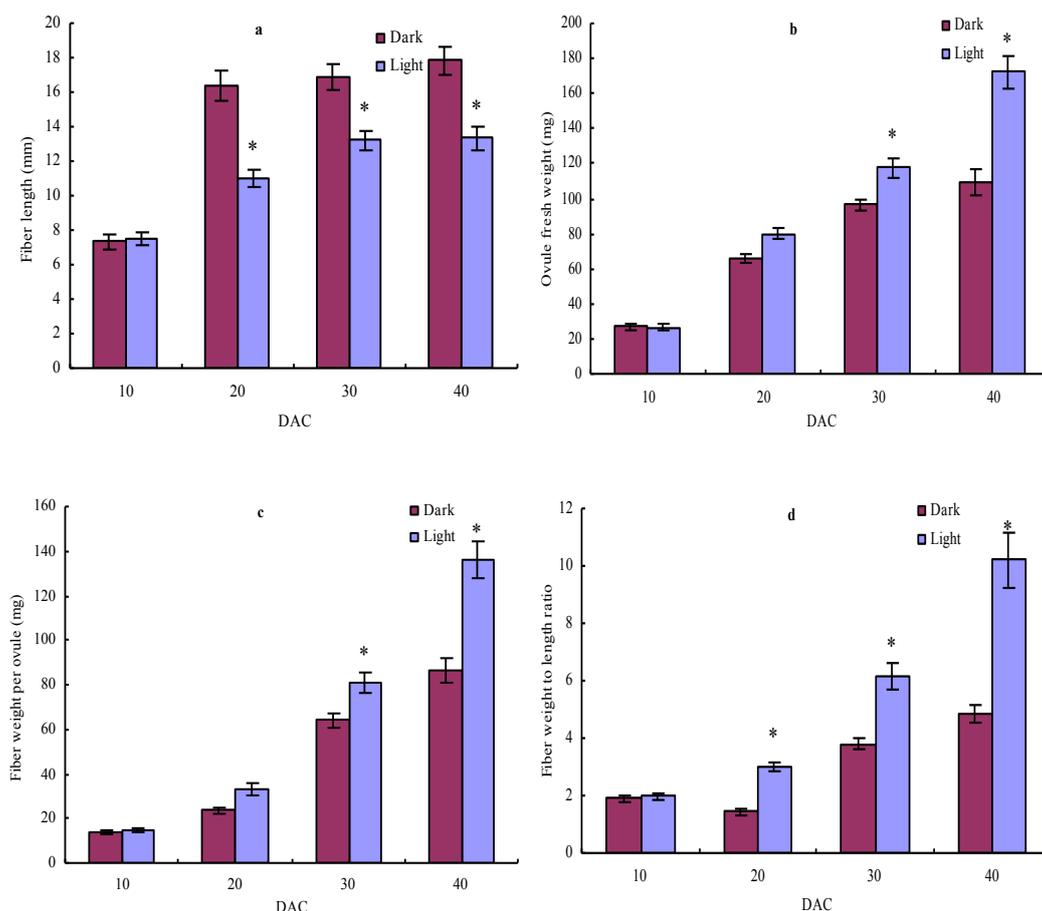
#### Fiber carbohydrate contents and carbohydrate transformation rates

The general trends were similar between the light and dark cultures regarding the changes in fiber carbohydrate contents (Tab. 2). The carbohydrate contents in the fibers in both the dark and light cultures decreased during fiber development. At 10 DAC, the fiber carbohydrate content in the light culture (26.54%) was higher than in the dark culture (16.47%). At 40 DAC, the fiber carbohydrate contents in the light and dark cultures were 4.57% and 4.05%, respectively, but the difference between these values was not significant. Differences in the fiber carbohydrate transformation

rate:  $[100 \times (10 \text{ DAC carbohydrate content} - 40 \text{ DAC carbohydrate content}) / 10 \text{ DAC carbohydrate content}]$ , were also observed between the light and dark cultures. The fiber carbohydrate transformation rate in the light culture was 82.76%, which was significantly higher than the rate of 75.45% measured in the dark culture.

**Tab. 1** Primer pairs used to amplify fiber genes in qPCR.

GenBank accession	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
AF043284	<i>GhEXPI</i>	GGTATGGAACGAGCACAG	GAACATAACAGGGACGATT
U73588	<i>GhSuSy</i>	AGTTGTAGGTGGTGATAGGC	CATTTCGGATTCTGTTCATT
D88416	<i>Ghβ-1,3-glucanase</i>	CGGCAACAATCTTCCATC	GTCGTAACCTTCGTCGTG
U58283	<i>GhCelA1</i>	GCTGAATCTGCCAACCCCT	CCTCCAAAGCCATAACCAT
U58284	<i>GhCelA2</i>	GCTGTTGTTGCGGAGGTT	TTGATTGGAGCGGACCCT
JN032297	<i>GhPAL</i>	TGCTGGTTGTGACCTTGT	GTTGGTGGCTTTGCTGAT
EF643507	<i>GhCHS</i>	GTGCTCGGAGATTACTGC	TGCTTCTACCAGGCTCTT
EF187440	<i>GhF3H</i>	GTTGAGGGCATTTAGGAT	AATCGCACCATCACTATCT
EF187441	<i>GhDFR</i>	GTGGTCGGTCCATTTATT	ATCTTTGAACTTGGTGGG
EF187442	<i>GhANS</i>	GTGGGTGACCGCTAAATG	AGCAAAGGTACGAGGAGG
EF187443	<i>GhANR</i>	TGCTGGTTGTGACCTTGT	GTTGGTGGCTTTGCTGAT
DQ116411	<i>GhUBQ7</i>	GAAGGCATTCCACCTGACCAAC	CTTGACCTTCTTCTTGTGCTTG



**Fig. 2** Differences in fiber and ovule growth parameters between the light and dark cultures of green cotton ovules cultured in liquid medium. **a** Fiber length (mm). **b** Ovule fresh weight (mg). **c** Fiber weight per ovule (mg). **d** Fiber weight-to-length ratio. The vertical bars represent the *SD*. “\*” indicates significant differences at 5%.

**Tab. 2** Differences in carbohydrate contents and carbohydrate transformation rates between the light and dark cultures.

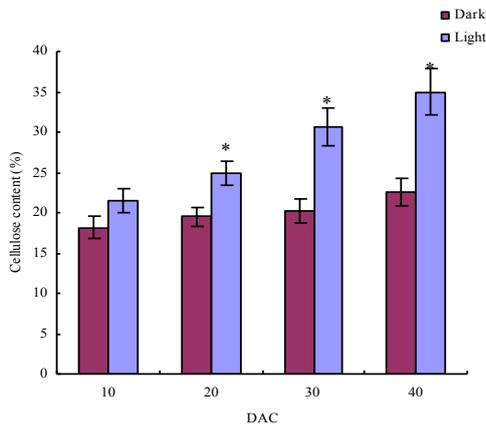
Cultivation mode	Carbohydrate content (%)				Carbohydrate transformation rate (%)
	10 DAC	20 DAC	30 DAC	40 DAC	
Dark culture	16.47 ± 1.43	15.83 ± 1.12	9.95 ± 0.56	4.05 ± 0.38	75.45 ± 2.36
Light culture	26.54 ± 1.65*	20.57 ± 1.34*	9.82 ± 0.68	4.57 ± 0.33	82.76 ± 3.81*

The data are given as means ± *SD*; “\*” shows the significant differences at 5%.

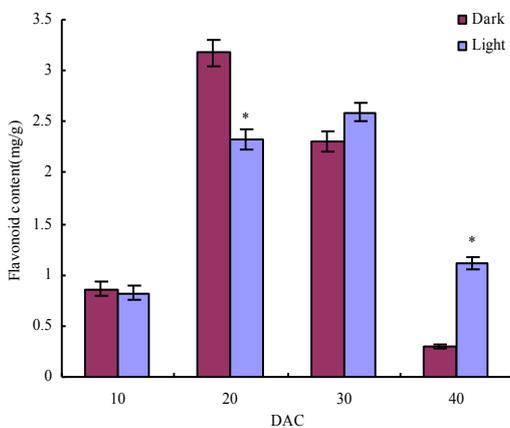
### Fiber cellulose and flavonoid contents

The patterns of cellulose accumulation in both the dark and light cultures were consistent with those in plant-grown fibers, with the cellulose contents increasing gradually from 10 DAC to 40 DAC (Fig. 3). However, the final cellulose contents in both the dark and light cultures were much lower than that in the in situ-grown fiber [30]. From 10 DAC to 40 DAC, the cellulose contents under the light culture were higher than under the dark culture. At 40 DAC, the cellulose content in the light culture was 34.94%, which was significantly greater than the content of 22.51% observed in the dark culture.

The peak flavonoid contents were obtained at 20 DAC in both the dark and light cultures (Fig. 4). At this time point, the flavonoid content under light culture was 2.33



**Fig. 3** Differences in fiber cellulose contents between the light and dark cultures of green cotton ovules cultured in liquid medium (%). The vertical bars represent the *SD*. “\*” indicates significant differences at 5%.



**Fig. 4** Differences in flavonoid contents between the light and dark cultures of green cotton ovules cultured in liquid medium (mg/g). The vertical bars represent the *SD*. “\*” indicates significant differences at 5%.

mg/g, which was significantly lower than the content measured under dark culture (3.17 mg/g). However, the flavonoid contents were higher in the light culture beyond 20 DAC, and significant differences were observed at 40 DAC.

#### Expression of genes related to fiber elongation and secondary wall biosynthesis

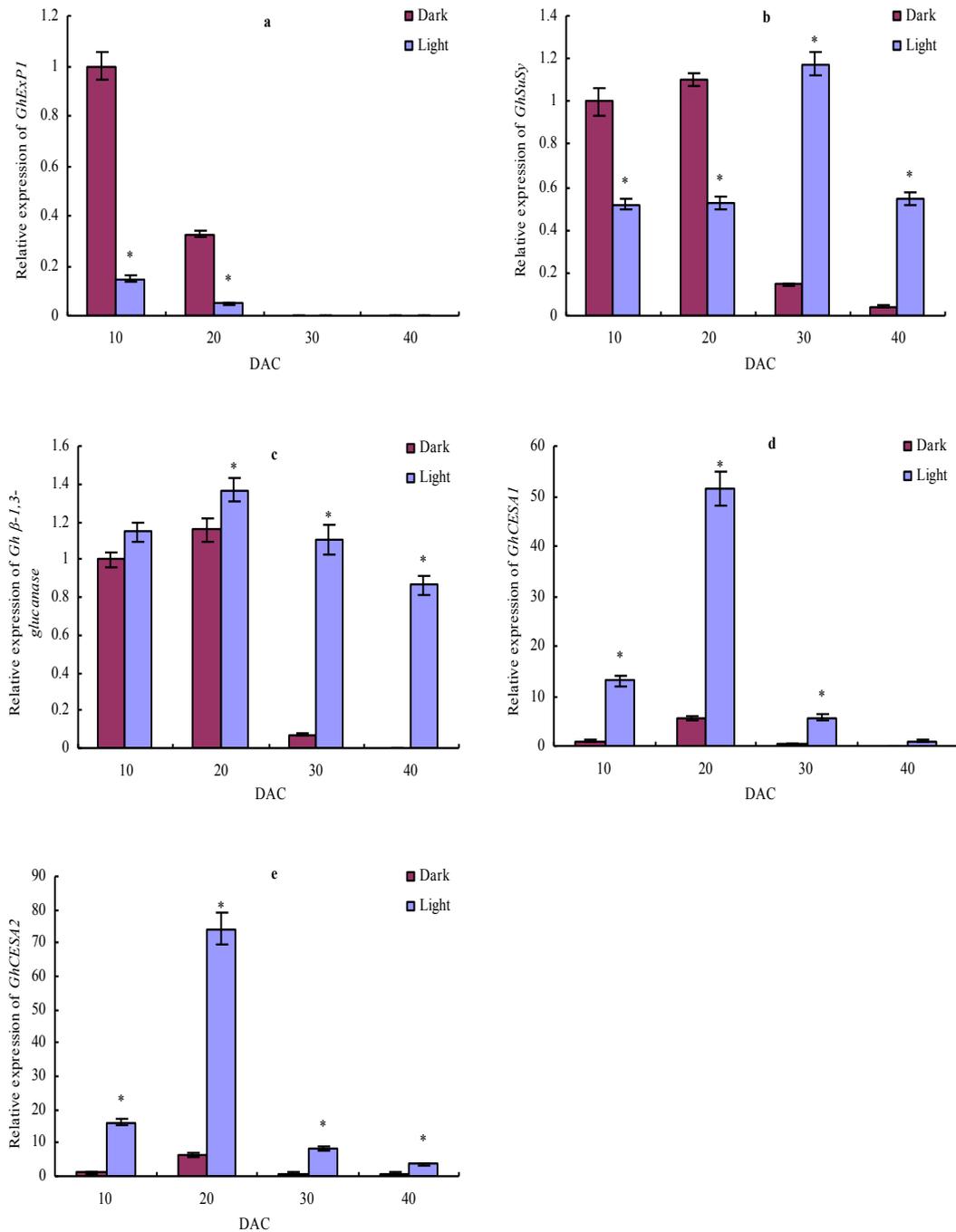
To further elucidate the influence of light on fiber development, the expression levels of the fiber elongation genes *GhEXPI* and *GhSuSy* and the secondary wall biosynthesis genes *GhSuSy*, *Ghβ-1,3-glucanase*, *GhCelA1*, and *GhCelA2* were measured from 10 DAC to 40 DAC. As shown in Fig. 5, the *GhEXPI* gene exhibited the highest expression levels at 10 DAC in both the dark and light culture, but this gene had negligible expression at 30 DAC and 40 DAC. Moreover, light culture was found to inhibit the expression of *GhEXPI*, as the expression levels of the *GhEXPI* gene at 10 DAC and 20 DAC were significantly lower than in the dark culture. The influence of light on the expression of *GhSuSy* differed from that on *GhEXPI*, as *GhSuSy* expression levels were significantly lower at 10 DAC and 20 DAC in the light culture than in the dark culture. However, the expression levels were significantly higher than under dark culture at 30 DAC and 40 DAC. Similar patterns of gene expression were observed for *Ghβ-1,3-glucanase*, *GhCelA1*, and *GhCelA2*. That is to say, the expression levels of these genes were highest at 20 DAC and then decreased gradually. The expression levels of *Ghβ-1,3-glucanase* in the light culture were significantly higher than in the dark culture at 30 DAC and 40 DAC, and those of *GhCelA1* and *GhCelA2* were significantly higher than in the dark culture from 10 DAC to 30 DAC. In addition, *GhCelA1* and *GhCelA2* exhibited higher expression levels only at 20 DAC in the light culture, and the levels of these genes in the other stages were all quite low. However, the expression levels of these two genes under dark culture were all quite low throughout fiber development.

#### Expression of genes related to flavonoid biosynthesis

The expression levels of six genes related to fiber flavonoid biosynthesis were measured in vitro (Fig. 6). From 10 DAC to 30 DAC, the *GhCHS*, *GhF3H*, *GhDFR*, *GhANS*, and *GhANR* genes exhibited high expression levels. However, at 40 DAC, the *GhPAL* and *GhCHS* exhibited moderate expression levels only under light conditions. In addition, a similar influence on the expression levels of the six genes was observed in both the dark and light cultures, with the expression levels of the six genes being inhibited in the light culture at 10 DAC and 20 DAC, while at 30 DAC, the expression levels of the six genes were higher than under dark culture.

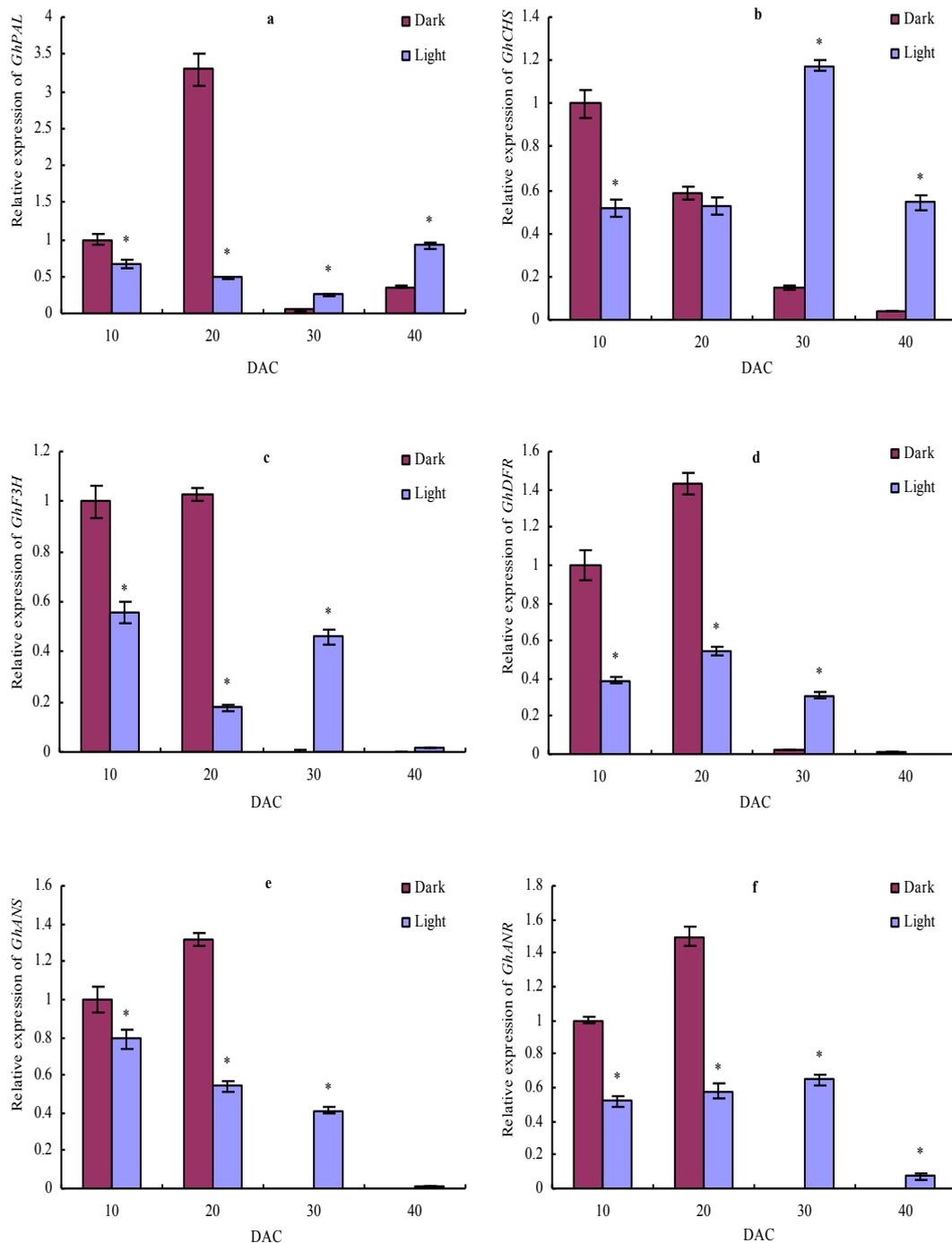
## Discussion

Phytochromes are plant photoreceptors that mediate physiological and developmental responses, such as germination, stem elongation, flowering, chloroplast and leaf development, and gene expression [31]. For example, cotton fibers exposed to a high red/far-red photon ratio during development were longer than those that received lower red/far-red ratio, implicating the involvement of a phytochrome [32]. In addition, cryptochromes mediate blue-light-dependent regulation of gene expression in addition to its effect on plant growth [33]. Our findings demonstrate that light culture



**Fig. 5** Relative expression levels of the main genes related to fiber development of green cotton under the dark and light culture: *GhEXP1* (a), *GhSuSy* (b), *Ghβ-1,3-glucanase* (c), *GhCelA1* (d), and *GhCESA2* (e). The vertical bars represent the SD. “\*” indicates significant differences at 5%.

increased the fresh weights of the ovule and fiber. The ovule and fiber fresh weights measured under the light culture from 20 DAC to 40 DAC were significantly higher than under the dark culture. The ratio of fiber weight to fiber length could reflect the degree of cell wall thickening because the main components of fiber are the cell wall materials [4]. From 20 DAC to 40 DAC, the ratios of fiber weight to length obtained in the light culture were significantly higher than in the dark culture, indicating that the light culture could enhance fiber cell wall thickening. To further study the effect of light on fiber biomass, carbohydrate concentrations and carbohydrate transformation rates were measured in both the dark and light cultures. The results indicate that the carbohydrate concentrations and carbohydrate transformation rates were higher in the light culture than in the dark culture during fiber development, promoting fiber weight gain.



**Fig. 6** Relative expression levels of genes related to flavonoid synthesis of green cotton under the dark and light culture: *GhPAL* (a), *GhCHS* (b), *GhF3H* (c), *GhDFR* (d), *GhANS* (e), and *GhANR* (f). The vertical bars represent the SD. “\*” indicates significant differences at 5%.

The results reported by Harmer et al. [7] indicate that *GhEXPI* is specifically expressed during fiber cell elongation and significantly influences fiber elongation. In addition, intracellular osmotic stress influences the elongation of plant cells, as cotton fiber cells maintain a certain level of intracellular osmotic stress that may promote fiber cell differentiation [34]. SuSy can catalyze the transformation of sucrose into fructose and UDPG, which can significantly increase intracellular osmotic stress. Previous research showed that the low SuSy activity in lintless mutant ovules results in a decrease in epidermal osmotic stress, preventing the differentiation of fiber cells [35,36]. In our experiments, the fiber lengths in the light culture were lower than in the dark culture, which might be related to the low levels of *GhEXPI* and *GhSuSy* expression during fiber cell differentiation in the light culture.

SuSy is not only a key enzyme regulating fiber cell differentiation but also a key enzyme regulating cellulose biosynthesis. In fact, SuSy can degrade sucrose to provide a substrate for cellulose biosynthesis [37,38]. From 30 DAC to 40 DAC, the levels of *GhSuSy* expression in the light culture were higher than in the dark culture, which could promote the biosynthesis of cellulose. Fiber cells contain high levels of  $\beta$ -1,3-glucan during secondary wall cellulose biosynthesis stages.  $\beta$ -1,3-glucanase can digest  $\beta$ -1,3-dextran glycosidic bonds, hydrolyze callose, and provide UDPG for the biosynthesis of cellulose [39,40]. Throughout the fiber growth period, the expression levels of  $\beta$ -1,3-glucanase in the light culture were higher than in the dark culture, with almost no expression being detected at 30 DAC and 40 DAC in the dark culture. These lower expression levels of  $\beta$ -1,3-glucanase in the dark culture resulted in less cellulose accumulation. *GhCelA1* and *GhCelA2* are two of the main genes responsible for the biosynthesis of cellulose and are highly expressed during secondary wall thickening. The results of this study demonstrate that the highest expression levels of *GhCelA1* and *GhCelA2* occurred at 20 DAC in both the dark and light cultures, and the expression levels of these genes in the light culture were significantly higher than in the dark culture throughout fiber development, indicating that light could promote the biosynthesis of cellulose.

Many studies have shown that light not only regulates plant physiological functions but can also regulate secondary metabolism [41,42]. Flavonoid biosynthesis genes are regulated both spatially and temporally in plant development and are induced by environmental stimuli, including light. The biosynthesis of flavonoid and the expression levels of related genes are regulated by phytochrome, cryptochrome, phototropin, and UV-B photoreceptors [43,44]. For example, flavonoid biosynthesis was suppressed by increasing the amount of far-red light of phytochrom [45], whereas UV-A / blue light induction of the flavonoids biosynthesis was mediated principally by cryptochrome [44], in addition, the biosynthesis of flavonoids and the expression levels of related genes are induced most effectively by UV-B light [46,47]. In fact, a white light source that contains all these wavelengths is usually used to induce flavonoid biosynthesis. Our findings revealed differences in flavonoid contents and in the expression levels of *GhPAL*, *GhCHS*, *GhF3H*, *GhDFR*, *GhANS*, and *GhANR* of green cotton fiber between the light and dark cultures. From 10 DAC to 20 DAC, the expression of these genes was inhibited in the light culture, and the measured flavonoid contents were lower than in the dark culture. However, the flavonoid contents and gene expression levels were higher in the light culture than in the dark culture from 30 DAC to 40 DAC. Flavonoid biosynthesis may be associated with osmotic stress in the cell, and high osmotic stress can promote the expression of genes related to flavonoid biosynthesis [48]. From 10 DAC to 20 DAC, the expression levels of *GhSuSy* in the dark culture were higher than in the light culture, which increased osmotic stress in the fiber cells and promoted the biosynthesis of flavonoids. However, opposite results were observed from 30 DAC to 40 DAC. In addition, carbohydrates are used not only to synthesize cellulose, but also to form flavonoids [49,50]. From 10 DAC to 20 DAC, cellulose biosynthesis was faster in the light culture than in the dark culture, which resulted in a large amount of carbohydrates being consumed by the fibers and a smaller amount of carbohydrates being used for flavonoid biosynthesis. However, during the later stage of culture (30–40 DAC), the speed of cellulose biosynthesis was low under light culture, and carbohydrates were mainly used for flavonoid biosynthesis.

## Conclusions

Our studies describe an analysis of the role of light in green cotton ovule culture development and flavonoid synthesis. The present results showed that fiber elongation is inhibited but secondary cell wall deposition enhanced when ovules are cultured in the light relative to in the dark. The effect of light on flavonoid biosynthesis depended on the developmental stage of the cotton fiber. The phenylephrine pathway leading to flavonoid biosynthesis is inhibited in the light-grown fibers at the early stages of development, but it is enhanced at the later stages of development. Our conclusions were drawn based on the light and dark culture of green cotton ovules, as the effects

of specific photoreceptors on green cotton fiber development and flavonoids biosynthesis need to be further investigated.

## References

1. Pan Z, Sun D, Sun J, Zhou Z, Jia Y, Pang B, et al. Effects of fiber wax and cellulose content on colored cotton fiber quality. *Euphytica*. 2010;173(2):141–149. <http://dx.doi.org/10.1007/s10681-010-0124-0>
2. de Moraes Teixeira E, Corrêa AC, Manzoli A, de Lima Leite F, de Oliveira CR, Mattoso LHC. Cellulose nanofibers from white and naturally colored cotton fibers. *Cellulose*. 2010;17(3):595–606. <http://dx.doi.org/10.1007/s10570-010-9403-0>
3. Al-Ghazi Y, Bourot S, Arioli T, Dennis ES, Llewellyn DJ. Transcript profiling during fiber development identifies pathways in secondary metabolism and cell wall structure that may contribute to cotton fiber quality. *Plant Cell Physiol*. 2009;50(7):1364–1381. <http://dx.doi.org/10.1093/pcp/pcp084>
4. Singh B, Cheek HD, Haigler CH. A synthetic auxin (NAA) suppresses secondary wall cellulose synthesis and enhances elongation in cultured cotton fiber. *Plant Cell Rep*. 2009;28(7):1023–1032. <http://dx.doi.org/10.1007/s00299-009-0714-2>
5. Chen J, Lv FJ, Liu JR, Ma YN, Wang YH, Chen BL, et al. Effects of different planting dates and low light on cotton fibre length formation. *Acta Physiol Plant*. 2014;36(10):2581–2595. <http://dx.doi.org/10.1007/s11738-014-1629-2>
6. Kende H, Bradford K, Brummell D, Cho HT, Cosgrove D, Fleming A, et al. Nomenclature for members of the expansin superfamily of genes and proteins. *Plant Mol Biol*. 2004;55(3):311–314. <http://dx.doi.org/10.1007/s11103-004-0158-6>
7. Harmer S, Orford S, Timmis J. Characterisation of six  $\alpha$ -expansin genes in *Gossypium hirsutum* (upland cotton). *Mol Genet Genomics*. 2002;268(1):1–9. <http://dx.doi.org/10.1007/s00438-002-0721-2>
8. Wang L, Ruan YL. Unraveling mechanisms of cell expansion linking solute transport, metabolism, plasmodesmata gating and cell wall dynamics. *Plant Signal Behav*. 2010;5(12):1561–1564. <http://dx.doi.org/10.4161/psb.5.12.13568>
9. Salnikov VV, Grimson MJ, Seagull RW, Haigler CH. Localization of sucrose synthase and callose in freeze-substituted secondary-wall-stage cotton fibers. *Protoplasma*. 2003;221(3–4):175–184. <http://dx.doi.org/10.1007/s00709-002-0079-7>
10. Saxena IM, Brown Jr RM. Cellulose synthases and related enzymes. *Curr Opin Plant Biol*. 2000;3(6):523–531. [http://dx.doi.org/10.1016/S1369-5266\(00\)00125-4](http://dx.doi.org/10.1016/S1369-5266(00)00125-4)
11. Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM. Higher plants contain homologs of the bacterial *celA* genes encoding the catalytic subunit of cellulose synthase. *Proc Natl Acad Sci USA*. 1996;93(22):12637–12642. <http://dx.doi.org/10.1073/pnas.93.22.12637>
12. Schmutz A, Jenny T, Amrhein N, Ryser U. Caffeic acid and glycerol are constituents of the suberin layers in green cotton fibres. *Planta*. 1993;189(3):453–460. <http://dx.doi.org/10.1007/BF00194445>
13. Schmutz A, Jenny T, Ryser U. A caffeoyl-fatty acid-glycerol ester from wax associated with green cotton fibre suberin. *Phytochemistry*. 1994;36(6):1343–1346. [http://dx.doi.org/10.1016/S0031-9422\(00\)89721-6](http://dx.doi.org/10.1016/S0031-9422(00)89721-6)
14. Zhao XQ, Wang XD. Composition analysis of pigment in colored cotton fiber. *Acta Agronomica Sinica*. 2005;31(4):456–462. <http://dx.doi.org/10.3321/j.issn:0496-3490.2005.04.010>
15. Zhang ML. Exploration of fiber differentiation and development and pigment component in colored cotton [PhD thesis]. Shandong: Shandong Agricultural University; 2013.
16. Tan J, Tu L, Deng F, Hu H, Nie Y, Zhang X. A genetic and metabolic analysis revealed that cotton fiber cell development was retarded by flavonoid naringenin. *Plant Physiol*. 2013;162(1):86–95. <http://dx.doi.org/10.1104/pp.112.212142>
17. Lee SH, Tewari RK, Hahn EJ, Paek KY. Photon flux density and light quality induce changes in growth, stomatal development, photosynthesis and transpiration of *Withania somnifera* (L.) Dunal. plantlets. *Plant Cell Tissue Organ Cult*. 2007;90(2):141–151. <http://dx.doi.org/10.1007/s11240-006-9191-2>

18. Rao AQ. An overview of phytochrome: an important light switch and photo-sensory antenna for regulation of vital functioning of plants. *Biologia*. 2015;70(10):1273–1283. <http://dx.doi.org/10.1515/biolog-2015-0147>
19. Pettigrew WT. Environmental effects on cotton fiber carbohydrate concentration and quality. *Crop Sci*. 2001;41(4):1108–1113. <http://dx.doi.org/10.2135/cropsci2001.4141108x>
20. Pan ZE, Du XM, Sun JL, Zhou ZL, Pang BY. Influences of boll shading on fiber color and fiber quality of colored cotton. *Cotton Science*. 2006;18(5):264–268. <http://dx.doi.org/10.3969/j.issn.1002-7807.2006.05.002>
21. Staneloni RJ, Rodriguez-Batiller MJ, Casal JJ. Abscisic acid, high-light, and oxidative stress down-regulate a photosynthetic gene via a promoter motif not involved in phytochrome-mediated transcriptional regulation. *Mol Plant*. 2008;1(1):75–83. <http://dx.doi.org/10.1093/mp/ssm007>
22. Reis A, Kleinowski AM, Klein FRS, Telles RT, do Amarante L, Braga EJB. Light quality on the in vitro growth and production of pigments in the genus *Alternanthera*. *J Crop Sci Biotechnol*. 2015;18(5):349–357. <http://dx.doi.org/10.1007/s12892-015-0074-0>
23. Kasperbauer MJ. Cotton fiber length is affected by far-red light impinging on developing bolls. *Crop Sci*. 2000;40(6):1673–1678. <http://dx.doi.org/10.2135/cropsci2000.4061673x>
24. Beasley CA, Ting IP. The effects of plant growth substances on in vitro fiber development from fertilized cotton ovules. *Am J Bot*. 1973;60(2):130–139. <http://dx.doi.org/10.2307/2441099>
25. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant*. 1962;15(3):473–497. <http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x>
26. Sun Y, Veerabomma S, Abdel-Mageed HA, Fokar M, Asami T, Yoshida S, et al. Brassinosteroid regulates fiber development on cultured cotton ovules. *Plant Cell Physiol*. 2005;46(8):1384–1391. <http://dx.doi.org/10.1093/pcp/pci150>
27. Li HS. *Experiment principles and techniques for plant physiology and biochemistry*. Beijing: Higher Education Press; 2000.
28. Viles Jr FJ, Silverman L. Determination of starch and cellulose with anthrone. *Anal Chem*. 1949;21(8):950–953. <http://dx.doi.org/10.1021/ac60032a019>
29. Song CQ. Secondary metabolites. In: Shanghai Institute of Plant Physiology, Chinese Academy of Sciences; Plant Physiology Society of Shanghai, editors. *Modern guidance for plant physiology experiments*. Beijing: Science Press; 1999. p. 222.
30. Teixeira EM, Corrêa AC, Manzoli A, Leite FL, Oliveira CR, Mattoso LHC. Cellulose nanofibers from white and naturally colored cotton fibers. *Cellulose*. 2010;17(3):595–606. <http://dx.doi.org/10.1007/s10570-010-9403-0>
31. Kong SG, Okajima K. Diverse photoreceptors and light responses in plants. *J Plant Res*. 2016;129(2):111–114. <http://dx.doi.org/10.1007/s10265-016-0792-5>
32. Kasperbauer MJ. Cotton fiber length is affected by far-red light impinging on developing bolls. *Crop Sci*. 2000;40(6):1673–1678. <http://dx.doi.org/10.2135/cropsci2000.4061673x>
33. Christian F, Roman U. Light-regulated interactions with SPA proteins underlie cryptochrome-mediated gene expression. *Genes Dev*. 2011;25(10):1004–1009. <http://dx.doi.org/10.1101/gad.2053911>
34. Ruan YL, Chourey PS. A fiberless seed mutation in cotton is associated with lack of fiber cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. *Plant Physiol*. 1998;118(2):399–406. <http://dx.doi.org/10.1104/pp.118.2.399>
35. Ruan YL, Llewellyn DJ, Furbank RT. Suppression of sucrose synthase gene expression represses cotton fiber cell initiation, elongation, and seed development. *Plant Cell*. 2003;15(4):952–964. <http://dx.doi.org/10.1105/tpc.010108>
36. Ruan YL, Llewellyn DJ, Furbank RT, Chourey PS. The delayed initiation and slow elongation of fuzz-like short fibre cells in relation to altered patterns of sucrose synthase expression and plasmodesmata gating in a lintless mutant of cotton. *J Exp Bot*. 2005;56(413):977–984. <http://dx.doi.org/10.1093/jxb/eri091>
37. Delmer DP, Haigler CH. The regulation of metabolic flux to cellulose, a major sink for carbon in plants. *Metab Eng*. 2002;4(1):22–28. <http://dx.doi.org/10.1006/mben.2001.0206>
38. Zhang W, Shu H, Hu H, Chen B, Wang Y, Zhou Z. Genotypic differences in some physiological characteristics during cotton fiber thickening and its influence on fiber strength. *Acta Physiol Plant*. 2009;31(5):927–935. <http://dx.doi.org/10.1007/s11738-009-0306-3>

39. Meier H, Buchs L, Buchala AJ, Homewood T. (1→3)-β-D-Glucan (callose) is a probable intermediate in biosynthesis of cellulose of cotton fibres. *Nature*. 1981;289:821–822. <http://dx.doi.org/10.1038/289821a0>
40. Shimizu Y, Aotsuka S, Hasegawa O, Kawada T, Sakuno T, Sakai F, et al. Changes in levels of mRNAs for cell wall-related enzymes in growing cotton fiber cells. *Plant Cell Physiol*. 1997;38(3):375–378. <http://dx.doi.org/10.1093/oxfordjournals.pcp.a029178>
41. Li HM, Xu ZG, Tang CM. Effect of light-emitting diodes on growth and morphogenesis of upland cotton (*Gossypium hirsutum* L.) plantlets in vitro. *Plant Cell Tissue Organ Cult*. 2010;103(2):155–163. <http://dx.doi.org/10.1007/s00438-002-0721-2>
42. Puspá RP, Ikuo K, Ryosuke M. Effect of red- and blue-light-emitting diodes on growth and morphogenesis of grapes. *Plant Cell Tissue Organ Cult*. 2008;92(2):147–153. <http://dx.doi.org/10.1007/s11240-007-9317-1>
43. González CV, Fanzone ML, Cortés LE, Bottini R, Lijavetzky DC, Ballaré CL, et al. Fruit-localized photoreceptors increase phenolic compounds in berry skins of field-grown *Vitis vinifera* L. cv. Malbec. *Phytochemistry*. 2014;110:46–57. <http://dx.doi.org/10.1016/j.phytochem.2014.11.018>
44. Zoratti L, Karppinen K, Luengo Escobar A, Häggman H, Jaakola L. Light-controlled flavonoid biosynthesis in fruits. *Front Plant Sci*. 2014;5(5):1–16. <http://dx.doi.org/10.3389/fpls.2014.00534>
45. Gerhardt KE, Lampi MA, Greenberg BM. The effects of far-red light on plant growth and flavonoid accumulation in *Brassica napus* in the presence of ultraviolet B radiation. *Photochem Photobiol*. 2008;84(6):1445–1454. <http://dx.doi.org/10.1111/j.1751-1097.2008.00362.x>
46. Namlı S, Işıkalın Ç, Akbaş F, Tokar Z, Tilkat EA. Effects of UV-B radiation on total phenolic, flavonoid and hypericin contents in *Hypericum retusum* Aucher grown under in vitro conditions. *Nat Prod Res*. 2014;28(24):2286–2292. <http://dx.doi.org/10.1080/14786419.2014.940588>
47. Huang X, Yao J, Zhao Y, Xie D, Jiang X, Xu Z. Efficient rutin and quercetin biosynthesis through flavonoids-related gene expression in *Fagopyrum tataricum* Gaertn hairy root cultures with UV-B irradiation. *Front Plant Sci*. 2016;7(63):1–11. <http://dx.doi.org/10.3389/fpls.2016.00063>
48. Gao JJ, Zhang Z, Peng RH, Xiong AS, Xu J, Zhu B, et al. Forced expression of *Mdmyb10*, a myb transcription factor gene from apple, enhances tolerance to osmotic stress in transgenic Arabidopsis. *Mol Biol Rep*. 2011;38(1):205–211. <http://dx.doi.org/10.1007/s11033-010-0096-0>
49. Hua SJ, Wang XD, Yuan SN, Shao MG, Zhao XQ, Zhu SJ, et al. Characterization of pigmentation and cellulose synthesis in colored cotton fibers. *Crop Sci*. 2007;47(4):1540–1546. <http://dx.doi.org/10.1007/s00438-002-0721-2>
50. Yuan SN, Hua SJ, Malik W, Bibi N, Wang XD. Physiological and biochemical dissection of fiber development in colored cotton. *Euphytica*. 2012;187(2):215–226. <http://dx.doi.org/10.1007/s10681-012-0653-9>