



Red light promotes cotton embryogenic callus formation by influencing endogenous hormones, polyamines and antioxidative enzyme activities

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Abstract

Genetic transformation through somatic embryogenesis is a major tool for improving cotton's (*Gossypium hirsutum* L.) development and defenses. However, the low efficiency of somatic embryogenesis seriously limits cotton's biotechnology-based breeding and gene functional analyses. In this study, the effects of different light qualities on callus morphology and embryogenic callus induction in cotton were evaluated. Embryogenic callus formation was affected by different light regimes during the induction and expression phases. Red light treatments during the induction phase significantly promoted the formation of embryogenic calli, reaching 94.0%, and the differentiation period was reduced to 30 days compared with more than 60 days under white light conditions. Calli were of a moderate size and had moderate peroxidase, superoxidase dismutase, and catalase activity levels under red light conditions, which may maintain the balance between callus growth and differentiation. The highest total polyamine, spermidine, and spermine contents, and the lowest putrescine content, were detected during red light treatments. Additionally, endogenous auxin levels were significantly greater in red light-treated calli, and the expression levels of the somatic embryogenesis marker genes AGAMOUS-like 15, LEAFY COTYLEDON1, LEAFY COTYLEDON2, BABY BOOM, and WUSCHEL were up-regulated under red light compared with white light conditions. Thus, red light applied in the induction phase favored embryogenic callus differentiation, possibly through physiological responses to light quality. A high-efficiency cotton somatic embryogenesis protocol using red light applications will help clarify the mechanism of somatic embryogenesis.

Keywords Embryogenic callus · Endogenous hormone · *Gossypium hirsutum* L. · Light quality · Polyamines

Abbreviations

ABA Abscisic acid
BL Blue light

CAT Catalase
CIM Callus induction medium
D Darkness
ECIM Embryogenic callus induction medium
FW Fresh weight
IAA Indole-3-acetic acid
LED Light-emitting diode
MSB Murashige and Skoog medium salts supplemented with B₅ organic compounds
PA Polyamine
POD Peroxidase
PPFD Photosynthetic photon flux density
Put Putrescine
qRT-PCR Quantitative reverse transcription-polymerase chain reaction
RL Red light
ROS Reactive oxygen species
SD Standard deviation
SOD Superoxidase dismutase

Ya Yu, Wenqiang Qin and Yi Li have contributed equally to this work.

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|-----|------------------------|
| Spd | Spermidine |
| Spm | Spermine |
| WL | White fluorescent lamp |
| ZA | Zeatin |

Introduction

Cotton (*Gossypium hirsutum* L.) is an important economic crop worldwide, providing natural fiber and edible oil. Cotton propagation using somatic embryos and detached organs has been widely used to genetically transform and maintain germplasm, create hybrid offspring between wild and domesticated species, and maintain mutated germplasm that cannot produce viable seeds (Cheng et al. 2016). However, the regeneration of plantlets for genetic transformation and bioengineering has only been successful in some cultivars having limited genotypes (Cheng et al. 2016; Wu et al. 2009a, b; Zhang et al. 2011). Therefore, the improvement of genetic transformation efficiency of commercially cotton cultivars remains a challenge.

The process of somatic embryogenic regeneration includes callus induction, embryogenic callus differentiation, somatic embryo development, and plantlet regeneration, of which embryogenic callus differentiation is particularly critical. It is, therefore, necessary to modulate the time and status of embryogenic callus differentiation and many environmental factors can affect the process.

Light, through changes in irradiance, is the most important factor affecting plant growth, morphology, physiology, and productivity (Yu et al. 2018). White fluorescent lamps (WLs) are widely used during *in vitro* cotton tissue culturing. The intensity and duration of red light (RL) and blue light (BL) have profound influences on plant growth and development by triggering physiological reactions (Bakk and Pachler 2013; Li et al. 2010; Lau and Deng 2010; Rikiishi et al. 2008; Cybularz-urban et al. 2007; Dong et al. 2006). Light-emitting diode (LED) lighting systems have several unique advantages compared with WLs, including the ability to control the spectral composition, their small size, durability, long operating lifetime, relatively cool emitting surfaces, and photon output. They are, therefore, ideal for use in plant tissue culturing, allowing wavelengths to be matched to plant photoreceptors, which optimizes production (Ye et al. 2017; Yang et al. 2017; Ferreira et al. 2016; Shimizu 2016; Chen et al. 2016; Nhut et al. 2015; Kwon et al. 2015; Li et al. 2010a, b). Research into LED light sources has focused on seed germination and plantlet growth in horticultural facilities (Li et al. 2010a, b; Shimizu 2016; Heringer et al. 2017). However, few reports have investigated the relationship between light quality and somatic embryogenesis in cotton.

Cotton somatic embryogenesis is still a bottleneck in the development of a high-efficiency regenerative protocol.

Many factors, such as the basal medium composition, plant growth regulators, culture conditions, and their interactions, can affect the efficiency of embryogenic callus induction (El-Esawi 2016; Elhiti and Stasolla 2016; Vondráková et al. 2015; Satish et al. 2016). Light conditions are particularly important for embryogenic callus formation, somatic embryo induction, and plant growth (Heringer et al. 2017; Chen et al. 2014, 2016; Nhut et al. 2015; Rodríguez-Sahagún et al. 2010; De-la-Peña et al. 2008; Onofrio et al. 1998; Michler and Lineberger 1987). For instance, Michler and Lineberger (1987) reported that the highest intensity white and blue light treatments were inhibitory to growth and somatic embryogenesis, while RL enhanced development to the heart stage. Both RL and BL spectra may be used to manipulate carrot cell cultures during growth optimization, while quince embryonic callus induction is influenced by light quality, achieving the highest rate under RL, followed by RL plus BL and then white light (Onofrio et al. 1998). Moreover, in *Panax vietnamensis*, the most suitable light conditions for embryogenesis are 60% RL plus 40% BL (Nhut et al. 2015). Bach and Pawlowska (2006) reported that RL facilitated callus differentiation in bulbous plants, whereas BL was inhibitory. However, no research on the effects of light quality on embryogenic callus induction in cotton has been conducted.

Therefore, in the present study, we investigated the effect of light quality on the rate of embryogenic callus differentiation in cotton. An assessment of calli under different light quality treatments was carried out, involving measurements of antioxidant enzyme activities, hormone levels and the polyamine (PA) content, as well as the expression levels of somatic embryogenesis marker genes. Our results suggest that RL treatments promote embryogenic callus formation in cotton.

Materials and Methods

Plant materials and growth conditions

Plants of the CRI24 cotton genotype, provided by the Institute of Cotton Research, Chinese Academy of Sciences, were used in this study. Seeds were surface-sterilized and germinated as previously described by Yu et al. (2011). Hypocotyls were dissected with sharp scissors to produce 5–8-mm long segments from 7-days-old seedlings, and the hypocotyl explants were used in the experiments. Explants were transferred to 100-mL flasks containing the callus induction medium (CIM) consisting of Murashige and Skoog medium (Murashige and Skoog 1962) and salts, supplemented with B₅ organic compounds (Gamborg et al. 1968). After ~40 days, calli were transferred onto embryogenic callus induction medium (ECIM). All media are listed

in Supplementary Tables 1 and 2. Cultures were maintained under a 16-h photoperiod at 28 ± 2 °C (Zhang et al. 2011).

Light treatments

Light treatments were designated as RLs (LEDs), BLs (LEDs), mixtures of RL plus BL (RL:BL = 3:1; RL:BL = 1:1; RL:BL = 1:3), WLs, and darkness (D). The spectral distributions of the RL (peak at 660 nm) and BL (peak at 460 nm) were measured using a spectrum analyzer (HR-350, HiPoint, Gaoxiong City, Taiwan Province, Taiwan). The WLs had an intensity of $30\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Other treatments were irradiated with the same photosynthetic photon flux density of $60\text{--}65 \mu\text{mol m}^{-2} \text{s}^{-1}$, which was measured using a spectrum analyzer. It was controlled by adjusting both the electric currents and numbers of light bulbs in the variable LED experimental system (XM-LEDX12T8D; Nanjing City, Jiangsu Province, China). Light parameters for each treatment are shown in Supplementary Table 3. The light regime was divided into the induction phase on CIM and the expression phase on ECIM.

Preliminary experiment

The objective of our study was to measure the effects of WL, monochromatic BL, monochromatic RL, and mixtures of RL and BL on the biochemistry and morphogenesis of cotton *in vitro*. In the preliminary experiment, six different light treatments were applied in the induction and expression phases. Initially, the first phase was treated with six types of light, and the second phase was illuminated with white light. Then, the first phase was illuminated with white light, and the second phase was treated with the same six types of light. Cotton embryogenic calli emerged ~40 days after calli were subcultured on ECIM. The differentiation rates were recorded at 40, 50, and 60 days after inoculation. In the main experiment, WL, RL, BL, and D treatments were applied in the induction phase, followed by WL in the expression phase. Embryogenic callus differentiation was observed and recorded 15, 25, and 30 days after inoculation.

Plant growth

Callus growth was estimated as the change in callus fresh weight (FW) every 3 days from 1 to 40 days of culturing. A random selection of ten explant samples was collected from the flasks. The experiment was repeated three times, and the results were averaged.

Chlorophyll content

To analyze the chlorophyll content, 0.1 g of a finely cut, well-mixed sample was extracted with 8 mL 95% acetone for

24 h at 4 °C in the dark and shaken 3–4 times until blanched. The absorbance was measured using a spectrophotometer (Shimadzu UV-2550, Kyoto, Japan) at 646.6, 663.6, and 450 nm after centrifugation of the mixture. Chlorophyll concentrations were calculated by a standard method (Porra et al. 1989) and expressed in mg g^{-1} FW.

PA content

PAs were analyzed as described by Silveira et al. (2013). Three samples (300 mg of FW each) were pulverized in 1.6 mL of 5% (v/v) perchloric acid (Merck). After 1 h of incubation at 4 °C, the samples were centrifuged at $20,000 \times g$ for 20 min at 4 °C. Free PAs were determined directly from the supernatant by derivatization with dansyl chloride (Merck) and subsequent high-performance liquid chromatography with a 5- μm reverse-phase column (Shimadzu Shin-pack CLC ODS). The gradient was developed by mixing increasing proportions of absolute acetonitrile (Merck) with 10% acetonitrile in water (pH 3.5). The gradient of absolute acetonitrile was programmed as follows: 65% over the first 10 min, from 65 to 100% between 10 and 13 min, and 100% between 13 and 21 min. The flow rate was 1 mL min^{-1} at 40 °C. The concentrations of total PAs, putrescine (Put) (Sigma-Aldrich), spermidine (Spd) (Sigma-Aldrich), and spermine (Spm) (Sigma-Aldrich) were determined using a fluorescence detector at 340 nm (excitation) and 510 nm (emission). Peak areas and retention times were measured by comparisons with PA standards.

Hormone content

After indole-3-acetic acid (IAA), zeatin (ZA), and abscisic acid (ABA) elution from SPE columns by pure methanol, each sample was evaporated to dryness and reconstituted in 50 μL methanol (Žur et al. 2015). Samples were then analyzed using a Supelco Ascentis RP-Amide column (7.5 cm \times 2.1 mm; 2.7 μm). Mobile phases consisted of 0.1% formic acid solution in water (solvent A) and acetonitrile:methanol (1:1; solvent B). Analyses were performed using a gradient elution at a flow rate of 1.5 mL min^{-1} with the gradient starting from 20% B at 0 min, rising to 80% B at 3 min, and returning to 20% B at 3.5 min. The total analysis time was 4 min. The high-performance liquid chromatography apparatus was an Agilent 1290 Infinity equipped with a 6460 Triple Quad LC/MS with Jet Stream (Agilent Technologies). The capillary voltage was set to 4000 V, and the two most abundant product ions were monitored using the Multiple Reaction Monitoring mode for each analyzed compound. The most abundant product ion was used for quantification, while the second ion was used to confirm the identities of the phytohormones. Mass Hunter

5 software (Agilent Technologies) was used for apparatus control, data collection, and processing.

Antioxidant enzyme activity

Calli (1 g) were ground with a mortar in 25 mL of 50 mM phosphate buffer solution, pH 7.8, containing 1% polyethylene pyrrole at 4 °C. The homogenate was centrifuged at 10,000×g at 4 °C for 15 min, and the supernatant was collected to measure enzyme activity levels. The activity of superoxidase dismutase (SOD) was assayed by monitoring its ability to inhibit the photochemical reduction of NBT (Beauchamp and Fridovich 1971). Peroxidase (POD) activity was estimated using the method of Thomas et al. (1982), while catalase (CAT) activity was measured by monitoring the reduction of H₂O₂ as described in Díaz-Vivancos et al. (2008). The specific antioxidant enzyme activity was expressed as U g⁻¹ FW, and the detailed procedures are those of Yu et al. (2017).

RNA isolation and real-time PCR analyses

Transcript levels of somatic embryogenic marker genes were measured by quantitative (q) reverse transcription (RT)-PCR. Total RNA extraction and cDNA synthesis were performed as previously described by Yang et al. (2014). Gene-specific primers are listed in Supplementary Table 4. Each SYBR Green qRT-PCR reaction consisted of 10 µL of SYBR Green PCR buffer, 0.5 µL of forward and reverse primer, 2 µL of template and 0.4 µL of ROX Dye. The final reaction volume was adjusted to 20 µL using double-distilled H₂O. qRT-PCR was carried out using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, USA). Thermal cycling was performed under the following conditions: initial denaturation at 95 °C for 30 s, followed by a two-step profile of 95 °C for 5 s and 60 °C for 15 s for 40 cycles. *AtUBQ10* was used as an internal control, and the data were processed using the 2^{-ΔΔC_t} method.

Statistical analysis

The experiments followed a completely randomized design. All results are expressed as mean values and standard deviations (SDs). Data represent the means of three replicates ± SDs. The analysis of variance and the Student's tests were performed using the Statistical Package for the Social Sciences Ver. 17.0 software (SPSS, Chicago, IL, USA). Tukey's HSD test for multiple comparisons was employed to detect differences between treatment means, and probability values of less than equal to 5% were considered to be significant. The effect of the light treatment on gene expression patterns was analyzed using Student's t-tests to determine

the significant differences. Differences of $P \leq 0.05$ were considered to be significant (* $P \leq 0.05$; ** $P \leq 0.01$).

Results

Effect of light quality on embryogenic callus induction in cotton

Explants were cultured under different light treatments (WL, RL, BL, D, RL:BL = 3:1, RL:BL = 1:1, and RL:BL = 1:3, Fig. 1) for callus or embryogenic callus induction (the induction or expression phase, respectively). As shown in Fig. 2a, b, the rates of embryogenic callus induction on ECIM under various light qualities were comparable to those under the WL treatment during the embryogenic callus induction phase; however, those on CIM treated with RL during the callus induction phase were significantly increased, reaching 77.2%, 85.3% and 94.0% at 40, 50, and 60 days after inoculation, respectively. Interestingly, the embryogenic calli also emerged much earlier with the RL treatment compared with the WL treatment.

We, therefore, investigated the effects of the RL treatment and observed a strong effect on embryogenic callus differentiation on CIM compared with the WL treatment at 20, 25, and 30 days (Fig. 2c). As shown in Fig. 2d, calli treated by RL readily formed yellow embryogenic calli and granular structures, while those grown under D conditions grew slowly, were small in size, white in color, and did not fully differentiate into embryogenic calli. When BL was used to treat the calli, they grew rapidly, were large, but rarely formed embryogenic calli. Under the normal WL treatment, the calli formed embryogenic calli, but showed less differentiation and were a pale yellow. Thus, RL applied during the callus induction phase significantly promoted embryogenic callus formation, suggesting that the responsiveness of embryogenic calli to light is determined by the light regime during the different phases.

Callus state under different light conditions

Different light qualities have variable effects on cotton callus proliferation (Fig. 2e). Under the BL/RL treatment, calli demonstrated a significantly greater growth rate than under the WL or D treatment from 21 to 40 days. (Fig. 2e). The fresh weights of callus under different light quality were significantly different in the 40 days, which was the largest under BL treatment, followed by RL, WL and D treatments. The color and texture of the calli also showed remarkable differences under various light sources. As shown in Fig. 2e, calli treated with RL were dark yellow and had a loose structure, while those treated with BL were more compact, and those receiving

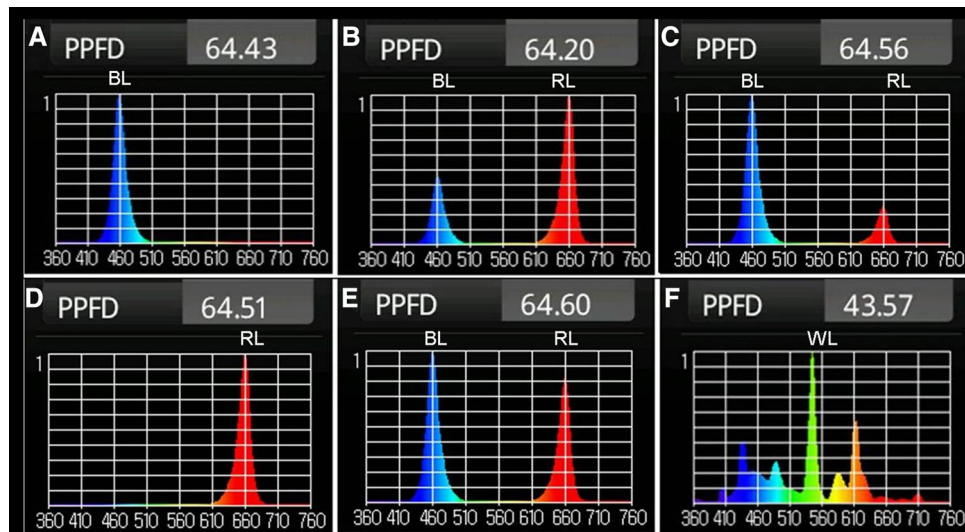


Fig. 1 Different light treatments. **a** BL indicates blue light-emitting diode light; **b** RL:BL=3:1 indicates that the proportion of photosynthetic photon flux density of red and blue LED is 3:1; **c** RL:BL=1:3 indicates that the proportion of photosynthetic photon flux density of red and blue LED is 1:3; **d** RL indicates red light-emitting diode light; **e** RL:BL=1:1 indicates that the proportion of photosynthetic photon flux density of red and blue LED is 1:1; **f** WL indicates white fluorescent lamp. The horizontal axis represents the spec-

tral luminous band. Photosynthetic photon flux density refers to the luminous flux density of the photosynthetic effective radiation. The unit of PPFD is $\mu\text{mol m}^{-2} \text{s}^{-1}$. The spectral distributions of the RL and BL peak at 660 nm and 460 nm, respectively at an intensity of $60\text{--}65 \mu\text{mol m}^{-2} \text{s}^{-1}$. The WL's relative spectral distribution was 380–760 nm at an intensity of $30\text{--}50 \mu\text{mol m}^{-2} \text{s}^{-1}$. BL blue LED light, RL red LED light. (Color figure online)

the D treatment were white and small. The callus color and texture determine its fate, embryogenesis or proliferation. Thus, a loose, light yellow callus readily differentiates into an embryogenic callus (Trolinder and Goodin 1987; Wu et al. 2004; Yang et al. 2014).

Additionally, the light quality directly determines subsequent somatic embryo differentiation. Calli treated with RL produced more somatic embryos in a shorter time than the other light treatments (data not shown). Collectively, the RL treatment can promote embryogenic cell formation and somatic embryo germination, which may be attributed to the physiological and biochemical changes induced by the light.

Antioxidant enzyme activity

During embryogenic callus formation, reactive oxygen species (ROS) maintain homeostasis in callus tissues. This is regulated by scavenging enzymes, including SOD, CAT, and POD. As shown in Fig. 3a, these enzymes showed different activity levels in calli treated with WL, RL, BL, and D. Under the BL treatment, calli exhibited the highest SOD and POD activities but the lowest CAT activity. Calli treated with D showed the highest CAT activity. The three scavenging enzymes showed moderate activity levels when calli were treated with RL. Thus, the RL treatment may maintain ROS homeostasis in calli by regulating scavenging enzyme activity levels.

Chlorophyll content

In our previous experiments, calli rich in chlorophyll rarely differentiated into embryogenic calli, as shown in Supplementary Fig. 1. Thus, we hypothesized that chlorophyll levels in calli were associated with embryogenic callus formation. To evaluate the light quality's function in embryogenic callus differentiation, we monitored the chlorophyll contents in calli receiving different light treatments. Calli treated with BL had the highest chlorophyll b content, followed by those treated with WL, RL, and then D. Meanwhile, there were no significant differences in the chlorophyll a contents among the calli treated with different light qualities (Fig. 3b).

Polyamine content

Total polyamines (PAs), including Put, Spd, and Spm, play important roles in embryogenic callus formation. Therefore, we assessed the levels of these three PAs and total PA in calli treated with WL, RL, BL, and D. As shown in Fig. 3c, the total PA content was greatest in calli treated with RL, as were the Spd and Spm contents. The total PA contents also differed among calli receiving the three other treatments, indicating that different light qualities affect PA biosynthesis and metabolism.

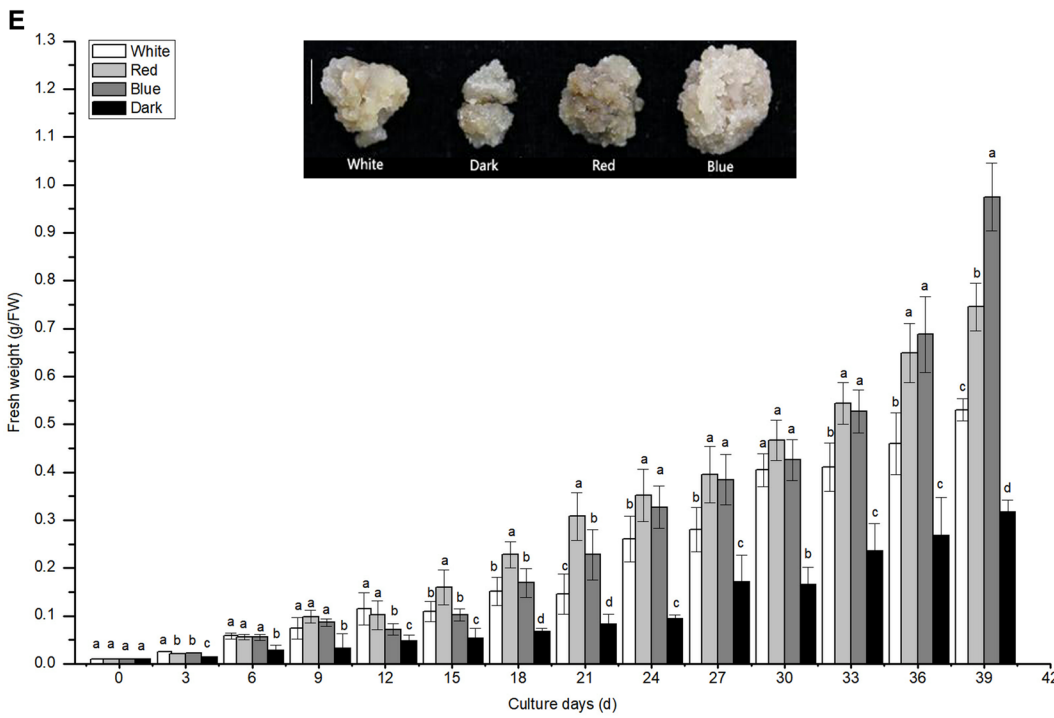
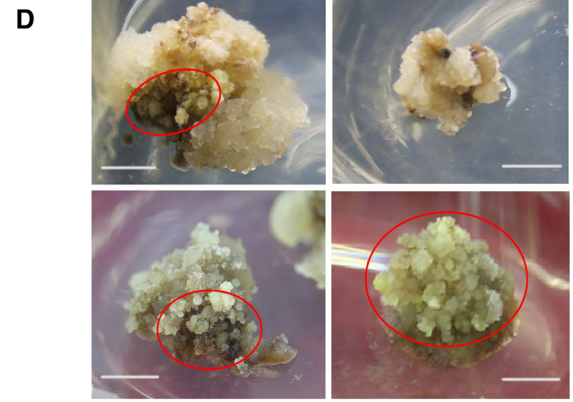
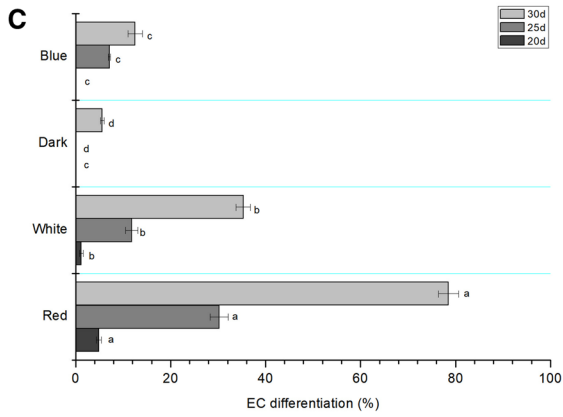
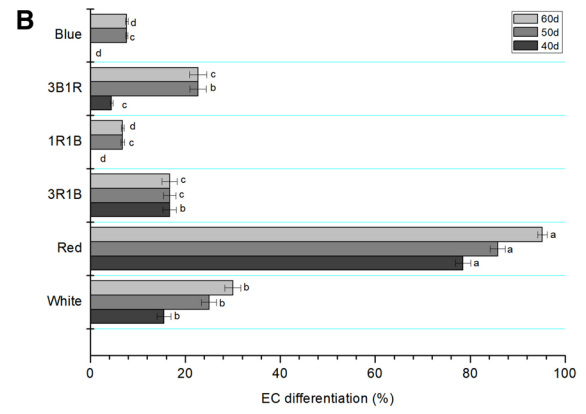
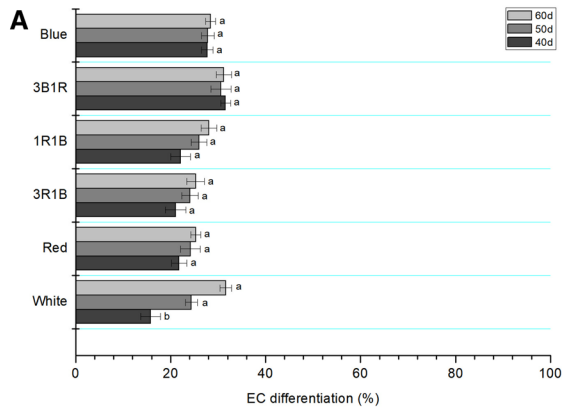


Fig. 2 Cotton embryogenic callus differentiation and proliferation under different light treatments. **a** Different light treatments were applied during the callus induction phase, then white light was applied during the embryogenic callus induction phase; $n=70$. **b** White light was applied during the callus induction phase, then different light treatments were applied during the embryogenic callus induction phase; $n=70$. **c** Four light treatments were applied during the callus induction phase, then white light was applied during the embryogenic callus induction phase; $n=70$. **d** The embryogenic callus at 30 days following the application of blue light (top left, bar=10 mm), white light (bottom left, bar=10 mm), or red light (bottom right, bar=10 mm) during the callus induction phase. Circles represent embryogenic calli. No embryogenic calli were observed following dark treatment (top right, bar=6 mm) at 30 days; **e** cotton callus proliferation under different light treatments during the callus induction phase. (top panel, bar = 10 mm) Callus proliferation (bottom panel) fresh weights of the calli from 0 to 40 days; $n=10$. In each graph, data represent the means of three replicates \pm SDs. Bars with different letter(s) are significantly different based on Tukey's HSD test ($P \leq 0.05$). (Color figure online)

Hormone content

Hormones are important in regulating somatic embryogenesis in plant tissue cultures. Thus, the promotion of cotton embryogenic callus formation by RL may be attributed to the endogenous hormone balance. As shown in Fig. 3d, calli receiving the RL treatment had significantly higher IAA levels than those receiving the BL or D treatment. Moreover, the IAA/ZA ratio in RL-treated calli was the highest among all of treated calli (Fig. 3e), while the endogenous ABA content was moderate in RL-treated calli. Thus, IAA or IAA/ZA may have positive effects on embryogenic callus formation.

Marker genes related to somatic embryogenesis

The expression levels of somatic embryogenesis marker genes, including AGAMOUS-like 15 (*AGL15*), LEAFY COTYLEDON1 (*LEC1*), LEAFY COTYLEDON2 (*LEC2*), BABY BOOM (*BBM*), and WUSCHEL (*WUS*), are associated with embryogenic callus formation. We used qPCR to determine the expression levels of these marker genes in calli receiving different light treatments. RL significantly up-regulated the expression of somatic embryogenesis-related genes compared with WL treatment, while BL resulted in a significant down-regulation of their expression levels compared with WL (Fig. 4). Thus, RL may regulate somatic embryogenesis-related gene expression, resulting in increased embryogenic callus formation.

Discussion

Early somatic embryogenesis involves the differentiation of somatic cells, the acquisition of cell totipotency, and the proliferation and dedifferentiation into embryogenic cells.

However, only cells with activated embryonic-associated genes can be transformed into embryogenic cells (Nomura and Komamine 1985). With the transformation of somatic cells into embryonic cells, the physiological cellular state changes greatly (Feher et al. 2003). Thus, the initiation of embryogenic calli is an important step for somatic embryo development and plantlet regeneration. In the RL treatment, embryogenic calli were well differentiated, and the subsequent somatic embryos were also well developed. Embryoids developed rapidly and grew into healthy plantlets compared with under the WL treatment. After the plantlets were acclimatized and transplanted to the field, their fertility was comparable to the control. The RL application provided a higher standardized cultivation rate than traditional WL treatment.

Effects of light quality and light regimes on embryogenic callus induction

Cotton plantlet regeneration through somatic embryogenesis is difficult because of the length of time required for embryogenic callus induction and the low rate of somatic embryo differentiation. Typical embryogenic callus induction takes 2–3 months and requires many subculture cycles. However, in this study, good quality embryogenic calli were obtained in 20–25 days under the RL treatment. Embryogenic callus formation has traditionally been divided into induction and expression stages, which include callus induction and embryogenic callus induction, respectively, in the formation of cotton embryogenic calli. In the present study, different light qualities applied during the embryogenic callus expression phase did not increase the differentiation rate of embryogenic cells, but a positive effect of RL was observed during the induction phase. Rodríguez-Sahagún et al. (2010) also reported that somatic embryogenesis was affected by the light regimes applied during the induction and expression phases in *Agave tequilana* Weber. Somatic cells acquire embryogenic characteristics by completely reorganizing the cellular state, including its physiology, metabolism, and gene expression (Feher et al. 2002). Because light regimes play an important role in somatic embryogenic callus induction, they should be considered when optimizing protocols for the mass production of somatic embryos in cotton.

We also found that callus growth and dedifferentiation were strongly influenced by different light treatments during the induction phase. As shown in Fig. 2d, the BL treatment significantly promoted callus proliferation but subsequently inhibited embryogenic callus formation. Moreover, the RL treatment significantly increased the percentage of embryogenic cells formed, enabling the production of more somatic embryos in a shorter time than the other light treatments. Thus, the callus state during the induction phase is

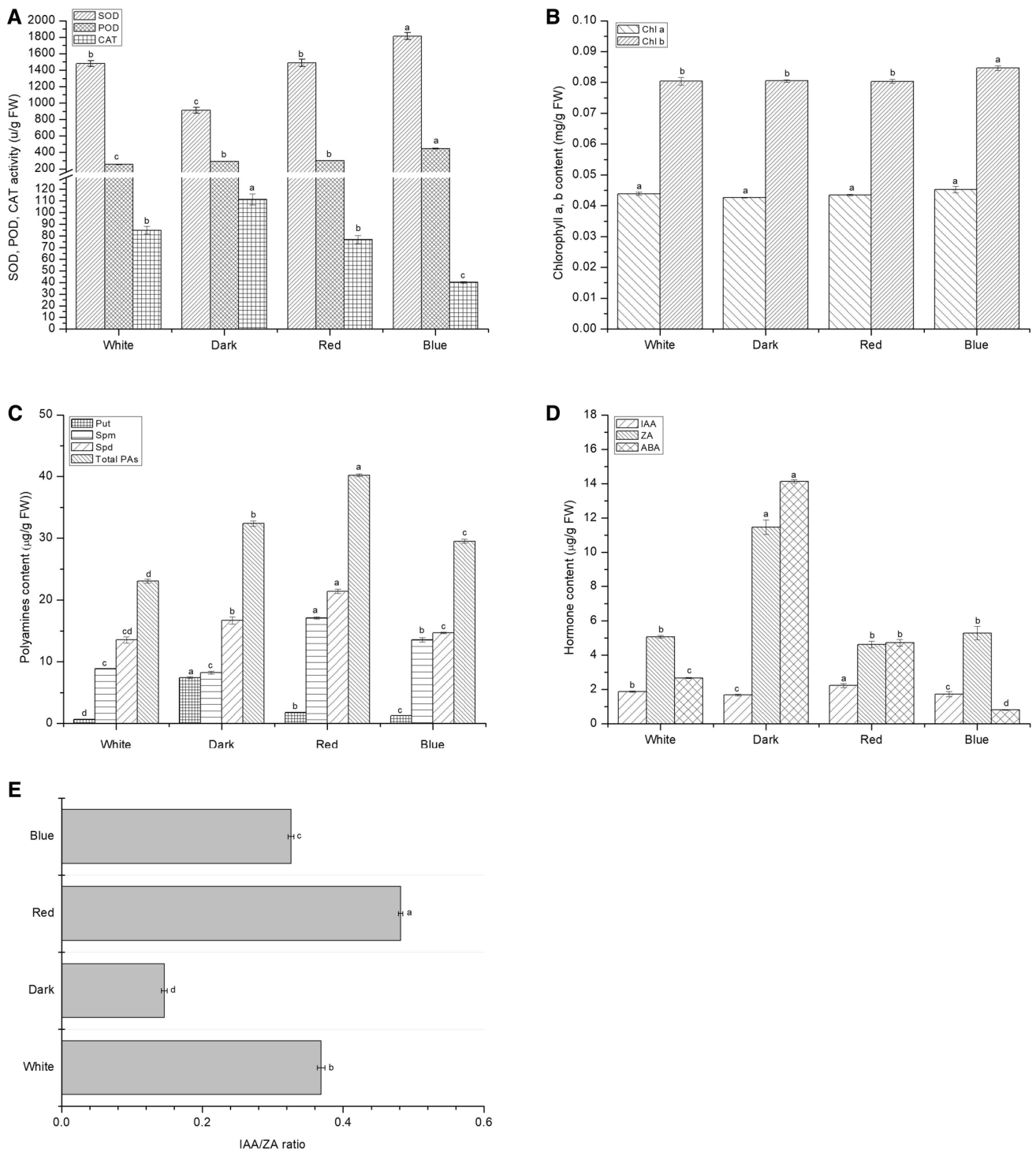
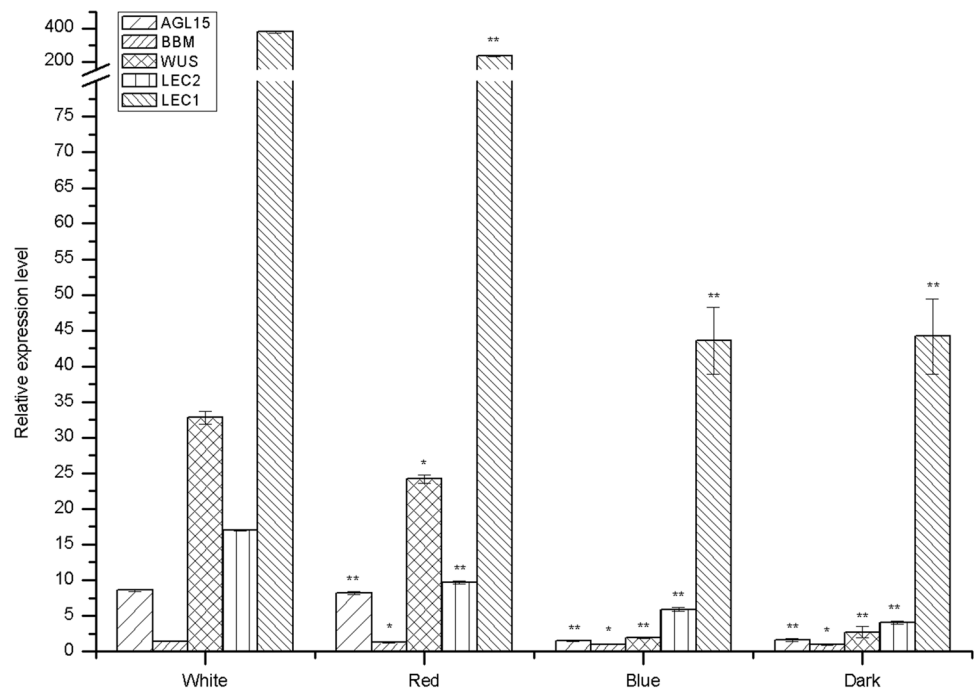


Fig. 3 Effect of antioxidant enzyme activity, chlorophyll content, polyamine content and hormone content in calli grown under different light treatments. **a** Peroxidase, superoxidase dismutase, and catalase activity levels; **b** chlorophyll contents; **c** polyamine contents; **d**

hormone contents; **e** IAA/ZA ratio. In each graph, data represent the means of three replicates \pm SDs ($n=3$). Bars with different letter(s) are significantly different based on Tukey's HSD test ($P \leq 0.05$)

Fig. 4 Expression levels of somatic embryogenesis-related marker genes in calli grown under different light treatments. Data represent the means of three replicates \pm SDs ($n = 3$). The effect of the light treatment on the gene expression pattern was analyzed by Student's t-test to determine the significant differences. Asterisks indicate statistically significant differences compared with the white control (* $P \leq 0.05$; ** $P \leq 0.01$ Student's t-test)



important in determining embryogenic callus induction, which is related to the applied light's quality.

Different plant species, including cotton, can produce various responses to light quality treatments, such as regeneration through somatic embryos. Onofrio et al. (1998) reported that the highest level of quince somatic embryogenesis occurs in cultures subjected to RL treatments and decreases progressively with the transition to RL plus BL and to WL. Nhut et al. (2015) reported that a combination of 60% RL (LED) and 40% BL (LED) promoted *P. vietnamensis* plant regeneration through embryogenic calli, while Baque et al. (2010) also showed that numerous *Morinda citrifolia* calli were induced by a combination of RL and BL.

Antioxidant enzyme activities

CAT, SOD, and POD scavenge harmful free radicals within cells and differences in their activity levels were reported during in vitro organogenesis and somatic embryogenesis in several plant species (Gupta and Datta 2003; Libik et al. 2005). Redox stress plays a vital role in embryogenesis through increased ROS levels (Feher et al. 2003).

Suitable embryonic calli should be moderate in size, and dark yellow or light gray in color under RL. Additionally, under BL, they should grow quickly and not differentiate into embryonic calli within a few days. In this study, POD and SOD activity levels were higher, reflecting rapid callus dedifferentiation and higher FWs. Mathieu et al. (2006) showed that an increase in SOD activity is positively correlated with somatic embryogenesis and the early development of conifers. Blazquez et al. (2009) also detected

increased SOD and CAT activity levels during the initial stages of morphological differentiation in saffron, reflecting changes to the antioxidant enzymatic system of the somatic embryos. Moreover, Rajeswari and Paliwal (2008) reported that in vitro shoot bud differentiation is accompanied by increases in POD and CAT activity levels in cultures kept under WL compared with D conditions. Because various changes in antioxidant enzyme activity levels occur in different plants or at different phases, we hypothesize that RL results in a moderate redox status and that high or low oxidative stress levels are not suitable for the potential competence of embryogenic cells.

Chlorophyll content

Our findings are consistent with previous reports in which BL promotes an increase in the chlorophyll content (Yamazaki 2010; Ye et al. 2017; Fan et al. 2013; Zheng and Van Labeke 2017). We found that calli rich in chlorophyll, appearing mostly green, rarely form embryogenic calli. Therefore, a low chlorophyll content should be a physiological marker in cotton embryogenic callus induction. In the present study, the RL treatment decreased the chlorophyll level of calli and increased the potential of embryogenic callus by modifying the physiological and biochemical states of the calli.

Endogenous hormones

Light regulates auxin-related gene expression partly through phytochrome action (Halliday and Fankhauser 2003), and

auxin is important in regulating the embryonic transitions of somatic cells (Wojcikowska and Gaj 2017; Xu et al. 2013; Padmanabhan et al. 2001; Yang et al. 2012; Michalczuk et al. 1992). Thus, optimal auxin levels are required for the induction and propagation of somatic embryos (Pandey and Chaudhary 2015). In the present study, both the IAA content and IAA/ZA ratio were higher following RL treatments than other treatments, which was consistent with previous reports during cotton somatic embryogenesis (Yang et al. 2012) and in *Cyathea delgadii*, in which the IAA contents of etiolated (capable) explants were higher than those of non-etiolated (incapable) explants (Grzyb et al. 2017).

In the present study, light quality affected the endogenous ABA content, which plays an important role in somatic embryogenesis (Rajasekaran et al. 1987). Ge et al. (2015) previously reported that ABA controls embryo development and maturation in cotton. Low endogenous ABA levels appear to be necessary for the acquisition and conservation of an embryogenic state in *Hevea brasiliensis* (Müll. Arg.) (Etienne et al. 1993), while ABA concentrations and LED light spectra influenced the number of somatic embryos induced and the proliferation of calli in *Peucedanum japonicum* Thunb. (Chen et al. 2016). We found that the ABA content was affected by different light qualities and that RL was conducive to embryonic callus formation, whereas D and BL inhibited formation.

PA content

PAs are present in all plant cells and function in the acquisition of somatic embryogenesis competence (De-la-Peña et al. 2008; Silveira et al. 2006; Shoeb et al. 2001; Minocha et al. 2004; Wu et al. 2009; Paul et al. 2009; Noceda et al. 2009; Nakagawa et al. 2011; Ray et al. 2014; Farias-Soares et al. 2014; Cheng et al. 2015; Anwar et al. 2015; Reis et al. 2016).

There was a high total amine content and high Spd and Spm concentrations in the somatic calli under the RL treatment. However, the Put content was low. When somatic calli transitioned into embryogenic calli, the Put concentration decreased further, while Spd and Spm levels successively increased, revealing their importance in embryogenesis callus and somatic embryo formation. Put levels were similarly low in potential embryogenic cultures and cell aggregates of *Ocotea catharinensis* (Silveira et al. 2006; Reis et al. 2016). In sugarcane var. SP79-1011, high observed levels of Spm could represent signaling required for the acquisition of embryogenic competence (Silveira et al. 2013). Moreover, increased Put, Spd, and Spm levels were reported in embryoids that differentiated early, while the Put content decreased and both Spd and Spm maintained their original levels during the embryoid period in cotton (Cheng et al. 2016a). Thus, PAs can modify somatic embryogenesis.

Somatic embryogenesis-related marker genes

Many genes involved in the initiation and progression of embryogenesis have been characterized, such as *BBM* (Silva et al. 2015), *WUS* (Zuo et al. 2002; Heringer et al. 2017), *LEC1* (Orłowska et al. 2016), *LEC2*, and *AGL15* (Yang et al. 2014; Radoeva and Weijers 2014). These could serve as developmental markers for improving the regeneration of more recalcitrant lines (Mahdavi-Darvari et al. 2014). Indeed, quantitative results in the present study showed that the expression levels of these marker genes were up-regulated in RL-treated calli.

Conclusion

Light quality significantly affects embryogenic callus induction, and the underlying mechanism is complex. Our findings and previous reports suggest that RL promotes an increase in the auxin, Spd, and Spm contents and a decrease in the Put content. Anwar et al. (2015) revealed an association between Spm and the auxin and cytokinin hormone signaling pathways, while changes to endogenous hormone and PA contents are key inductive factors for embryogenesis. RL affected the activity levels of SOD, POD, and CAT in the antioxidative system, enabling the calli to achieve a moderate redox state for cotton embryogenesis. The mechanism by which RL affects endogenous hormones, PAs, and antioxidative enzyme activity levels is still under study. Nevertheless, this study represents the first investigation into the relationship between light quality and the acquisition of embryogenic potential in cotton tissue cultures.

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Author contributions Conceived and designed the experiments: FL and XG. Performed the experiments: YY, WQ, and YL. Analyzed the data: WQ, and CZ. Contributed reagents/materials/analysis tools: YW and ZY. Wrote the paper: YY. Revised the paper: YY, WQ, and YL.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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