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Article

Cadmium-Induced Structure Change of Pigment Glands and the Reduction of the Gossypol Content in Cottonseed Kernels

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Abstract: The risk of cotton production on arable land contaminated with heavy metals has increased in recent years. Cotton shows stronger and more extensive resistance to heavy metals, such as cadmium (Cd) than that of other major crops. Here, a potted plant experiment was performed to study Cd-induced alterations in the cottonseed kernel gossypol content and pigment gland structure at maturity in two transgenic cotton cultivars (ZD-90 and SGK3) and an upland cotton standard genotype (TM-1). The results showed that Cd accumulation in cottonseed kernels increased with increasing Cd levels in the soil. The seed kernel Cd content in plants grown on Cd-treated soils was 10-20 times greater than the amount in the corresponding controls. There was a significant difference in Cd accumulation in cottonseed kernels at the 400 and 600 μM Cd levels. Cd accumulation was higher in SGK3 and ZD-90 than in TM-1. However, the gossypol content in cottonseed kernels was lower in SGK3 and ZD-90 than in TM-1. There was a negative correlation ($r = 0.550$) between Cd accumulation and the gossypol content in cottonseed kernels. The density of cottonseed kernel pigment glands decreased under Cd stress. This is consistent with the change in gossypol content, which decreased under Cd stress. The damage of the cultivars ZD-90 and SGK3 from Cd poisoning was relatively low under Cd stress, while TM-1 was seriously affected and exhibited Cd sensitivity. Further studies are necessary to understand the cause of the reduced gossypol content in cotton seeds under Cd stress.

Keywords: Cottonseeds; cadmium; gossypol; pigment gland

1 Introduction

Heavy metal pollution in the soil is a global environmental and agricultural production safety problem. In particular, as a nonessential element of plant growth, the heavy metal cadmium (Cd) is the most harmful to humans and animals [1,2]. Cd input into agricultural production occurs through the discharge of industrial solid waste and the use of pesticides and fertilizers that contain Cd [3,4]. It has been shown that Cd can be absorbed and accumulated by plants from the soil and can also exhibit toxicity to plants. High Cd concentrations in the soil affect not only crop yield and quality but also the health of humans and animals through the food chain. Furthermore, many diseases and even death have been caused by Cd uptake by humans [5,6]. Attention has been drawn to the risk of Cd, and more importance was attached to Cd

research and management after the occurrence of itai-itai disease caused by the Cd pollution of rice in Japan in the 1960s. In recent years, the phytoremediation of Cd-contaminated soils by using hyperaccumulator plants has been increasing [7,8]. However, the application of phytoremediation techniques is still very limited due to the low biomass level of hyperaccumulator plants [9].

Cotton (*Gossypium hirsutum* L.) is an important industrial crop with a considerable magnitude of biomass, and it can be cultivated on industrially polluted soils [10,11]. Fiber is the major product of cotton, and the seed is the main byproduct. Cottonseed kernels consist of 30-45% protein and 25-40% oil, making them an important resource for edible oil and protein [12]. Approximately 10-11 million metric tons of cottonseed protein are produced worldwide each year [13]. However, cottonseed has not been widely used as a highly nutritious food resource because it contains gossypol. The gossypol content in upland cotton seeds is usually 0.6%-2% [14]. If there were no gossypol present, cotton seeds would contain enough protein to meet the basic protein needs (50 grams/person) daily of more than 600 million people for one year [13]. Gossypol is a characteristic chemical substance in cotton, which is synthesized in the roots and then transported to the aerial parts of the plant and stored in the pigment glands of cotton [15,16]. It is poisonous to humans and mono-gastric animals because of the six hydroxyl groups and three carboxyl groups in its chemical structure, which can combine with amino acids, especially lysine. To date, a wide range of antiviral, anticancer and antifungal functions of gossypol have been demonstrated in medicine [17]. The steroidogenesis metabolism pathway could be affected by gossypol, which disturbs energy production [18], signal transduction [19], DNA synthesis and repair [20,21], and a variety of cellular enzymes needed by these metabolism pathways. Thus, the growth of the injured cells could be inhibited by gossypol. Pigment glands, which are distributed in the epidermis of cotton plants, also called ‘internal glands’, ‘black glands’, ‘oil glands’, or ‘gossypol glands’, have brown contents and appear as dark opaque spots that are spread all over the plants of Gossypium except on the pollen and stigmas [22]. Gossypol is secreted from pigment glands as an important natural protection substance because of the antagonistic effect on pathogens and pests through the accumulation of the sesquiterpene aldehyde gossypol and related sesquiterpene aldehydes, which are the major toxins in cotton [22,23]. However, no study on gossypol content and pigment gland structure under Cd stress has been reported.

In this study, we investigated the effect of Cd on cottonseed pigment glands in three cotton cultivars ZD-90, SGK3 and TM-1. Our main objectives were to identify cotton genotypes with low seed Cd accumulation in the seeds and tolerance to Cd toxicity, as well as to provide a theoretical basis for improving the comprehensive utilization of cottonseed kernels and for elucidating the mechanism of phytoremediation of Cd-contaminated soil by cotton cultivars.

2 Materials and Methods

2.1 Plant Materials

Three cotton cultivars (TM-1, ZD-90 and SGK3) were used in the present experiment. ZD-90 is a transgenic glyphosate-tolerant cotton from a germplasm with *EPESP-G7* derived from our laboratory; SGK3 is an insect-resistant cultivar obtained from the Biotechnology Center of the Chinese Academy of Agricultural Sciences, and TM-1 is the upland cotton genetic standard line obtained from the USDA, ARS, College Station, Texas, USA.

2.2 Experimental Design and Cd Treatment

The study was carried out at the experimental station of Zhejiang University and repeated for two years. Plants were grown in pots (35 cm diameter × 40 cm depth) outdoors in a large and rainproof greenhouse. Uncontaminated soil (15 kg) with a pH = 6.2, 2.99% organic matter, 1.35% total N (162.58 mg/kg available N), 0.13% total P (23.82 mg/kg available P), and 0.83% total K (112.79 mg/kg available K) was placed in each pot. Five seeds per pot were sown on the 5th of May in 2015 and 2016. Only one resistant seedling

survived in each pot after two weeks. Cd in the form of $CdCl_2 \cdot 2.5H_2O$ was added and maintained to achieve the relatively stable concentrations of 0 (control), 200, 400 and 600 μM during the whole growing period. There were three replications per treatment, which were arranged in a completely randomized experimental design.

2.3 Determination of Cd Content

For the quantification of Cd in cotton seeds, samples were dried to a constant weight at 80°C and ground into a fine powder. They were then wet digested for 4-5 hours by adding a mixture of 4-5 ml HNO_3 and 0.5 ml H_2O_2. Each digested sample was transferred at a constant volume to a 50 ml flask for Cd determination. Cd was quantified using an ICP-mass spectrometer (7500a, Agilent).

2.4 Measurements of Gossypol, Oil, Protein and Cys Contents in Cottonseed Kernels

After ginning, the cotton seeds were delinted with H_2SO_4 and then shelled and milled. Powder samples of the cottonseed kernels were used to determine the gossypol, oil, protein and Cys contents by near-infrared reflectance spectroscopy on a NIR Systems model 5000 instrument (NIR Systems, Inc., Silver Spring, MD, USA) with routine analysis and calibration development carried out according to the WINISI II manual (ISI FOSS NIR Systems/TECTOR, Infra soft International, LLC.) [24].

2.5 Observation of the Cell Structure of the Kernel Pigment Glands

The cotton seeds were delinted with sulfuric acid and then dried after washing with water. Seeds were later shelled, soaked for 12 hours at 30°C, placed on filter paper for 48 hours to allow for germination and then fixed with FAA (70% ethanol:acetic acid:formaldehyde at 90:5:5). The fixed samples were washed with 70% ethanol and dehydrated by passing them through gradient concentrations of ethanol (75%→80%→85%→90%→95%→100%→100%). Dehydrated samples were embedded in paraffin wax, stained and cleared, based on the following steps: 1/2 100% ethanol + 1/2 xylene→xylene→xylene→1/2 xylene + 1/2 paraffin wax→paraffin wax→paraffin wax at an interval of 2~3 hours for each step. The embedded samples were cut with a microtome at a thickness of between 8 and 10 μM. The sliced sections were then dewaxed (xylene→xylene→1/2 100% ethanol + 1/2 xylene→100% ethanol) and rehydrated with gradient concentrations of ethanol (100%→95%→90%→85%→80%→75%→70%→50%→30%→H_2O) for 10~15 minutes at each level. Tissues were stained for 15 minutes with hematoxylin and dehydrated with gradient ethanol, after which a clearing agent (xylene) was added, before the tissues were finally embedded into permanent paraffin sections.

2.6 TEM Analysis of the Ultrastructure of the Kernel Pigment Gland

Seeds of randomly selected plants were fixed overnight in 2.5% glutaraldehyde (v/v) in 0.1 M PBS (phosphate buffered saline, pH 7.0) and washed three times with PBS with fifteen-minute intervals between each washing. The samples were postfixed in 1% OsO_4 (osmium (VIII) oxide) for 2 hours and then washed three times in 0.1 M PBS (pH 7.0) for 15 minutes each time. They were then dehydrated with a gradient ethanol series (50, 60, 70, 80, 90, 95, and 100% ethanol) in 30-minute intervals and, finally, by absolute acetone for 40 minutes. The samples were then infiltrated and embedded in Spurr's resin overnight. After heating the specimens at 70°C for 9 hours, ultrathin sections (80 nm) were prepared and mounted on copper grids for viewing in a transmission electron microscope (H-7650TEM) at an accelerating voltage of 80.0 kV. The analysis of Cd was conducted with energy dispersive X-ray analysis (EDAX GENESIS XM2) combined with TEM.

2.7 Statistical Analysis

Data used for statistical analysis were the mean values of 10 plants in each treatment from the two test years. Statistical analysis was performed using SAS v.9 software and Microsoft Office Excel 2003 with statistical significance at $p \leq 0.05$. All results are expressed as the mean ± SE of triplicate values.

3 Results

3.1 Cd Accumulation in Cottonseed Kernels

Cd accumulation in cotton seeds affects not only the germination rate of the seeds and the survival rate of seedlings but also their comprehensive utilization. The mean value of Cd uptake by seed kernels of the three cotton cultivars is depicted in Fig. 1. The results clearly showed that the amount of Cd accumulated by seed kernels increased with Cd supply and varied between cultivars. Cd accumulation was ten to twenty times that of the control under Cd stress conditions. Cd accumulation was significantly different at all Cd levels compared with that of the controls for all three cotton cultivars. There was a significant difference between Cd accumulation of SGK3 and that of the other cultivars, but there was no significant difference between that of ZD-90 and SGK3 at the 200 μM Cd level. Moreover, the Cd accumulation in seed kernels of SGK3 (10.47 $\mu g \cdot g^{-1}$) was almost twice that in seed kernels of TM-1 (4.29 $\mu g \cdot g^{-1}$). These cultivars responded differently regarding Cd uptake, and the response was in the order TM-1 < ZD-90 < SGK3.

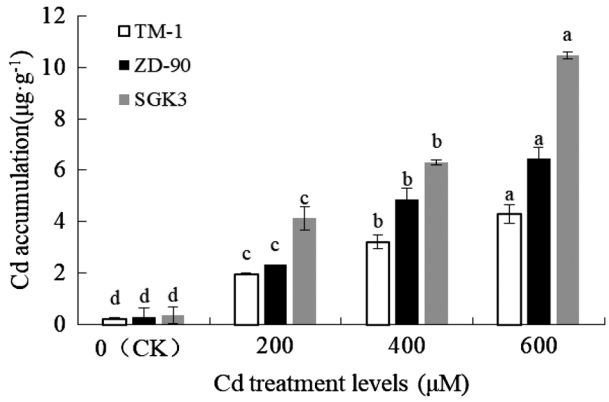

Figure 1: Cd accumulation in seed kernels of three cotton cultivars/lines treated with different Cd levels ($\mu g \cdot g^{-1}$). Different letters for the same cultivar indicate significant differences between different treatments within the same cultivar ($p < 0.05$)

3.2 Gossypol Content in Cottonseed Kernels under Cd Stress

Gossypol has antagonistic effects on pathogenic bacteria and pests in cotton, but it is poisonous to humans and animals. The gossypol content of the three cotton cultivars is shown in Fig. 2. There was a

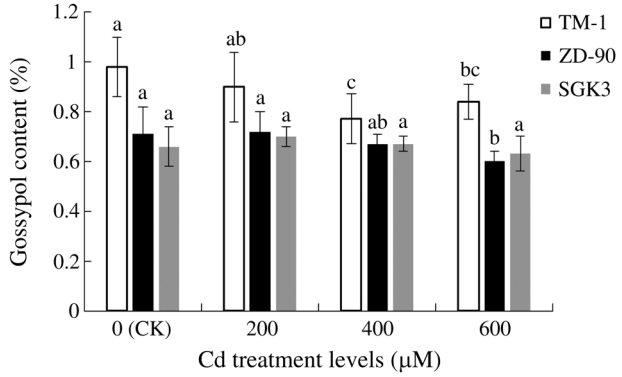

Figure 2: Gossypol content (%) of three cotton cultivars/lines under treatments with different Cd levels. Different letters for the same cultivar indicate significant differences between different treatments within the same cultivar ($p < 0.05$)

relatively high effect of Cd stress on the gossypol content, with a wide difference in the three cotton cultivars. The average gossypol content of the two studied years decreased with increasing Cd levels. The gossypol content in ZD-90 and SGK3 was lower than in TM-1, and it significantly decreased by 21.43% at the 400 μM Cd level in the kernels of TM-1 and by 15.49% at 600 μM in ZD-90 compared with that of the controls, and no significant difference was observed in SGK3. However, a small increase (5.71%) was found in SGK3 at the 200 μM Cd level compared to that of the control. The gossypol content of cottonseed kernels decreased in three cotton varieties (lines) under Cd stress.

3.3 Correlation Analysis of Gossypol Content and other Components under Cd Stress

A correlation study between the gossypol content and Cd accumulation, oil content, protein content and Cys content in the seed kernels of three cotton cultivars is shown in Fig. 3. A significant positive correlation (r = 0.924**) was observed between the average gossypol and oil contents of all cultivars. This correlation was higher in TM-1 (r = 0.93) than in SGK3 (r = 0.22). In contrast, there was a negative correlation (r = -0.550) between the gossypol content and Cd accumulation. Thus, the reduction of gossypol content in cotton seeds may be caused by Cd stress. Cd stress had a higher influence on the gossypol content in TM-1 (r = -0.84) and ZD-90 (r = -0.884) than in SGK3 (r = -0.524). There was a significant negative correlation (r = 0.924**) between the gossypol content and protein content, which was higher in TM-1 (r = -0.987*) than in SGK3 (r = -0.412). A negative correlation (r = -0.868**) was also detected between the gossypol content and Cys content. However, a positive correlation (r = 0.229) was found in SGK3. There was a reduction in the correlation between the gossypol content and other components, especially Cd accumulation and Cys content, in cottonseed kernels under Cd stress.

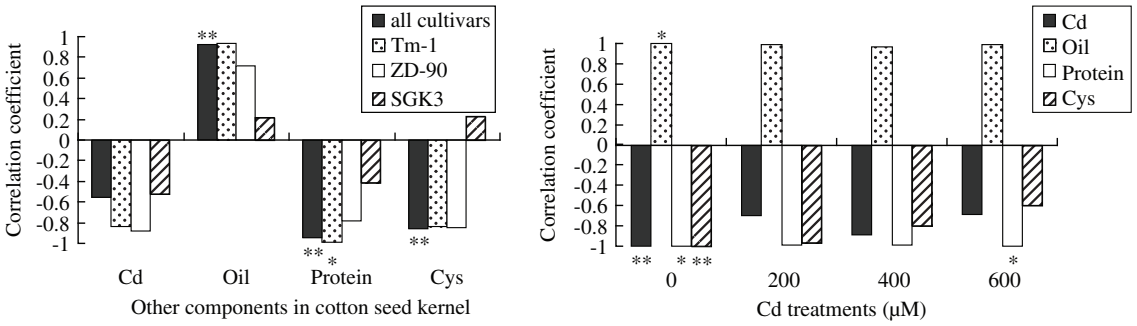

Figure 3: Correlation coefficients between gossypol content and Cd accumulation, oil content, protein content and Cys content in the seed kernels of three cotton cultivars (TM-1, ZD-90, SGK3). Note: * and ** represent significance at levels of 0.05 and 0.01, respectively

3.4 Morphological Characteristics of Seed Pigment Glands under Cd Stress

The pigment gland is a specific structure in cotton and its related plants. The main substances, up to 35-50%, in the pigment gland are gossypol and its derivatives. A linear correlation was found between pigment gland density and the gossypol content in cotton seeds, though they were not generally equal. The morphological observation of cotton seed pigment gland structure under Cd stress is shown in Fig. 4. Pigment glands are shown as dots that could be visible to the naked eye, and their shapes were almost globular or ovoid. The density of the dots decreased gradually in cottonseed kernels with increasing Cd levels. This effect was especially obvious at 600 μM Cd. The density of the dots on SGK3 and TM-1 declined slightly at the 200 μM Cd level, and their sizes were larger than that of those in the control. Moreover, the seed kernels of ZD-90 became black. The surface of the seed kernels became smoother, and the density of its pigment glands was lower in TM-1 at the 400 μM Cd level than at the 600 μM Cd

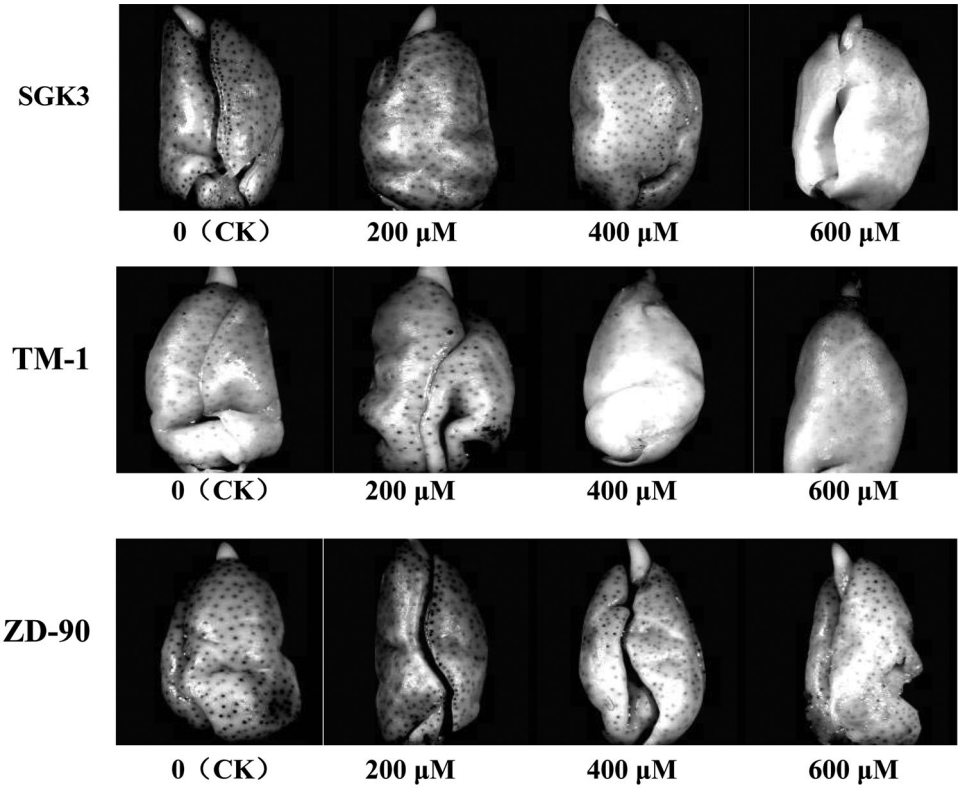

Figure 4: Morphological characteristics of seed pigment glands in the three cotton cultivars/lines treated with various Cd levels

level. The density of pigment glands decreased gradually with increasing Cd levels, and the change in pigment gland content was basically consistent with the gossypol content in the cottonseed kernels.

3.5 Cellular Microstructure Analysis of Seed Pigment Glands under Cd Stress

Analysis of paraffin sections was performed to observe the structure of the pigment glands more clearly under Cd stress (Figs. 5–8).

The cell structure of the pigment glands was globular or ovoid in the control cottonseed kernels. The cavity was rich in solution, mainly containing gossypol and its derivatives. Cells were arranged very closely along the cavity and assumed the shape of a long ellipse by extrusion, while other the cells were normal (Figs. 5A–5C; 5a–5c).

The size of the kernel pigment glands became larger at the 200 μM Cd level in SGK3 and ZD-90 than that of the glands in the corresponding controls, while the change in size was insignificant in TM-1. The shape of the pigment glands and the cells around the glands was similar to those in the control. A slight phenomenon of plasmolysis occurred in the other cells (Figs. 6A–6C; 6a–6c).

It was found that the pigment gland was partially damaged, some cells became loose around the cavity, and the color of the pigment glands became deeper at the 400 μM Cd level in the seed kernels of ZD-90 and TM-1 compared with those in the control. However, less obvious effects were observed in SGK3. Severe plasmolysis occurred in the other cells in the three cotton cultivars (Figs. 7A–7C; 7a–7c).

The damage to the pigment glands was most serious in seed kernels and was even fatal at the 600 μM Cd level in all cotton cultivars. Although there was no significant change between SGK3 and the other cultivars

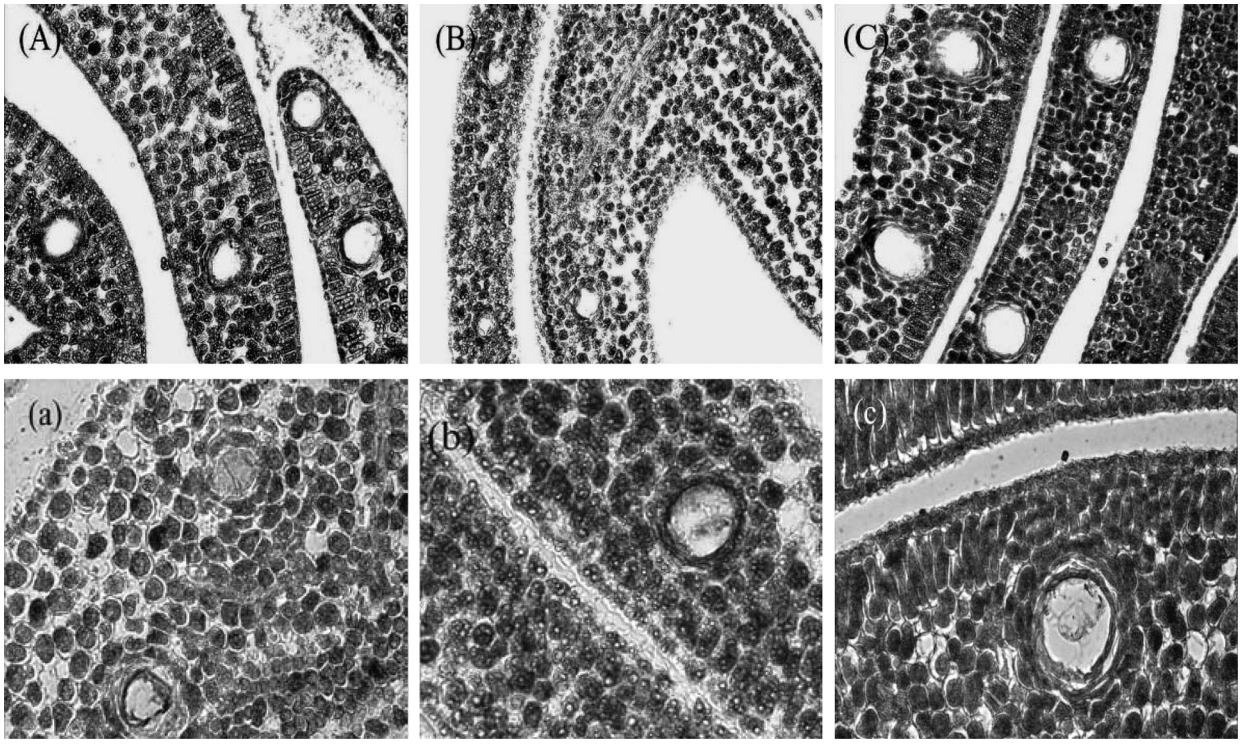

Figure 5: Cell structure of seed pigment glands in the three cotton cultivars/lines treated with 0 μM Cd level. (A, a: SGK3; B, b: ZD-90; C, c: TM-1; capital letters: 20 times magnification; lower-case letters: 50 times magnification)

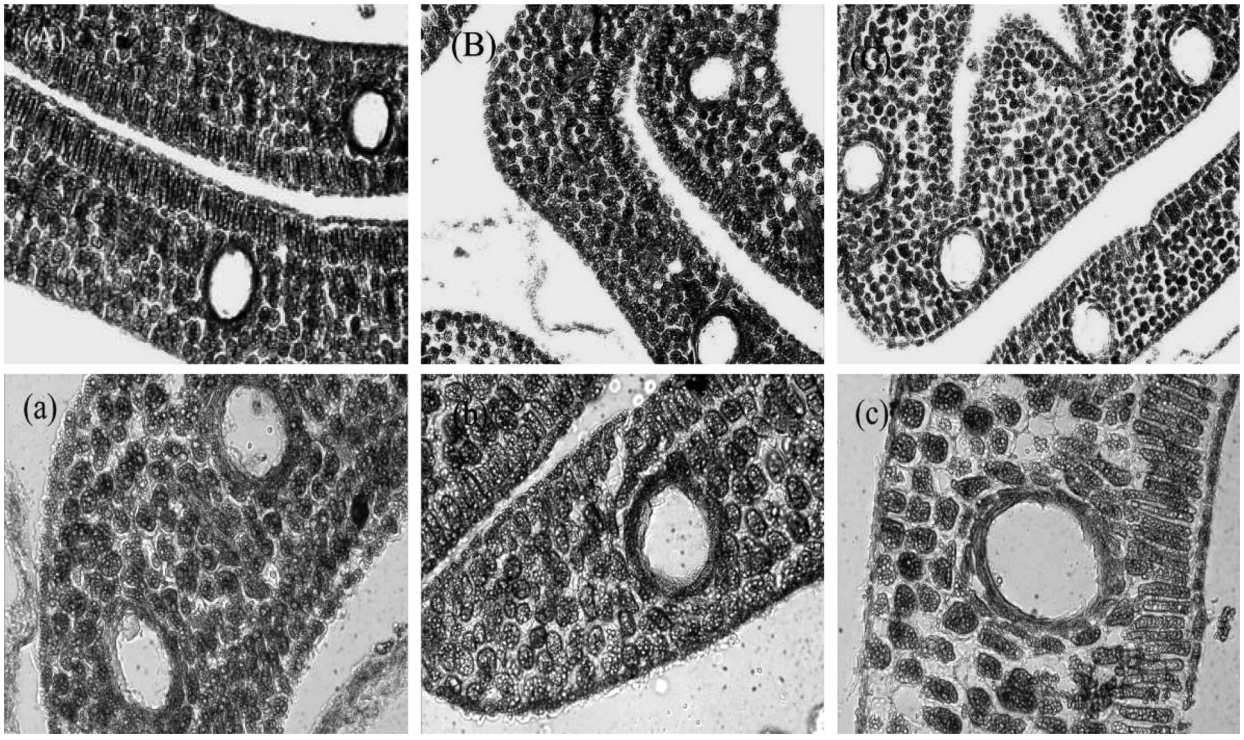

Figure 6: Cell structure of seed pigment glands in the three cotton cultivars/lines treated with 200 μM Cd. (A, a: SGK3; B, b: ZD-90; C, c: TM-1; capital letters: 20 times magnification; lower-case letters: 50 times magnification)

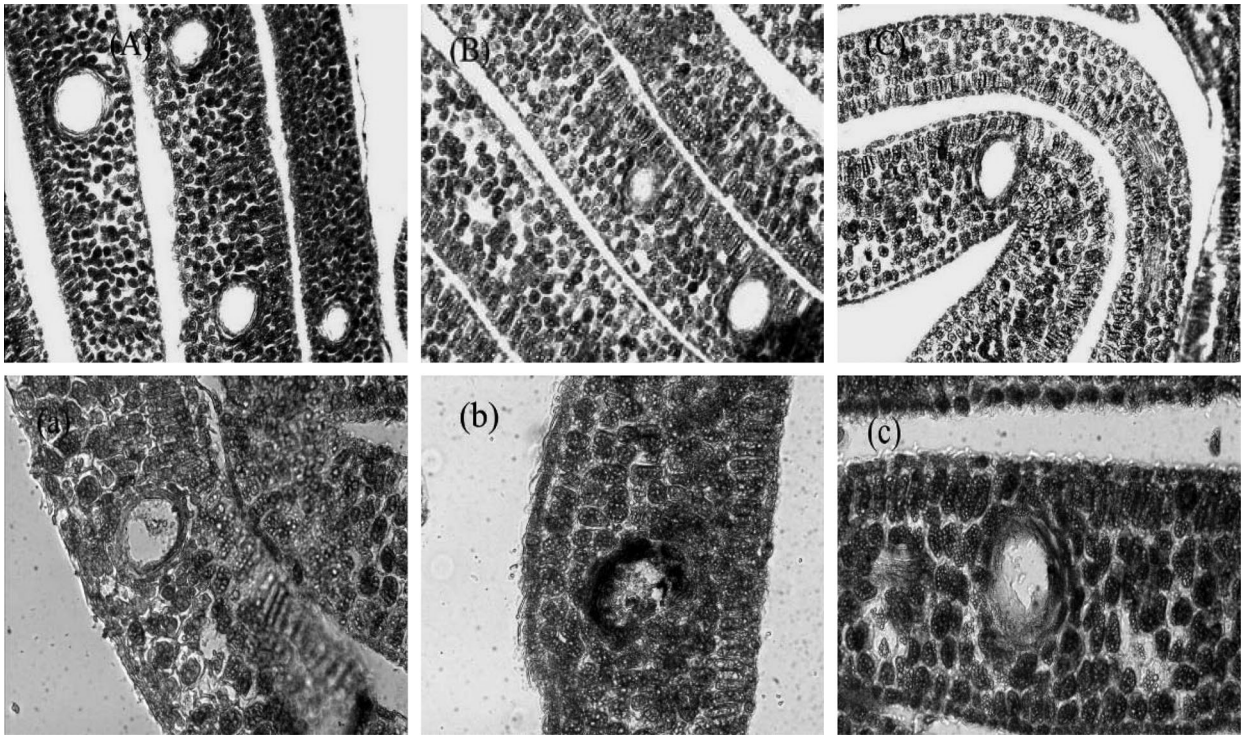

Figure 7: Cell structure of seed pigment glands in the three cotton cultivars/lines treated with 400 μM Cd. (A, a: SGK3; B, b: ZD-90; C, c: TM-1; capital letters: 20 times magnification; lower-case letters: 50 times magnification)

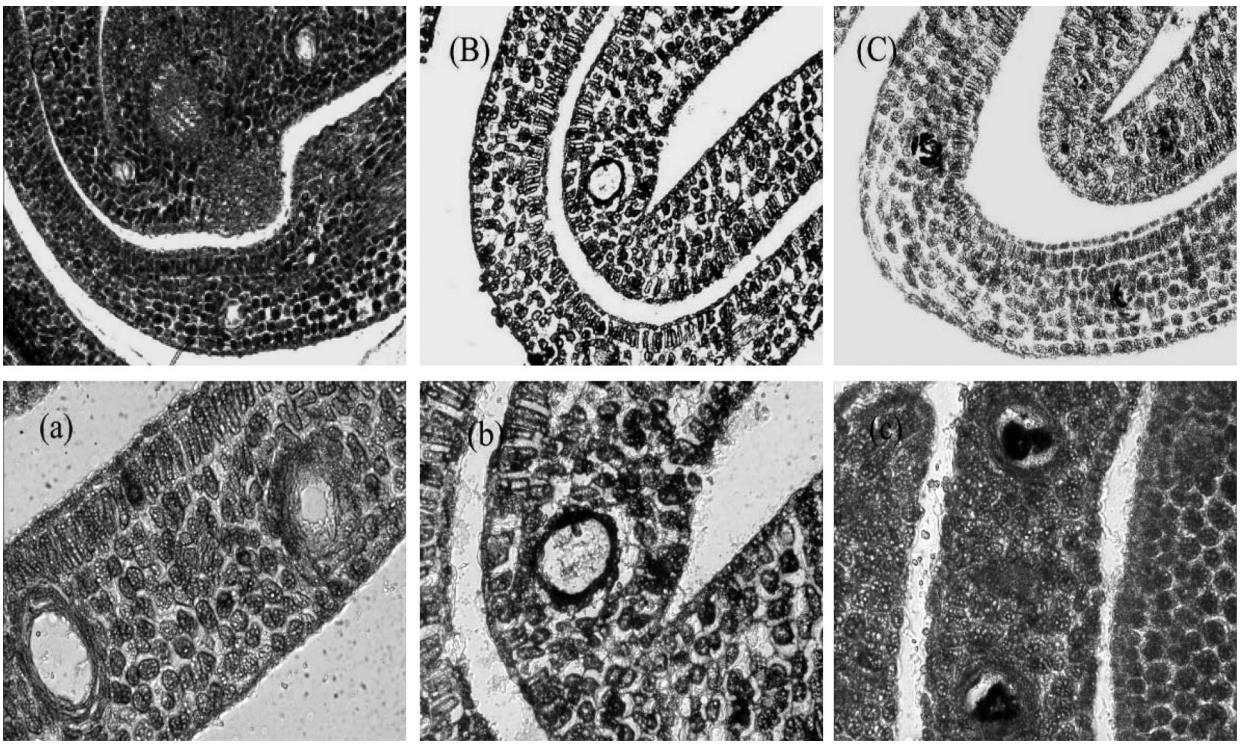

Figure 8: Cell structure of seed pigment glands in the three cotton cultivars/lines treated with 600 μM Cd. (A, a: SGK3; B, b: ZD-90; C, c: TM-1; capital letters: 20 times magnification; lower-case letters: 50 times magnification)

at the 600 μM Cd level, the gland structure was partially affected, and the size decreased. A hollow cavity was formed around which the cells became much closer (Figs. 8A and 8a). The color of the gland walls became deeper, and there were liquid inclusions condensed in the cavity of ZD-90 (Figs. 8B and 8b). The volume of the kernel pigment glands decreased and the color became deeper at the 600 μM Cd level in TM-1. Additionally, the gland cavity was rich in black inclusions (Figs. 8C and 8c). The color of the gland wall became deeper, and the damage to the kernel pigment gland was most serious at the 600 μM Cd level in all cotton cultivars.

3.6 Ultrastructure Analysis of Seed Pigment Glands under Cd Stress

Cd stress had an obvious influence on the seed kernel gossypol content. Gossypol mainly exists in the pigment glands, so its content changes can be inferred from the changes in the pigment gland. The ultrastructure of the seed pigment glands of SGK3 at all Cd levels is shown in Fig. 9.

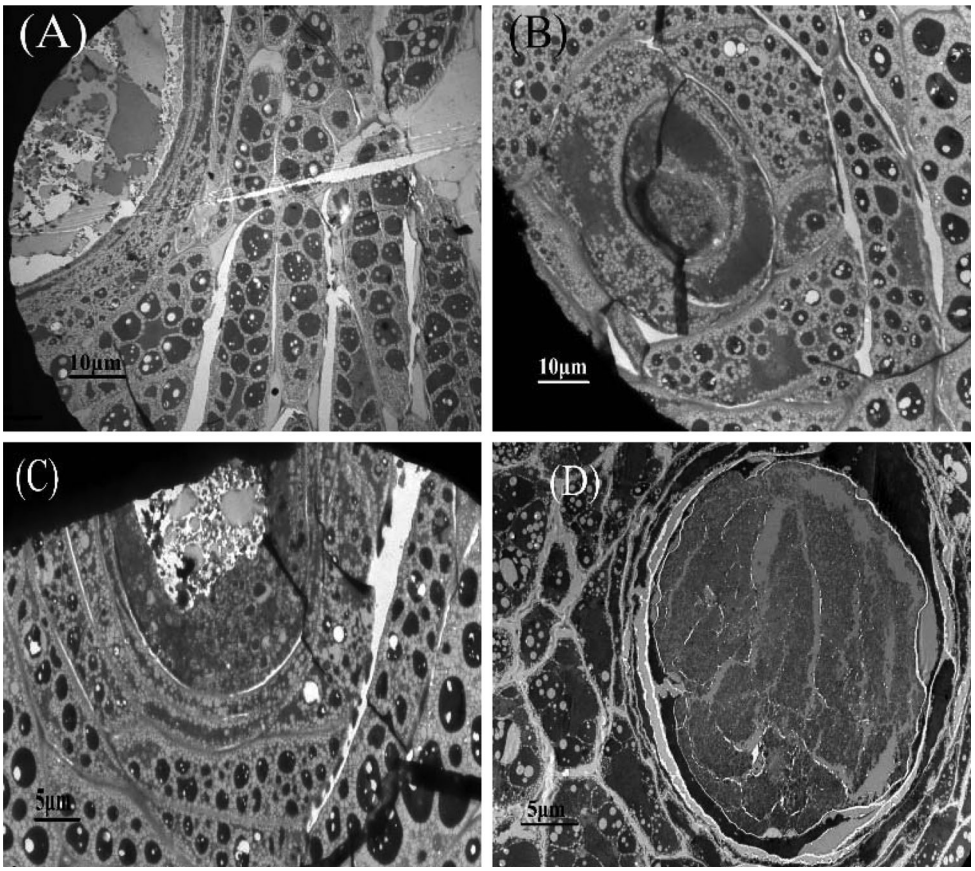

Figure 9: Ultrastructural analysis of seed pigment glands of cotton cultivar SGK3 treated with various Cd levels. (A: control; B: 200 μM; C: 400 μM; D: 600 μM). Scale bars: A = 10 μm; B = 10 μm; C = 5 μm; D = 5 μm

The cavity of the pigment gland was rich in a solution containing gossypol and its derivatives. Cells arrayed very closely near the cavity and were different in size compared to that of cells in the control. The peripheral cells of the cavity were a long ellipse by extrusion, and the nucleus was clearly observed (Fig. 9A). The ultrastructure of seed pigment glands was similar to that of the gland primordium and exhibited a dark color at the 200 μM Cd level compared to that of the control (Fig. 9B). The color of the

gland became dark, and the solution inside the cavity condensed and congregated at the 400 μM Cd level (Fig. 9C). At 600 μM Cd, the color of gland cavity became obviously dark, and liquid inclusions inside the cavity condensed and congregated significantly. An indication of plasmolysis of the gland cavity is shown in Fig. 9D. Furthermore, cells around the gland cavity became closer, and cell organelles were seriously damaged. The cell nucleus was almost invisible.

4 Discussion

4.1 Transgenic Cotton has a High Capacity for Cd Accumulation

Cd is a nonessential nutrient element for plants, but it is one of the most deleterious heavy metals in contaminated soils. Therefore, Cd accumulation varies according to the type of crop and its genetic composition. There was no significant difference in the contents of Pb, Cd, Cu, Cr, and Fe between herbicide-resistant transgenic rapeseed and nontransgenic rapeseed [25,26]. A higher capacity of Cd accumulation was found in transgenic cotton seeds in this study, which was inconsistent with that of Wei et al. [25,26], who found that the Cd content in transgenic cotton seeds was equal to that in nontransgenic cotton seeds. However, our current finding is supported by the results of transgenic cotton seedlings that were more resistant to Cd stress than nontransgenic cotton in our previous research [27,28]. This might be due to gene expression and function in the plants and might also be due to the formation of disulfide, which occurs largely by the function of a foreign gene. The disulfide in phytochelatins (PCs) and metallothioneins (MTs) can be used to chelate Cd very well. It has been reported that PCs can combine 90% Cd absorbed by higher plants [29]. PCs are synthesized enzymatically from glutathione (GSH) by phytochelatin synthase (PCS). They play a key role in detoxification and metabolism in plants by chelating and sequestering heavy metals. The toxicity effect of heavy metals on plants could be reduced by the reduced amount of dissociative metal ions due to the chelation of PCs and metal ions [30]. Therefore, it is very important to study the phytoremediation of Cd-polluted soils using cotton. However, the reason for the high capacity of Cd accumulation in transgenic cotton has to be discussed further.

4.2 The Gossypol Content Decreased as a Result of Cd Stress

Gossypol is a kind of polyphenolic compound that occurs naturally in the pigment gland of cotton. A negative correlation was observed between the gossypol content and Cd concentration in the seed kernels in our study. The reduction of gossypol content might be due to the influence of excessive Cd in the cotton plants. Studies have shown that gossypol is synthesized in the roots and then transported to the aerial parts of the plant [31] and stored in the pigment glands of each organ of the plant. The ability to synthesize gossypol and transport it to other organs is affected by environmental factors. When disease and insects occur, elicitors can induce the expression of genes related to gossypol synthesis, so gossypol plays a very important role in plant growth and development [32]. A good ability to absorb Cd in the roots and then transport Cd from the roots to the shoots in cotton plants was observed [11]. Therefore, we inferred that the decrease in gossypol content may be due to the accumulation of Cd affecting the synthesis or transport capacity of gossypol to some extent, just as when the plant faces pests and diseases. Therefore, it could be speculated that the mechanism of gossypol synthesis under Cd stress in cotton plants is similar to its mechanism known in medicine.

4.3 Cd Stress Results in Decreased Density and Structural Damage of the Pigment Glands

The density of pigment glands generally has a positive correlation with the gossypol content in cotton seeds and other parts of the cotton plant [33], but they are not equivalent (Shi et al.) [34]. In this study, the density of the pigment glands decreased gradually in cottonseed kernels with increasing Cd levels. This result was basically consistent with that of the gossypol content in cottonseed kernels. The analysis results of the paraffin section of the control were consistent with those from the study of Hu [35], whose research showed that the cell structure of the pigment glands in cottonseed kernels was globular or ovoid in the control. The

color of the gland walls became deeper, and the damage to the kernel pigment glands was most serious at the 600 μM Cd level in all cotton cultivars. Viewed in connection with the decreased gossypol content at the 600 μM Cd level, we suggest Cd-induced damage to the pigment gland cavity, which caused a decline in gossypol accumulation in the seed kernels. If all glands in the cotton plant were seriously damaged under Cd stress so that they could not store gossypol, the plant would then be similar to glandless cotton, which has no organ or structure to store gossypol. As a result, the gossypol content was very low in the seed kernels. This finding was consistent with that of the study of Liu [36], whose research showed that the nuclei and condensed chromosomes were located in the pigment glands.

5 Conclusions

In this study, Cd accumulation increased with increasing Cd levels in the seed kernels. The density of the seed kernel pigment glands decreased under Cd stress. This is consistent with the change in gossypol content, which decreased under Cd stress in the three cotton cultivars. The gossypol content was lower in ZD-90 and SGK3 than in TM-1. We also found that there was a significant genotype difference in Cd resistance among the three cultivars. The damage to ZD-90 and SGK3 under Cd stress was relatively low, while TM-1 was the most severely damaged and Cd-sensitive. Overall, cadmium induced a reduction in the gossypol content and pigment gland density in this study. However, the cause of the reduced gossypol content under Cd stress is not clear. It may be caused by the density reduction of the pigment glands or by influence on pigment gland genes, thus affecting gossypol synthesis and metabolism, either because gossypol was involved in a special signal transduction pathway as one of the secondary metabolites itself or in other pathways affected by gossypol synthesis. The functional mechanism of gossypol under Cd stress is highly complex and still needs to be further discussed.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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