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Full Length Article

Correlation of Pigment Gland Formation and Gossypol Biosynthesis in Glanded and Glandless Trispecific Cotton Progenies

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Abstract

The pigment gland is a specific organizational structure of cotton (*Gossypium* spp.). The gossypol is a type of sesquiterpene substance that exists in pigment glands and its synthesis is closely related to pigment glands. In order to provide certain basis for studies on the mutual regulation of pigment glands and gossypol as well as utilize the pigment gland trait, Using glanded line GI_1 (glanded both in seed and plant) and GI_2 (glandless both in seed and plant) as research objects, the formation process of pigment gland was observed, the content and degree of accumulation of gossypol in the process of pigment gland formation by high performance liquid chromatography (HPLC) and lead acetate deposition were measured. Cytological observation showed that the pigment gland cells of GI_1 firstly disintegrated into the pigment gland primodium on 25 DPA, while primodium disintegrated into the pigment gland cavity on 29 DPA. Afterwards, the pigment glands appeared on the surface of the cotyledon. There was no pigment gland primodium and pigment gland cavity on the surface of cotyledon from GI_2. HPLC showed that the gossypol content of GI_1 was higher than that in GI_2; while during embryogenesis, the content of gossypol were correlated without significance related to the density and diameter of pigment glands; During seed germination period, the content of gossypol was not correlated with the density or diameter of pigment glands. The result of lead acetate deposition showed that the gossypol of GI_1 deposited not only in the gland primodium and pigment gland cavity, but also in the intercellular space; while the gossypol in GI_2 was deposited only in the intercellular space. The results showed that the pigment gland was not the only storage place of gossypol. © 2019 Friends Science Publishers

Keywords: Cotton; Pigment glands; Gossypol; Near-isogenic lines
Abbreviations: HPLC, High Performance Liquid Chromatography; PCD, programmed cell death**;** HE, Hematoxylin-Eosin; DPA, Days Post-anthesis

Introduction

Pigment glands and gossypol are important traits of cotton (Dong *et al*., 2010; He *et al*., 2011). Present as black or brown red spots, pigment glands spread over the various organs of cotton except pollens and seed coat (Qiu and Liu, 2008; Wang *et al*., 2016). The gossypol is a specific secondary metabolite in cotton, which can enhance the plant's ability to resist pest and disease (Townsend *et al*., 2005; Scheffler and Romano, 2008; Sun *et al*., 2010). However, due to the gossypol, the cotton seed is harmful to human and ruminant animals, which limits the use of cotton seed as protein and oil resources (Hedin *et al*., 1992; Stipanovic *et al*., 2006; Cai *et al*., 2009; Benbouza *et al*., 2010; Sun *et al*., 2013; Tao *et al*., 2013).

The pigment gland is closely related to the synthesis and accumulation of gossypol (Zhu and Chen, 2005). Studies on the origin and development of cotton pigment glands and correlation with synthesis and accumulation of gossypol have already been reported (Bell and Stipanovic, 1977; Ding *et al*., 2004). Cotton pigment glands originate from a mass of the meristematic cells, develop into group of secretory cells after division, and mature pigment glands are composed of a secreted cavity structure with secretion surrounded by 1 to 3 layers of secreted cells (Standford and Viehoever, 1918). The process of pigment glands formation includes the pigment gland cell firstly disintegrated into the gland primodium, then the gland primodium disintegrates into the pigment gland cavity (Zhu *et al*., 1993, 1998). Different material and plant organization result at different time from the pigment gland primodium disintegration to pigment gland cavity formation (Ding *et al*., 2003, 2004). Studies have shown that the formation of pigment gland is via the programmed cell death (PCD) in cotton (Liu *et al*., 2010). The PCD of pigment gland cell is accompanied by the synthesis and accumulation of gossypol; therefore, the product of pigment gland PCD may be used in the synthesis of gossypol. Gossypol, its derivatives and other secondary metabolism product in the pigment gland were the main substances to make the pigment gland and to show their color (Liu *et al*., 2011).

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Pigment glands are important in the synthesis and accumulation position to gossypols. The questions, a) whether or not the formation of pigment gland participate in the gossypol biosynthesis, b) whether or not the gossypol biosynthesis starts at the initial stage of pigment gland formation and influences pigment gland development and c) where the gossypol is detected and stored in glandless cotton while it does not have pigment gland cavity remain important to answer. In order to provide basis for the mutual regulation of pigment glands and gossypol as well as the utilization of pigment glands trait, we studied the pigment gland formation process and accumulation dynamics of gossypol accumulation during the seed germination and embryogenesis periods.

Materials and Methods

Plant Materials

The near-isogenic lines GI_1 (glanded both in seed and plant) and GI_2 (glandless both in plant and seeds) of cotton (*Gossypium* spp.) were studied. Genetically the lines were trispecific hybrid progenies of (*G. arboreum* × *G. bickii*) × (*G. hirsutum*).

Measurement of Pigment Gland Density and Diameter

For this purpose, the flowers were tagged. The embryos of materials from 22, 25, 29, 35, 40, 45 and 50 DPA were collected. The mature seeds of the lines GI_1 and GI_2 were collected and short fluff was removed by concentrated sulfuric acid and cleaned by sterilized water and then dipped in sterilized water for 12 h. The seeds were germinated on plates containing filter paper and water at 28℃ in an illuminated incubator. The cotyledons were also taken at 2, 4, 6, 10, 12, 24, 36, 48, 60 and 72 h. The diameter and density of the pigmented glands were measured using stereoscopic microscope for 0.25×0.25 cm area.

The Microstructure observation of Pigment Glands

The cotyledons at 2, 12, 36 and 72 h and embryos from 22, 25, 29, 35 and 50 DPA were collected and fixed in FAA. The tissues were dehydrated in increasing concentrations of ethanol, embedded in paraffin and cut into section. The tissue sections were stained by HE after deparaffinized in xylol and dehydrated with decreasing grades of ethanol. The histological structure of pigment glands were observed under microscope (Li, 2007).

Determination of Gossypol Content

The embryos at 22, 25, 29, 35, 40, 45, 50 DPA and the cotyledons at 2, 4, 6, 10, 12, 24, 36, 48, 60 and 72 h were collected, respectively. The extraction of the gossypol was done using previously established method (Chen *et al*., 2012). A 0.5 g samples were grounded into powder in liquid nitrogen and put into 10 mL centrifuge tube with 3~10 mL 70% acetone and stored at room temperature to extract gossypol for 30 min. Subsequently, the gossypol was extracted by ultrasonic sound for 45 min (40℃, 90 W), cooled and centrifuged. The supernatant was diluted up to 25 mL and then filtered through a 0.45 μm (the diameter: 13 mm) microporous membrane. A 5 μL duplicate sample was taken for measurement. At the same time, the standard gossypol sample 10 mg (Sigma) was completely dissolved in pure acetone, and diluted to a final volume 100 mL to make the concentration 100 μg/mL. The standard curve for gossypol was constructed from five increasing concentrations for the standard gossypol of 1, 10, 20, 30, 60×10^{-6} g/mL, using 5 μL of samples. The optimized HPLC method (Luan *et al*., 2010) was used for the determination of the gossypol content, using Agilent1260 chromatograph machine (Santa Clara, CA, American, American Agilent) with inertsil 5 μm ODS-18 (4.6 mm ×150 mm) at 25℃ in methanol/1% phosphoric acid solution 85:15 (v/v) at flow rate of 1.0 mL/min for UV detection at 238 nm. A model was deduced from the linear regression curve analysis with horizontal axis of the peak area and the vertical axis of mass concentration (n=5): $y = 58.137x-11.862$ ($R^2=0.9998$).

Deposition of Gossypol

The method of gossypol deposition was followed as reported previously (Liu *et al*., 2011). The embryos from 22, 25, 29, 35, 50 DPA and the cotyledons at 2, 12, 36 and 72 h were taken and fixed by glutaraldehyde (2.5%) in dimethyl sodium arsenate buffer (0.1 mol/L) with 3% lead acetate (pH=8.0) solution. The tissue section was made for histological determination according to the method of Li (2007).

Statistical Analysis

Difference for the data from Table 1, Fig. 3, 9 and 10 have been statistically treated between GI_1 and GI_2 (n=3 replicates, 10 samples/replicate). The statistical analysis of variance was done determined by using SPSS 19.0 Statistics Software.

Results

The Density and Diameter Observation of the Pigment Gland

Morphological observation: All through the period of embryo formation, the change in pigment glands on the cotyledon surface of GI_1 was as follows: At 22 and 25 DPA , there was no pigment gland on the cotyledon surface (Fig. 1a and b); at 29 DPA, the pigment gland on the cotyledon surface of GI_1 became black (Fig. 1c); at 30 DPA, the pigment gland on the cotyledon surface of GI_1 was light brownish red (Fig. 1d); and at 35 to 50 DPA, the color of pigment glands on the cotyledon surface of GI_1 grew deeper and turned

Table 1: The gossypol content during the period of embryo

Days post-anthesis (DPA)	Gossypol (%)	
	GI_1 ($\bar{x}$±SD)	GI_2 ($\bar{x}$±SD)
29	0.079±0.049*	0±0
35	0.708±0.170**	0.036±0.031
40	1.392±0.507**	0.033±0.012
45	0.845±0.250*	0.161±0.084
50	3.300±1.613*	0.360±0.606

Note: *indicate the significant difference at the 0.05 level, ** indicate the significant difference at the 0.01 level

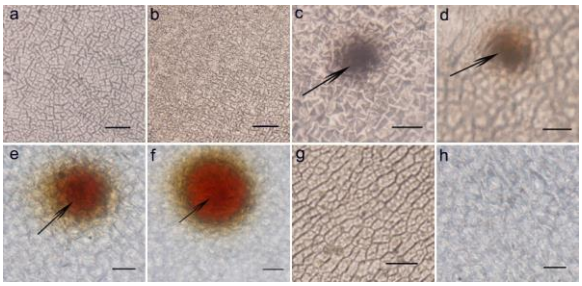

Fig. 1: The pigment gland morphology in GI_1 and GI_2 during the period of embryo formation. The pigment gland morphology in GI_1 (a–f). a 22 DPA; b 25 DPA; c 29 DPA; d 30 DPA; e 35 DPA; f 50 DPA. The pigment gland morphology in GI_2 (g–h). g 29 DPA; h 50 DPA. Bar (a–h) = 20 μm

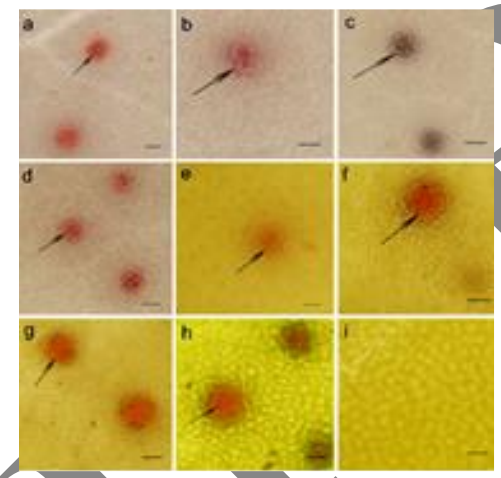

Fig. 2: The pigment gland morphology in GI_1 and GI_2 during the period of seed germination. The pigment gland morphology in GI_1 (a–h) 2, 6, 10, 12, 24, 48, 60 and 72 h. The pigment gland morphology in GI_2 (i). i 24 h. Bar (a–i) = 20 μm

brownish red (Fig. 1e and f). All through the period of embryo formation in GI_2, from 22 to 50 DPA, no pigment gland was seen on the cotyledon surface of GI_2 (Fig. 1g and h). From 2 to 6 h after the seed germination, the pigment gland on the cotyledon surface of GI_1 showed orange color (Fig. 2a and b), then became brown at 10 h after germination (Fig. 2c), Afterwards, the color became deeper and gradually turned to brownish red (Fig. 2d–h). No pigment gland was seen on the cotyledon surface of GI_2 during seed germination (Fig. 2i).

Density and diameter of pigment glands during embryogenesis period: During embryogenesis, the changes in pigment gland density of GI_1 were noted as follows (Fig. 3a): No pigment gland was seen on the cotyledon surface before 29 DPA; while at 29 DPA, pigment glands were clearly seen on the cotyledon surface. From 29 to 50 DPA, the pigment gland density indicated a fluctuating trend *i.e*., it decreased initially then increased and finally decreased. At 35 DPA, the density of pigment gland decreased (48/cm^2); at 40 DPA, the density of pigment glands reached its maximum (102/cm^2) while at 50 DPA it decreased again (64/cm^2). The pigment gland density difference in GI_1 reached its maximum between 40 DPA and other periods except at 50 DPA. The diameter of pigment glands of GI_1 showed an increasing trend. At 50 DPA, the pigment gland diameter of GI_1 reached its maximum, and the difference was significant compared with other periods except for 45 DPA. A correlation coefficient of 0.038 ($P > 0.05$), indicated no association between the density and the diameter of the pigmented glands (Fig. 3b).

Density and diameter of pigment glands during seed germination: During seed germination, the density and diameter of GI_1 pigment glands changed irregularly. The pigment gland density of GI_1 reached its maximum at 2 h after the seed germination (141.8/cm^2), decreased 6 h after the seed germination to its lowest level (74.8/cm^2). The difference in pigment gland density was significant between at 2, 6, 48, and 60 h (Fig. 4a). During the course of seed germination, the pigment gland diameter of GI_1 changed slightly. From 2 to 36 h, the pigment gland diameter lied between 59.4–64.8 μm. At 48 h after germination, the diameter of pigment glands increased sharply, reaching 81.6 μm and then at 72 h after seed germination, the diameter of pigment glands reached 89.2 μm (Fig. 4b). During seed germination period, the correlation coefficient of 0.095 ($P > 0.05$) indicated no association between the pigment gland density and diameter.

Microstructural Observations on the Pigment Gland

Microstructure of pigment gland during the embryogenesis: The morphological change of pigment glands during the embryo formation period was as follows (Fig. 5): At 22 DPA, the cotyledon cells of GI_1 were closely arranged and no special cells were formed (Fig. 5a). At 25 DPA, the gland primodium started to appear in the cotyledon cells. The gland primodium was composed of a dozen of cells that had dark color and dense protoplasm. The gland primodium was spherical, and its cells were closely arranged in 2 to 3 layers. The number of central cells was generally 2 to 3, which were relatively larger with clearly visible cell wall and nucleus. Compared with the central cells, the peripheral cells were squashed into long ovals (Fig. 5b). At 29 DPA, different types of pigment glands began to emerge on the cotyledon of GI_1; some were gland primodia that were likely to disintegrate, while some had already disintegrated into

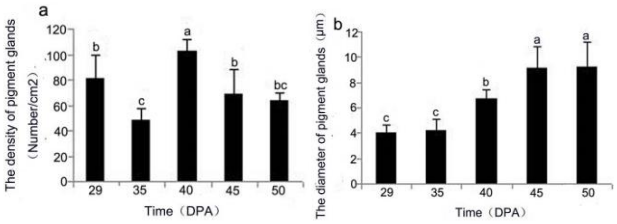

Fig. 3: The density and diameter of pigment gland during the period of embryo formation. a The denisity of pigment gland. b The diameter of pigment gland

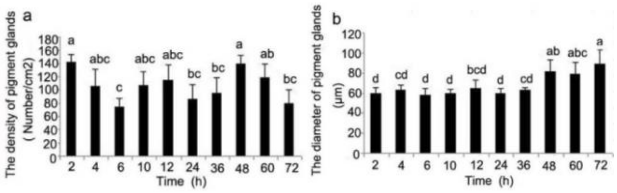

Fig. 4: The density and diameter of pigment gland during the period of seed germination. (a) density of pigment gland and (b) diameter of pigment gland

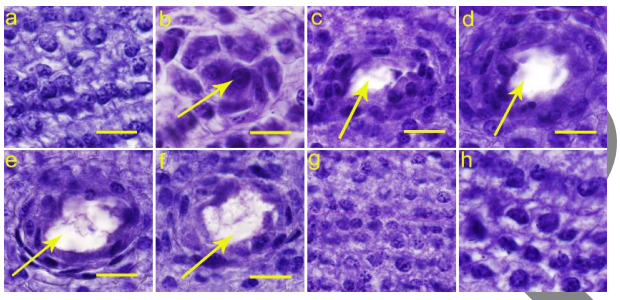

Fig. 5: The formation stages of pigment glands during the period of embryo formation (yellow arrow head). The formation of pigment gland in GI_1 (a–f). a The cell arrangement at 22 DPA; b The formation of the pigment gland primodium at 25 DPA; c The pigment gland primodium beginning to disintegrate at 29 DPA; d The pigment gland cavity at 29 DPA; e the pigment gland cavity at 35 DPA; f the pigment gland cavity at 50 DPA. The formation of pigment gland in GI_2 (g–h). g at 29 DPA; h at 50 DPA. Bar (a–h) = 50 μm

pigment gland cavity. The central cells of gland primodia that would disintegrate were irregular. They began to disintegrate and the cell wall and nucleus became blurred. Under pressure, the peripheral cells became slender thin strip and showed a discontinuous state (Fig. 5c). The cell in the pigment gland cavity became blurred and its nucleus degraded. Upon staining, a deep thick wall was clearly visible, which might have formed from the cell debris after disintegration. The peripheral cells were squeezed into long strips and some filamentous objects could be seen in the cavity (Fig. 5d). From 30 to 50 DPA, the central cells further disintegrated, and the diameter of pigment gland cavity continued to increase (Fig. 5e). At 50 DPA, a secretory cavity was formed in the central position of the pigment gland. A large number of secreting cells in disintegration gathered around it (Fig. 5f). In the embryos formation period, from 22 to 50 DPA, the cells in the young cotyledon of GI_2 were closely arranged; neither gland primodium nor pigment gland cavity appeared (Fig. 5g and h).

Microstructure of the pigment gland at the seed germination stages: At 2 h after seed germination, the pigment gland cavity on the cotyledon surface of GI_1 could be clearly seen. It was comprised of 2 to 3 layers of cells. The peripheral cells were like long strips due to extrusion (Fig. 6a). From 12 to 36 h after seed germination, the peripheral and central cells of the pigment gland cavity were still long strips. The boundary between the cell nucleus and cell wall was even more blurred and some filamentous material was faintly visible in the pigment gland cavity (Fig. 6b and c). At 72 h after seed germination, the material in the pigment gland cavity increased and its color became deeper. Throughout the germination stage, the diameter of the pigment gland cavity changed a little (Fig. 6d). From 2 to 72 h of seed germination, the cells in GI_2 were closely arranged, neither the pigment gland primodium nor the pigment gland cavity appeared (Fig. 6e and f). The main reason that color difference of pigment gland between seed germination and embryo formation was due to difference in staining time.

Gossypol Deposition in the Process of Pigment Gland Formation

Gossypol deposition during embryogenesis: During the embryo formation period, the gossypol in cotyledons of GI_1 mainly existed in the intercellular space and the pigment gland cavity (Fig. 7). At 22 DPA, the gland primodium had not formed, only a small amount of gossypol was seen (Fig. 7a). At 25 DPA, the pigment gland primodium began to form. A small amount of gossypol could be seen in the intercellular space and the newly formed pigment gland primodium (Fig. 7b). At 29 DPA, a large amount of gossypol was deposited not only in the pigment gland primodium (beginning to disintegrate and the pigment gland cavity that was formed by the disintegrated gland primodium) but also in the intercellular space (Fig. 7c and d). From 35 to 50 DPA, the amount of the gossypol in pigment gland cavity continued to grow, nearly filling the entire pigment gland cavity. However, the amount of gossypol deposition in some of the pigment gland cavity was relatively lesser (Fig. 7e and f). From 22 till 50 DPA, only a small amount of gossypol could be seen in the intercellular space in GI_2 (Fig. 7g and h).

Gossypol deposition in seed germination period: From 2 to 72 h after seed germination, the gossypol deposition was clearly seen in the pigment gland cavity and intercellular space of GI_1. The amount of the gossypol deposition was less in the intercellular space; most of the gossypol deposited in the pigment gland cavity (Fig. 8). The gossypol deposition in the pigment gland cavity was not evenly distributed; some cavities were almost completely filled with the gossypol while others were partially filled (Fig. 8a–d).

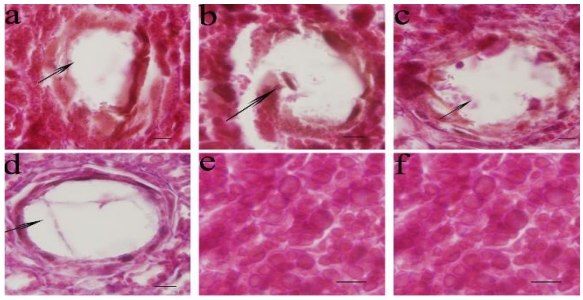

Fig. 6: The formation stages of pigment glands during the period of seed germination (black arrow head). The morphogenesis of pigment gland in GI_1 (a–d). a The pigment gland cavity at 2 h; b The pigment gland cavity at 12 h; c The pigment gland cavity at 36 h; d The pigment glands cavity at 72 h. The morphogenesis of pigment gland in GI_2 (e–f). e The pigment gland cavity at 2 h; f The pigment gland cavity at 72 h. Bar (a–f) = 50 μm

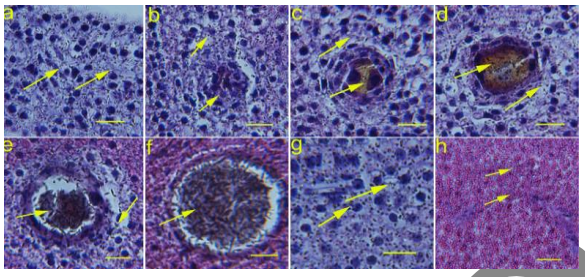

Fig. 7: The gossypol deposition during the period of embro formation (yellow arrow head). The gossypol deposition in GI_1 (a–f). a The gossypol deposition at 22 DPA; b The gossypol deposition at 25 DPA; c The gossypol deposition at 29 DPA; d The gossypol deposition at 29 DPA; e The gossypol deposition at 35 DPA; f The gossypol deposition at 50 DPA. The gossypol deposition in GI_2 (g–h). g The gossypol deposition at 22 DPA; h The gossypol deposition at 50 DPA. Bar (a–h) = 20 μm

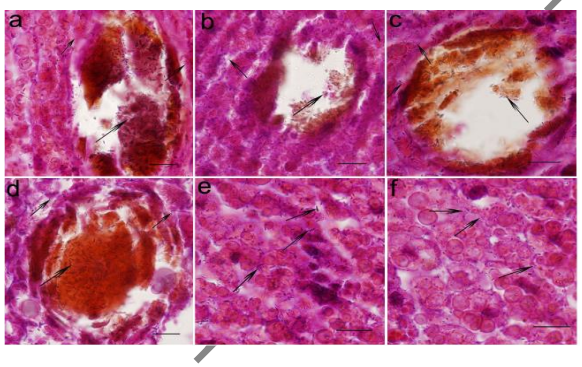

Fig. 8: The gossypol deposition during the period of seed germination (black arrow head). The gossypol deposition in GI_1 (a–d). a The gossypol deposition at 2 h; b The gossypol deposition at 12 h; c The gossypol deposition at 36 h; d The gossypol deposition at 72 h. The gossypol deposition in GI_2 (e–f). e The gossypol deposition at 2 h; f The gossypol deposite at 2 h. Bar (a–f) = 50 μm

During seed germination of GI_2, a small amount of gossypol was found in the intercellular space (Fig. 8e and f). The main reason of color difference in pigment gland between seed germination and embryo formation was due to difference in their staining with HE.

Gossypol Content during Pigment Gland Formation

Gossypol content during embryogenesis: In seed embryo formation period, the gossypol content of GI_1 was higher than that of GI_2 (Table 1). The difference in gossypol content of the two materials reached extremely significant level between 35 and 40 DPA. The changing trend of the gossypol content from GI_1 was identical with that of GI_2 *i.e.*, increased initially, then decreased and finally increased again. At 22 and 25 DPA, the amount of gossypol deposition in GI_1 was relatively smaller and no gossypol was detected by HPLC. At 29 DPA, the gossypol content was 0.079%; from 29 to 40 DPA it increased while at 45 DPA, although decreased but it was still higher than that measured at 29 DPA. At 50 DPA, the gossypol content increased and reached up to 3.3%. The difference of gossypol content reached highly significant level between 50 and 29 DPA (Fig. 9a).

No gossypol was detected from 22 to 29 DPA in GI_2; At 35 DPA, the gossypol content in GI_2 showed an increasing trend and reached 0.036%. At 40 DPA, the content of gossypol decreased slightly, which may be due to heterogeneity of the sampling. From 45 till 50 DPA, the gossypol content again showed an increasing trend. At 50 DPA, the content of the gossypol reached 0.360%. The gossypol contents showed no significant difference at various stage (Fig. 9b).

Drawing relationship in the gossypol content of the pigment gland diameter and its density in embryo formation period of GI_1 showed that the correlation coefficient between gossypol content and pigment gland density was 0.85 (P >0.05) and that between gossypol content and pigment gland diameter was 0.698 (P>0.05). The result indicated that the gossypol content and pigment gland density as well as diameter were not correlated.

Gossypol content during seed germination: In seed germination period, the gossypol content of GI_1 was higher than that of GI_2. The difference between the two materials reached highly significant level except the 36 h after seed germination (Table 2). The gossypol content of GI_1 initially increased, then decreased, then increased again and decreased in the end. From 2 to 4 h after germination, the content of gossypol continued to increase and reached its maximum (1.84%); from 6 h after germination, it began to decrease (0.744%) and was at the lowest level till 36 h after germination, and then increased again. At 72 h after germination, the content of gossypol reached to 1.512%. The difference in gossypol content was significant between 4 and 36 h, There was no significant difference among other stages during the seed germination period (Fig. 10a).

The changing trend of gossypol content in GI_2 showed a decrease initially, then increased and decreased in the end. At 2 h after germination, the content of gossypol was 0.017%; while at 4 h it declined to the lowest level (0.013%) and thereafter it began to increase and reached its maximum value

Table 2: The gossypol content during the period of seed germination

Germination time (h)	Gossypol (%)	
	GI_1 ($\bar{x} \pm SD$)	GI_2 ($\bar{x} \pm SD$)
2	1.121±0.024**	0.017±0.003
4	1.848±0.883*	0.013±0.002
6	1.486±0.085**	0.028±0.032
10	1.713±0.321**	0.024±0.021
12	1.585±0.201**	0.028±0.022
24	1.629±0.274**	0.031±0.014
36	0.744±0.415	0.077±0.031
48	1.206±0.042**	0.050±0.002
60	1.313±0.052**	0.042±0.005
72	1.512±0.191**	0.029±0.010

Note: * indicate the significant difference at the 0.05 level, **indicate the significant difference at the 0.01 level

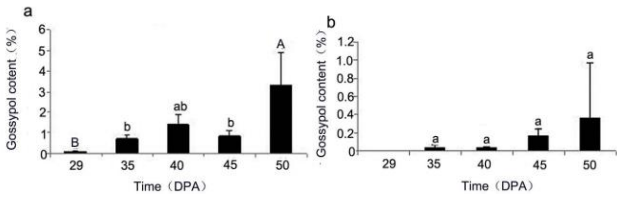

Fig. 9: The gossypol content during the period of embro formation. a The gossypol content in GI_1. b The gossypol content in GI_2

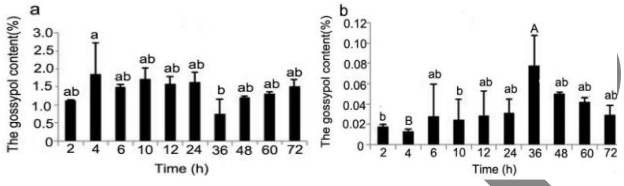

Fig. 10: The gossypol content during the period of seed germination. The gossypol content in GI_1 (a) and gossypol content in GI_2 (b)

(0.077%) till 36 h after germination. At 48 h after germination, the gossypol content decreased again. The gossypol continued to decrease and dropped to 0.029% 72 h after germination. The difference of gossypol content was highly significant ($P < 0.01$) in GI_2 between 4 and 36 h after germination (Fig. 10b).

The correlation analysis among the gossypol content, pigment gland diameter and pigment gland density during the seed germination period of GI_1 showed that correlation coefficients between the gossypol content and pigment gland density as well as diameter were 0.269 ($P > 0.05$) and 0.122, ($P > 0.05$), implying that no correlation existed between gossypol content and the density as well as diameter of the pigment gland.

Discussion

As regards pigment gland morphogensis, this study showed that of GI_1 and GI_2 were obtained from the trispecific hybrid progenies of (*G. arboreum*× *G. bickii*)× (*G. hirsutum*). The gland primodium of GI_1 was formed at 25 DPA; the pigment gland primodium began to disintegrate into pigment gland cavity at 29 DPA. The pigment gland formation process of GI_1 was different from that of *G. bickii* and *G. hirsutum*, the time which the pigment gland primodium disintegrated into the pigment gland cavity was later than that of *G. hirsutum* but earlier than that of *G. bickii*. We speculate this may be related to genetic background, different genetic background induced different forms of genetic interaction. Neither pigment gland primodium nor pigment gland cavity existed in the cotyledon of GI_2 during the embryo formation process and seed germination period. No pigment glands could form on the surface of the cotyledon because of lacking the tissue structure condition, which formed the pigment gland.

The development of pigment glands is a dynamic process and it differs because of different research materials. Ding *et al*. (2004) studied the pigment gland formation process in *G. stocksii* and found that the time of the pigment gland primodium emergence was short and the pigment gland cavity could appeared on 17 DPA. Zhu *et al*. (1999) and Ding *et al*. (2003) studied the pigment gland formation process of *G. bickii* and found the pigment gland primodium in the cotyledon of *G. bickii* appeared on 19 DPA, and the pigment gland cavity appeared on 30 DPA. The reason that pigment glands were not seen on the dormant seed surface of *G. bickii* was only the gland primodium was formed. Liu *et al*. (2013) reported that in *G. bickii*, the pigment glands of its dormant seeds could be placed into two kinds; one kind was pigment gland primodium without secretory cavity, while the other was pigment glands cavity without black secretory substance. The reason that no pigment glands appeared on the dormant seeds surface of *G. bickii* was assigned to the absence of black secretory substance. Related research showed that the time from pigment gland primodium to pigment gland cavity of *G. hirstum* was short; the pigment gland cell had already started to disintegrate into the pigment gland cavity when most pigment gland primodium were not formed (Tong *et al*., 2005).

Earlier studies showed that there was a positive correlation between the gossypol content and the pigment gland density in each part of the plant (Singh and Weaver, 1972; Wilson and Smith, 1976). Contrarily, Xiang *et al*. (1988) thought that there was no positive correlation between the gossypol and the density of the pigment gland. The glanded cotton has low gossypol cotton and the glandless cotton may be gossypol bearing. After careful analysis of five Australian wild species of *Gossypium*, Zhu *et al*. (1997) found no significant correlation between the gossypol content and the density of pigment glands in the cotton organ except the hypocotyl and petal, which showed a significant positive correlation. In this study, in the course of seed embryo formation and seed germination period, the gossypol content of GI_1 was higher than that in GI_2. In the seed embryo formation period, the gossypol content was not correlated with the density and diameter of the pigment gland.

Likewise, during seed germination period, the gossypol content was not related to the density and diameter of the pigment gland. This may be due to different porportion of the pigment gland cavity with less gossypol.

Smith (1974) believed that the root tip was the site of gossypol synthesis. The gossypol synthesized in the root was transported to aerial plant parts and stored in the pigment glands; therefore, the pigment gland was the storage organ of gossypol. Zhu *et al*. (1999) found that in the cotyledon from five wild species of *Gossypium* during the seed germination, the gossypol could be detected until 3 to 4 days after pigment gland formation was completed. It may be inferred that the pigment gland formation and gossypol synthesis were not synchronic. Liu *et al*. (2011) reported that the black reaction only occurred in the part in which the pigment gland formed, and there was no black reaction in other tissue site; and the phenol-quinones substance was in the secreted cells. In this study, the gossypol deposition could be seen in the intercellular space before the gland primodium and the pigment gland cavity were formed. Furthermore, when the pigment gland primodium disintegrated to form the pigment gland cavity, a large amount of gossypol deposition could be clearly seen in the pigment gland cavity as well as in the intercellular space in GI_1 (Fig. 7a–d). Though neither the pigment gland primodium nor the pigment gland cavity was formed, the gossypol deposition was detected in the intercellular space in GI_2 (Fig. 7g and h). The results showed that the pigment gland was not the only place where gossypol was stored. However, whether or not the gossypol influence the pigment gland morphogenesis formation still need further studied.

Conclusion

The time from the pigment gland primodium to the pigment gland cavity was different between GI_1 and other materials. The gossypol content of GI_1 was higher than that in GI_2 and there was no correlation between the gossypol content and pigment gland density as well as diameter. Moreover, the gossypol of GI_1 deposited not only in gland primodium and pigment gland cavity, but also in the intercellular space indicating that the pigment gland was not the only storage place of gossypol.

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