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SHORT COMMUNICATION

The accumulation of pigment in fiber related to proanthocyanidins synthesis for brown cotton

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Abstract Brown cotton is a kind of naturally colored cotton. Because of less processing and little dying, it is more friendlier to environment than white cotton. For brown cotton, pigment accumulation in fiber is one of the most important characteristics. In this study, we selected a brown fiber line and a white fiber cultivar to determine the factor that affects the pigmentation in brown fiber. Accordingly, fibers were collected to verify the presence of PAs by *p*-dimethylaminocinnamaldehyde (DMACA) and toluidine blue O (TBO) staining. The PAs content and related genes expressions were determined. As a result, there were obvious differences on the aspect of PAs synthesis in fiber between white cotton and brown cotton. For white fiber, the PAs content reached maximum at 5 DPA, and then gradually decreased to zero. But for brown fiber, the PAs content was increased from 5 to 15 DPA stage, and reached the maximum at the 15 DPA stage, then gradually decreased from 15 to 40 DPA stage. On the contrary, in white cotton, PAs were synthesized in the whole developmental stage from 5 to 40 DPA. And PAs content in brown fiber were far more than that in white fiber, which may be the reason why the brown pigment accumulated in brown fiber.

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Abbreviations

PAs	Proanthocyanidins
DPA	Days post anthesis
DMACA	*p*-Dimethylaminocinnamaldehyde
TBO	Toluidine blue O
CHS	Chalcone synthase
DFR	Dihydroflavonol reductase
ANS	Anthocyanidin synthase
F3H	Flavanone 3-hydroxylase
ANR	Anthocyanidin reductase
PUB	Polyubiquitin

Introduction

Colored cotton has a naturally pigmented fiber that grows in shades of green and brown. Because of less processing and dying procedures, it will induce less harmful chemical effects and is more friendlier to environment than white cotton (Fox 1987).

Brown cotton is a kind of naturally colored cotton, which accumulates brown pigment in fiber. Recently, extraction and analysis of chemical properties primarily demonstrated that the pigment in brown fiber might belong to flavonoids (Hua et al. 2007). Several flavonoid structural genes, including encoding dihydroflavonol reductase (*DFR*), anthocyanidin synthase (*ANS*), flavanone 3-hydroxylase (*F3H*), and anthocyanidin reductase (*ANR*) were cloned from brown fiber (Xiao et al. 2007). This indicated that the cloned flavonoid structural genes and proanthocyanidins (PAs, also called condensed tannins) were involved in the pigmentation in brown fiber.

PAs are colorless polymers of flavan-3-ol units, which are synthesized from the first metabolites via the shikimate and flavonoid pathway (Tian et al. 2008). They present in fruits, leaves, seeds and bark of many species. They usually act as powerful antioxidants with their monomers, such as epicatechin, catechin and epigallocatechin (Tian et al. 2008). Owing to their beneficial effects on cardiac health, immunity and longevity, PAs have recently attracted more attention. Till date, the pathways for the biosynthesis of PAs are essentially clear, and the structural genes such as *DFR*, *ANS*, *F3H*, and *ANR* were cloned in many plant species (Pang et al. 2007; Robbins et al. 2003; Bogs et al. 2006; Sharma and Dixon 2005).

According to reports, the *Arabidopsis thaliana* exhibits brown color which is caused by the oxidation of flavonoids, particularly PAs in developing seed coat (Pourcel et al. 2005). However, to our knowledge, the chemical character of brown pigment in fiber is still unknown; the relationship between PAs and brown pigment had not yet been reported. In this study, two cotton lines including a brown fiber line (Zongcaixuan No. 1) and a white fiber cultivar (Simian No. 3) were selected to test the effect of PAs content on pigment accumulation in brown cotton. Specifically, the presence of PAs in fiber was observed by histochemical staining with *p*-dimethylaminocinnamaldehyde (DMACA) and toluidine blue O (TBO), the PAs content in fiber during different development stages were determined, the relationship between PAs synthesis and brown pigment accumulation in fiber was discussed.

Materials and methods

Plant materials

A cotton line Zongcaixuan No. 1 (brown fiber line) and a cotton cultivar Simian No. 3 (white fiber cultivar) were cultivated in the farm of Anhui Agricultural University, People's Republic of China in the year 2010. The white cultivar was selected as control. The cotton bolls were collected at 3, 5, 10, 15, 20, 25, 30, 35 and 40 days post anthesis (DPA) for the following experiments.

Histochemical staining

p-dimethylaminocinnamaldehyde staining of cotton fiber

The presence of PAs was detected by staining fresh fibers with DMACA solution i.e. 0.1% DMACA in 6 N HCl:95% ethanol = 1:1. About 0.1 g fiber was used and stained with 1 ml DMACA solution. After staining for 10 min, the fibers were washed with 5 ml distilled water thrice. The staining result was observed and documented using a digital camera (HX5C, Sony, Japan).

Toluidine blue O staining of cotton fiber

The fresh fibers from 3, 5 and 25 days were fixed with 0.1 mol/L phosphate buffer (pH 6.8) including 2% glutaraldehyde and 1% polyoxymethylene, then dehydrated with 10–90% aqueous ethanol solution. The fibers were immersed in an aqueous solution containing 0.5% of TBO in 0.1 M phosphate buffer at pH 6.8 for 20 s and washed with distilled water for 1–2 min. The staining result was observed under the microscope and documented using the digital camera (Leica CTR6000, Germany).

PAs contents determination

Determination of PAs content was according to the method described by Ikegami et al. (2009). Briefly, 100 mg of fresh fiber was ground into powder in 5 ml of 80% methanol and sonicated for 30 min. After centrifugation at 4,500×*g* for 10 min at 4°C, the "soluble PAs" in supernatant was collected. The residue was resuspended with 5 ml of 1% HCl in methanol and incubated at 60°C for 1 h, the "insoluble PAs" in the supernatant was collected after centrifugation at 4,500×*g* for 10 min at 4°C. The PAs contents were determined according to DMACA color reaction methods (Li et al. 1996; Ayako et al. 2009). The amount of PAs was calculated as equivalent to (+)-catechin.

Analysis of *m*RNA expression of the structural genes for PAs synthesis by reverse transcription PCR

Total RNA was isolated using CTAB method (Murray and Thompson 1980) and reverse transcribed into *c*DNA using M-MLV Rtase cDNA synthesis kit (Takara, China). The primers to specifically amplify the structural genes: chalcone synthase (*CHS*), *DFR*, *F3H*, *ANS* and *ANR* are as listed in Table 1. Polyubiquitin (*PUB*) gene was amplified as RNA standard. PCRs were amplified for 30 cycles.

Results

Histochemical staining results for brown cotton and white cotton

To observe the presence of PAs, the fiber from development stages: 5–40 DPA were collected every 5 days. As shown in Fig. 1, there are obvious differences between brown and white cotton. For brown cotton, after 35 DPA stage, the brown pigment was gradually accumulated in fiber, and the color became darker and darker. When the

Table 1 The primers of structural genes for PAs synthesis

Genes	Probe sequences	Primer sequence (5′–3′)
GhCHS	*Gossypium hirsutum* (EF643507)	GCNGCNACNAARGCNATHAA
		TGNCCRTCDATNGCNCCRTC
GhF3H	*Gossypium hirsutum* (EF187440)	GGNAARAARGGNGGNTTYAT
		TTNACNACRTTNGCNCCYTC
GhDFR	*Gossypium hirsutum* (EF187441)	ATHAARCCNACNGTNAAYGG
		GGNACRTTRTAYTCNGGRTA
GhANS	*Gossypium hirsutum* (EF187442)	CCNCARGTNCCNACNATHGA
		GGYTGNGGRCAYTTNGGRTA
GhANR	*Gossypium hirsutum* (EF187443)	GCNTGYGTNGTNGGNGGNAC
		GCRAAYTTCCANGCNGCYTT
PUB	*Gossypium barbadense* (AY375335)	CAACGCTCCACCTTGTCCTT
		CGTAAAACCAGAAAACCCAC

fiber was stained with DMACA, the strong blue coloration was observed in Fig. 1b, which indicated the accumulation of a large amount of PAs in brown fiber. Moreover, from 5–15 DPA stage, the gradually darkened coloration explained the increase of PAs content. In contrast with brown cotton, when the white fiber was stained with DMACA, the light blue color was detected merely at 5, 10 and 15 DPA stage (Fig. 1d). After 15 DPA stage, the blue color gradually disappeared. These results indicated that only little amount of PAs was accumulated in white fiber from 5–15 DPA stage. Furthermore, there was no PAs accumulation in a later stage after 15 DPA for white fiber.

To make the difference of PAs accumulation in fiber between brown cotton and white cotton clear, the TBO staining method was selected to observe the accumulation of PAs. As shown in Fig. 2, the fiber was protuberated from the surface of episperm at 3 DPA stage. After staining, it appeared blue, which showed the presence of PAs. Between brown and white cotton, there was no obvious difference on the front and side of protuberant fiber. However, at 5 DPA stage, the distinction was presented. In contrast to the light blue on white fiber, the brown fiber took dark blue on the front and side. Moreover, the coloration difference became distinct at 25 DPA stage. What draws special attention is that there was no blue color on white fiber when compared with brown fiber, which indicated that there was no PAs accumulation in white fiber.

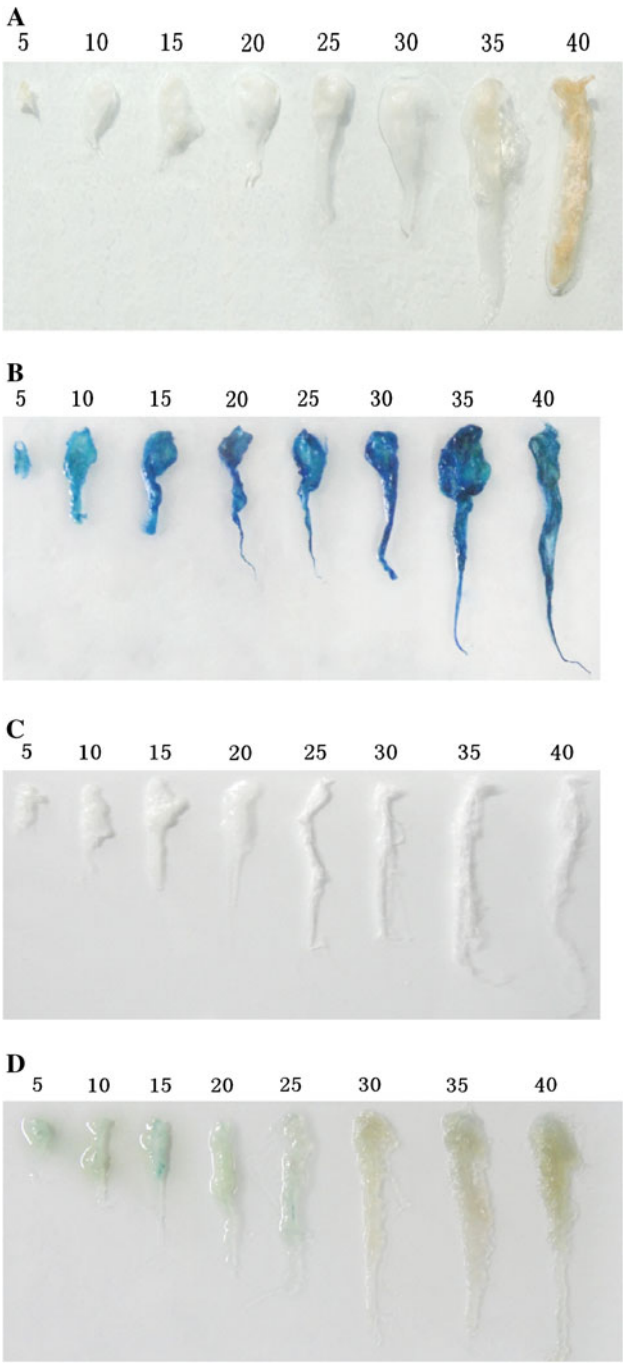

Fig. 1 DMACA staining to show synthesis and accumulation of PAs in fiber of brown cotton and white cotton. **a** brown cotton before DMACA staining; **b** brown cotton after DMACA staining; **c** white cotton before DMACA staining; **d** white cotton after DMACA staining. The numbers *5*, *10*, *15*, *20*, *25*, *30*, *35* and 40 indicated the collection stages (DPA)

PAs content in fiber during different development stages

To further clarify the difference of PAs accumulation between brown cotton and white cotton, PAs content in fiber were determined and calculated as equivalent to (+)-catechin. Figure 3 shows that significant differences were detected between brown cotton and white cotton from 5 to 40 DPA stage. For brown fiber (Fig. 3a) PAs content was increased first and decreased afterwards. At 15 DPA stage, it reached maximum, which contained 20.67 mg/g of soluble PAs and 16.27 mg/g of insoluble PAs in brown fiber. But for white fiber, (Fig. 3b), the PAs content reached

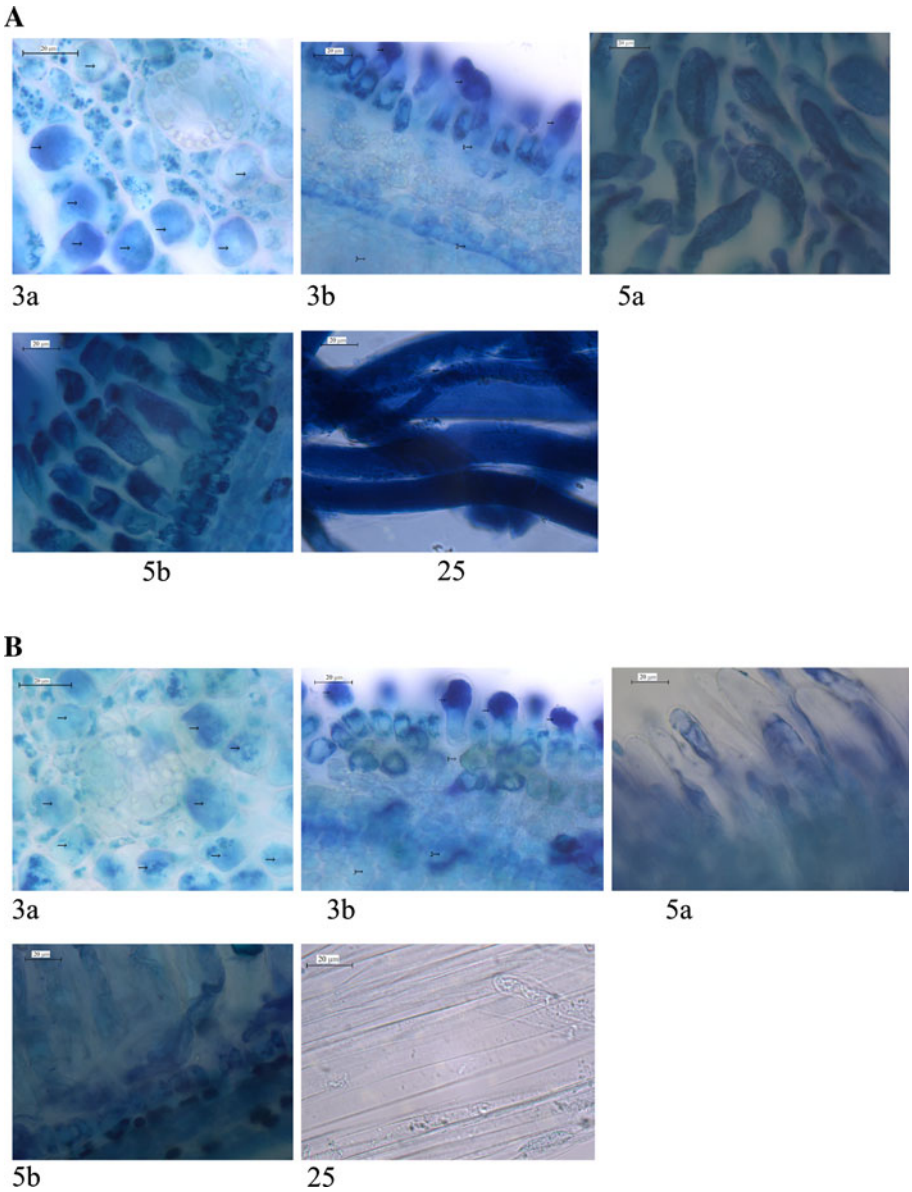

Fig. 2 TBO staining to show presence of PAs in fiber of brown cotton and white cotton. **a** TBO staining result for brown cotton; **b** TBO staining result for white cotton;. The numbers *3*, *5*, and *25* indicated the collection stages (DPA), the letters *a* and *b* showed the fiber initiation on the right surface and side figure for sectioned episperm. The numbers *1*, *2*, *3* in Fig. 3b show the partial structure of cotton seed coat cross-section. *1* epidemis layer, *2* outer pigment layer, *3* palisade layer

maximum at 5 DPA stage. There were only 0.08 mg/g of soluble PAs and 0.14 mg/g of insoluble PAs in fiber, which were 10–12 fold less than that in brown fiber. After 5 DPA stage, the PAs content was then gradually decreased to zero at 20 DPA stage.

*m*RNA expression of the structural genes for PAs

To determine whether the structural genes *GhCHS*, *GhF3H*, *GhDFR*, *GhANS* and *GhANR* were related to the PAs accumulation in cotton fiber, we detected their transcript levels in different development stages. As shown in Fig. 4, for brown fiber, all these genes exhibited high transcript levels in developing fibers, which reached maximum at 15 DPA, and decline at 20–25 DPA. However, for white fiber, five structural genes were only detected in 5 DPA. This observation indicated that these structural genes expressed preferentially in brown fibers, and their temporal expression profiles were consistent with the time course of PAs content accumulation.

Discussion

PAs are present in fruits, leaves, seeds, and bark of many species, such as legume, grape, persimmon and arabidopsis (Tian et al. 2008; Pang et al. 2007; Robbins et al. 2003; Bogs et al. 2006; Sharma and Dixon 2005). They can be oxidized into brown complexes which contribute to the typical brown color of testa in seed (Zhao et al. 2010; Pourcel et al. 2005). Fiber is a kind of specialized epidermis cell. Brown pigment accumulation in fiber may be

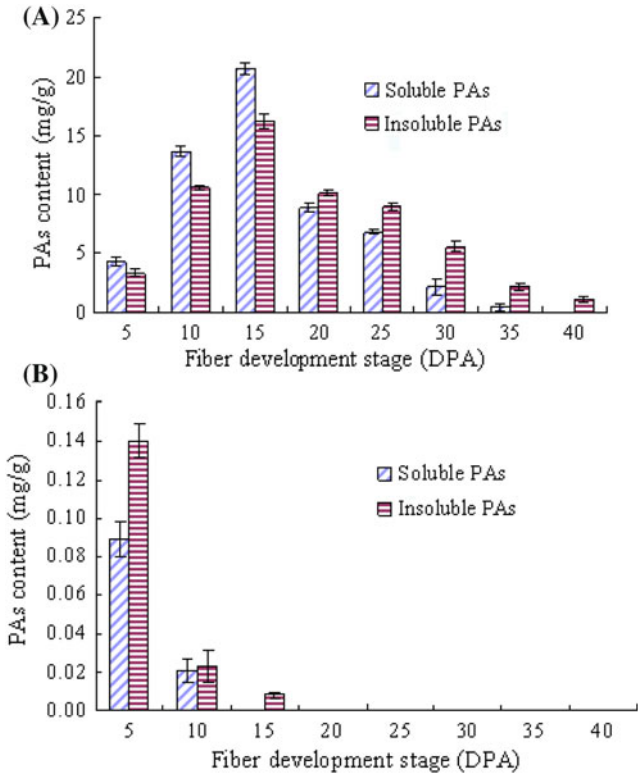

Fig. 3 Changes of PAs content in the brown fiber (**a**) and the white fiber (**b**) during the development stage from 5 to 40 DPA

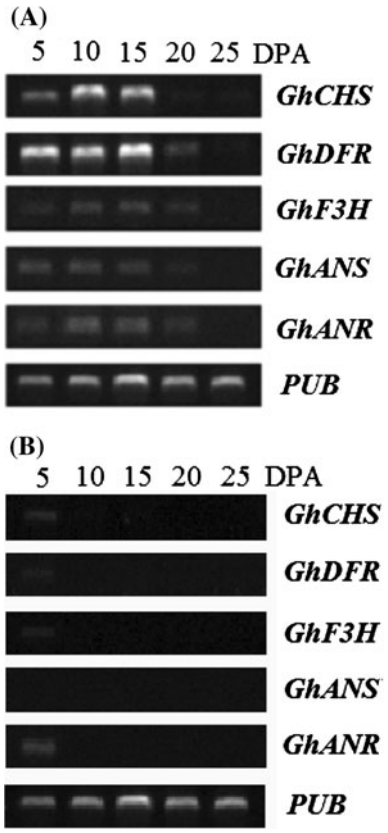

Fig. 4 Structural gene expression of PAs synthesis in fiber of brown cotton (**a**) and white cotton (**b**). The numbers *5*, *10*, *15*, *20*, *25* indicated the collection stages (DPA)

related to PAs synthesis. It has been reported that the pigment in brown fiber belongs to flavonoid (Hua et al. 2007). Furthermore, several structural genes to synthesize PAs such as *F3H*, *DFR*, *ANS* were cloned from brown fiber (Xiao et al. 2007). In the experiment, two cotton lines: a brown fiber line (Zongcaixuan No. 1) and a white fiber cultivar (Simian No. 3) were selected to clearly indicate how the pigment synthesized and accumulated in brown cotton. Our data showed that there are obvious differences in the aspect of PAs synthesis between white cotton and brown cotton. The structural genes to synthesize PAs expressed preferentially in brown fibers. PAs content in brown fiber was far more than that in white fiber. For white fiber, the PAs content reached maximum at 5 DPA, then gradually decreased to zero. But for brown fiber, the PAs content was increased first and decreased afterwards. It reached the maximum at the 15 DPA stage, then gradually decreased in late stages. It is interesting to note that the decrease of soluble and insoluble PAs in late stages was accompanied with the appearance of brown pigment in the fiber. Thus, the brown pigment accumulation in fiber may be caused by oxidation of PAs.

In plants, browning reactions of seed coat are usually caused by oxidation of phenolic compounds and result in the formation of quinones. For arabidopsis, seed coat browning is caused by the oxidation of flavonoid, particularly proanthocyandins. To explain pigmentation of brown in testa, two models were proposed. For early seed coat browning, it could be caused by the enzymatic oxidation of phenolic compounds. For late browning in seeds, it could be caused by chemical oxidation with air molecular oxygen (Pourcel et al. 2005; Turlapati et al. 2010). For two models, direct experimental verification is still lacking, and it is still not clear whether this process is enzymatic or non-enzymatic (Sharma and Dixon 2005; Pourcel et al. 2005). In this context, the brown pigment was gradually shown in fiber only in late development stage when the boll is opening and fiber is exposed to sunshine and air. During senescing and desiccation period of fiber, the brown pigment was gradually accumulated. This may be caused by oxidization reaction of PAs with air molecular oxygen.

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