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# Morphological and cytological assessments reveal pollen degradation causes pollen abortion in cotton cytoplasmic male sterility lines

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## Abstract

**Background** Understanding the mechanism of male sterility is crucial for producing hybrid seeds and developing sterile germplasm resources. However, only a few cytoplasmic male sterility (CMS) lines of cotton have been produced due to several challenges, like inadequate variation of agronomic traits, incomplete sterility, weak resilience of restorer lines, and difficulty in combining strong dominance. Therefore, the morphological and cytological identification of CMS in cotton will facilitate hybrid breeding.

**Results** Two $F_2$ segregating populations of cotton were constructed from cytoplasmic male sterile lines (HaA and 01A, maternal) and restorer lines (HaR and 26R, paternal). Genetic analysis of these populations revealed a segregation ratio of 3:1 for fertile to sterile plants. Phenotypic analysis indicated no significant differences in traits of flower bud development between sterile and fertile plants. However, sterile plants exhibited smaller floral organs, shortened filament lengths, and anther atrophy on the flowering day in comparison with the fertile plants. When performed scanning electron microscopy (SEM), the two $F_2$ populations revealed morphological variations in the anther epidermis. Cellular analysis showed no significant differences in pollen development before pollen maturation. Interestingly, between the pollen maturation and flowering stages, the tapetum layer of sterile plants degenerated prematurely, resulting in abnormal pollen grains and gradual pollen degradation.

**Conclusion** The results of this study suggest that fertility-restoring genes are controlled by a single dominant gene. Sterile plants exhibit distinctive floral morphology, which is characterized by stamen atrophy and abnormal anthers. Pollen abortion occurs between pollen maturity and flowering, indicating that premature tapetum degradation may be the primary cause of pollen abortion. Overall, our study provides a theoretical basis for utilizing CMS in hybrid breeding and in-depth investigation of the dominant configuration of cotton hybrid combinations, mechanisms of sterility, and the role of sterile and restorer genes.

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**Keywords** Cotton, Cytoplasmic male sterility, Genetic analysis, Morphological characteristics, Pollen development

## Background

Heterosis, also known as hybrid vigor, refers to the phenomenon where the offspring of hybrid varieties of a species or across different species exhibit enhanced traits such as greater biomass, accelerated developmental rates, and higher fertility than their parent plants (Birchler et al. 2010; Groszmann et al. 2013). Production of hybrid seeds is a crucial step for heterosis utilization, which requires minimizing self-pollination and promoting crossbreeding between parent plants through commonly used techniques, such as artificial and chemical emasculation as well as male sterility (Kempe et al. 2011; Colombo et al. 2017; Yahaya et al. 2020; Zhang et al. 2023).

Male sterility has been first observed by the German botanist Joseph Gottlieb Kölreuter in 1973 (Mayr 1986; Zhang et al. 2021). Plant male sterility refers to the inability to produce dehiscent anthers, functional pollen, and viable male gametes, whereas female fertility remains unaffected (Shikanai et al. 1988; Chen et al. 2014; Farinati et al. 2023). Male sterility can be classified into three categories based on the causes: cytoplasmic male sterility (CMS) which is caused by the interaction between mitochondrial and nuclear genes, genic male sterility (GMS) which is regulated by nuclear genes alone, and environment-sensitive GMS (EGMS) which is attributed to the interactions between nuclear genes and environmental factors, including photoperiod-sensitive GMS (PGMS), temperature-sensitive GMS (TGMS), and photoperiod and temperature-sensitive GMS (PTGMS) (Vedel et al. 1994; Chen et al. 2014; Sun et al. 2022; Ranaware et al. 2023).

Cotton, a member of the *Gossypium* genus of the *Malvaceae* family exhibits heterosis in hybrids, which often exceeding the control cultivars in yield by up to 5%–15% (Shahzad et al. 2020; Chen et al. 2022; Zhang et al. 2023). The cotton genus contains approximately 46 diploid species (2n = 2x = 26), including *Gossypium herbaceum*, *G. arboreum*, etc., and seven allotetraploid species (2n = 4x = 52), including *G. hirsutum*, *G. barbadense*, etc. (Zhang et al. 2020; Kushanov et al. 2021; Lima et al. 2021; Jan et al. 2022). Cotton CMS lines are found in various species like *G. harknessii*, *G. trilobum*, *G. hirsutum*, *G. barbadense*, *G. arboreum*, *G. bickii*, *G. anomalum*, and other species (Suzuki et al. 2013; Han et al. 2017; Hamid et al. 2020; Zhao et al. 2020; Li et al. 2022). Among these, certain CMS materials exhibit stable sterile character with broad applications, including *G. harknessii* (CMS-D2), *G. trilobum* (CMS-D8), *G. hirsutum* (CMS-(AD)1), and *G. barbadense* (CMS-(AD)2) (Han et al. 2017; Li et al. 2022). The sterile cytoplasm of *G. harknessii* is the primary cytoplasm source in the "three-line system" (the sterile line, restorer line, and maintainer line) hybrid breeding in cotton, leading to the creation of superior varieties, facilitating cotton improvement, and Chinese CMS cotton cultivars are utilized in research and breeding application globally (Nie et al. 2020; Li et al. 2023). However, the negative effects of cytoplasmic sterility on anther development and yield-related traits impose limitations in cotton breeding (Zhang et al. 2019; Zhang et al. 2022; Zuo et al. 2022).

The cotton flower is hermaphroditic, composed of six parts: the peduncle, receptacle, sepal, petal, stamen, and pistil. The development of mature and fertile pollen is imperative for successful fertilization, and consequently for crop yield (Zhang et al. 2022). Yang et al. (2018) has reported that Zhong41A and Zhong41B has no obvious differences in flower appearance except for the smaller corolla in Zhong41A. However, upon closer examination, after removing the petals, the CMS line Zhong41A exhibited shorter stamen filaments and stigma, indehiscent and light yellow authers, and absent of pollen grains. Similarly, a study by Xuan et al. (2022) focused on CMS-D2 line SI3A and its restorer line 0-613-2R has found no morphological differences in vegetative growth among those materials. However, during the flowering period, anthers of SI3A and 0-613-2R have showed clear differences in appearance and development, and the pollen grains of SI3A remain unstained.

Sanders et al. (1999) has argued that abnormal anther structures and aberrant pollen development could affect plant fertility. Laser et al. (1972) reports that pollen abortion can occur at any stage of pollen development. Pollen development comprises of three major stages: (i) microsporogenesis, which involves the differentiation of sporogenous cells and meiosis; (ii) post-meiotic development of microspores; (iii) microgametogenesis, which involves the microspore mitosis (Chaudhury 1993; Gómez et al. 2015; Halbritter et al. 2018). A study by Li et al. (2021) has found that differences between CMS line J4A and its maintainer line J4B begin to appear at the start of meiosis. In J4A, the middle cell layers do not degenerate, and tapetum cells fail to undergo mitosis and are unable to

provide nutrients for microspore development, leading to microspore abortion (Li et al. 2021). Further, Kong et al. (2017) has observed H276A microspores and has reported that neither the degradation of nuclei during the tetrad stage nor the degradation of tapetal cells occur during microspore development.

In this study, we used the CMS lines as the maternal parents and the restorer lines as the paternal parents. Two distinct $F_2$ segregating populations were constructed as the experimental materials. Genetic analysis was conducted on the fertility survey results. Further, we employed phenotypic investigations, scanning electron microscopy (SEM) analysis, and paraffin section to determine the morphological features, cytological characteristics, and sterility of the experimental materials.

## Materials and methods

### Plant materials and field experiments

Two $F_2$ segregating populations of cotton were established using cytoplasmic sterile lines (HaA and 01A, maternal) and restorer lines (HaR and 26R, paternal), designated as Y66 (HaA×HaR) and Y73 (01A×26R), respectively. The CMS material both are *G. harknessii* (CMS-D2) from the Biotechnology Research Institute, Chinese Academy of Agricultural Sciences. All experimental materials were grown in a teaching test field at Shihezi University (Shihezi, Xinjiang Uygur Autonomous Region, China; 85.94°E, 44.27°N; altitude 436 m). The cultivation followed standard production practices, including regular fertilization and irrigation. The segregating populations were established in 2019, in the procedure of conducting crosses to obtain the $F_1$ generation at first, followed by self-crossing to produce the $F_2$ generation. Morphological and cytological analyses based on fertility were conducted on the $F_2$ segregating populations in 2021.

### Fertility investigations and characteristics flower morphology

To evaluate fertility, each $F_2$ population was assessed during the flowering stage. Plants were categorized and labeled as fertile or sterile based on pollen production, and the data was recorded. To ensure accuracy, assessments were conducted at (nearly) the same time of the day under similar weather conditions. The survey was repeated five times, with at least three flowers per plant were examined each time (Liu et al. 2018). Fertility data were analyzed using IBM SPSS Statistics v.26 to establish the $\chi^2$ tests and *P*-values. The flower buds and blooming flowers from sterile and fertile plants were sampled at the same growth stage to avoid discrepancies caused by incomplete development or organ deformities. The morphology of the floral organs was observed and photographed three times at various developmental stages.

### Sampling and fixation

We selected sterile and fertile flower buds at stages of pollen maturation, pre-flowering, and flowering. Paraffin sections were immersed in an FAA fixative solution (anhydrous ethanol: glacial acetic acid: 37% formaldehyde solution: distilled water=10:1:2:7, v/v), vacuumed, and slowly deflated. This step was repeated three times for 5–10 min. After vacuuming, sections were immersed in a fresh FAA fixation solution and temporarily stored at 4 ℃. After a 24 h incubation period, samples were transferred to 70% ethanol and stored at 4 ℃ (Yang et al. 2012). SEM samples were soaked in a 2.5% glutaraldehyde fixation solution, vacuumed, and slowly deflated. And this process was repeated three times, 5–10 min each time. Samples were then stored in a fresh 2.5% glutaraldehyde fixation solution at 4 ℃ (Wu et al. 2015).

### SEM observations of cotton anthers

Fixed anthers were first immersed in a 2.5% glutaraldehyde fixation solution, followed by soaking in 0.1 mol·$L^{-1}$ phosphate buffer (pH=7.2) for 20 min. This process was repeated three times at room temperature. Subsequently, anthers underwent dehydration using a series of ethanol concentrations: 50%, 70%, 80%, 90%, and 100%, each was incubated at 4 ℃ for 20 min. Next, a fully automated critical-point dryer (Leica EM CPD300, Germany) was used for the $CO_2$ drying. The treated anthers were mounted on a sample platform using double-sided adhesive tape and coated with a metal layer by sputter deposition (Chang et al. 2016; Tian et al. 2018; Liu et al. 2019). Observations and micrograph acquisition were performed using a scanning electron microscope (Hitachi SU8010, Japan) at the Analysis and Testing Center of Shihezi University.

### Paraffin section observation of cotton anthers

The anthers, previously immersed in FAA fixative solution, underwent stepwise dehydration and waxed leaching in the following solution: 75% alcohol for 5 h, 85% alcohol for 2.5 h, 90% alcohol for 2.5 h, 95% alcohol for 1.5 h, and anhydrous ethanol for 30 min, with each step repeated twice. Subsequently, they were soaked in xylene twice for 10 min each time, followed by immersion in paraffin three times for 1 h each time. The wax-soaked samples were then embedded in an embedding machine. The melted wax was poured into the embedding frame and cooled at -20 ℃ on a freezing table. Once the wax

was solidified, samples were sliced into 4–6 μm sections using a microtome. The tissue slides were mounted on slides and dried for 1 h. After dewaxing in xylene twice for 8–10 min each time, samples were rehydrated in ethanol for 10 min. Subsequently, samples were placed in a 0.5% Toluidine Blue O staining solution for 5 min and dehydrated in an alcohol gradient series (Min et al. 2014). Finally, the samples were rinsed in xylene three times for 5 min each time, and the tissue sections were mounted. Finally, an automatic digital slide scanner (3DHISTECH Panoramic MIDI II, Hungary) was used for observation and imaging (Shao et al. 2022).

## Results

### Genetic analysis of the CMS segregating population

In the $F_2$ segregating population, we observed two phenotypes of the Y66 and Y73 plants, one was fertile and the other was sterile. Fertility phenotype was assessed based on pollen during the flowering stage (Table 1). In the Y66 $F_2$ population, we identified 224 fertile plants and 71 sterile plants, consistent with a 3:1 segregation ratio. Similarly, the Y73 $F_2$ population had 239 fertile plants and 88 sterile plants, also following a 3:1 segregation ratio.

### Phenotype observation of the $F_2$ populations

Throughout the growth and development, there were no significant differences in the morphological composition of the floral organs between fertile and sterile plants. However, sterile plants exhibited male sterile flower organs. On the day of flowering, there was no noticeable difference in the color of the floral organs between the sterile and fertile plants, and all petals were in light yellow (Fig. 1 A, E, I, and M). However, there were variations in morphological size, whereas floral organs, petals, and sepals of sterile plants are significantly smaller than the fertile plants among Y66 and Y73 (Fig. 1). Upon removing the petals, noticeable differences in anther histology were observed between sterile and fertile plants. Sterile plants possessed small shrivelled anthers with short and tiny filaments. No pollen was observed in the sterile plants, and the anther surface appeared concave and uneven in light yellow or light brown (Fig. 1D and L). Nevertheless, in fertile plants, we noticed plump and spherical anthers with dispersed pollen which eventually turning to glossy yellow (Fig. 1H and P).

### SEM observation of the anther epidermis in $F_2$

Based on the phenotypic observations, differences were observed in anthers between sterile and fertile plants of Y66 and Y73 on the flowering day, and anthers of the sterile plants displayed deformities. Further observation using SEM revealed that anthers of sterile plants were smaller than those of the fertile plants. Moreover, there were obvious differences in the morphological characteristics of the anther epidermis, including the peak and valley of the fold (Fig. 2). In Y66 sterile plants, the anther epidermis exhibited tight wrinkling and indentation, the epidermis was wavy, with irregular and loosely arranged folds, and smooth peaks and valleys (Fig. 2A–C). Y66 fertile plants displayed fully expanded anther epidermis with prominent scale-like folds arranged in a closed structure, with high peaks and deep valleys, resulting in a relatively rough surface (Fig. 2D–F). Similarly, in Y73 sterile plants, the area between the vascular bundle and the anther chamber contracted tightly. The anther epidermis was irregularly folded and loosely arranged, with relatively mild peaks and valleys (Fig. 2G–I). The anther epidermis of the fully mature Y73 fertile plants was fully expanded, with recognizable wavy folds, and the epidermal folds were closely packed with steep peaks and deep valleys (Fig. 2J–L).

### Paraffin sections of anthers from the $F_2$ populations

To further analyze the sterile characteristics of the $F_2$ segregating CMS population, we prepared paraffin sections of anthers from sterile and fertile plants at stages of pollen maturation, pre-flowering, and flowering (Fig. 3). At the pollen maturation stage, we observed a similar degree of tapetum degradation in anthers of sterile and fertile plants in both Y66 and Y73. The pollen grains were displayed clear spinules on their surface, with no obvious difference been observed (Fig. 3A, E, I, and M). During this stage, there were no differences in pollen grain development between sterile Y66 and Y73, or between their fertile counterparts. In the pre-flowering stage, noticeable differences were found in the degree of tapetum degradation between sterile and fertile plants in Y66 and Y73. The anther chambers of the sterile plants were lack of degradation residues of tapetum cell, with pollen grains exhibited abnormalities and degradation, resulting in empty pollen grains. Furthermore, the nucleolus and cytoplasm were disappeared, eventually forming empty

**Table 1** Segregation ratio of fertile plants and sterile plants in $F_2$ populations

| Materials | Total | Fertility | Sterility | Expected ratio | $\chi^2$ | *P* |
|---|---|---|---|---|---|---|
| Y66 | 295 | 224 | 71 | 3:1 | 0.137 | 0.712 |
| Y73 | 327 | 239 | 88 | 3:1 | 0.637 | 0.425 |

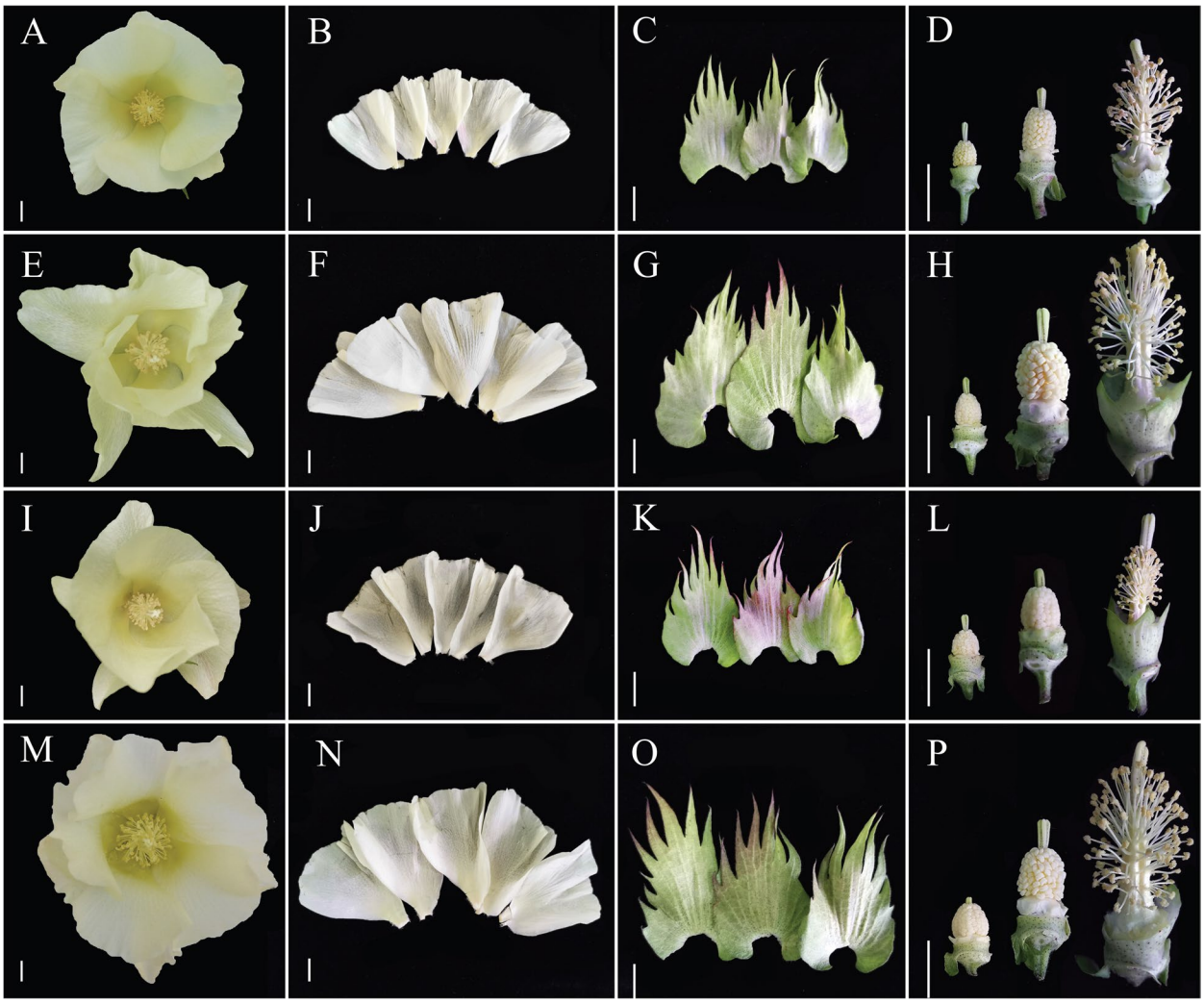


**Fig. 1** Phenotypic observation of Y66 and Y73. Floral organs' morphology, including flower, petal, calyx, and anther at different developmental stages of Y66 sterile plants (**A**-**D**), Y66 fertile plants (**E**–**H**), Y73 sterile plants (**I**-**L**), and Y73 fertile plants (**M**-**P**). Scale bar: 1 cm

pollen grains, with reduction of the sharpness of the pollen grain spinules. In addition, degradation was observed in the cells in the anther chamber and anther vascular bundle area (Fig. 3B, C, J, and K). In fertile plants, fully developed pollen grains showed normal morphology and structure, and the degraded tapetum was evenly distributed (Fig. 3F, G, N, and O). On the day of flowering, anthers from sterile plants showed inward shrinkage of the anther chamber, while those from fertile plants were full expanded (Fig. 3D, H, L, and P).

## Discussion

### Potential of the male sterility for producing hybrid seeds

Hybrid seeds have better characteristics in terms of yield, environmental adaptability, and disease resistance (Groszmann et al. 2013; Du et al. 2020). The selection of the parental line is the key for hybrid seed production (Mukri et al. 2022). Male sterile line is effective for ensuring the purity of hybrid seeds (Chen et al. 2014; Kim et al. 2018). Compared with artificial and chemical emasculation, using male sterile cultivars can significantly reduce labor costs and effectively utilize materials and financial resource (Colombo et al. 2017; Sekiguchi et al. 2023). In addition, the male sterile is a straightforward and effective trait for large-scale seed production. The higher degree of sterility, the greater purity of the hybrid line. The method to utilize male sterility in hybrid seed production include the "three-line system" and the "two-line system" (the sterile line and the restorer line) (Ashraf et al. 2020; Liu et al. 2024). Restorer lines are used for produce commercial hybrid seeds, while maintainer lines are used to propagate the seeds of sterile lines (Wang 2019; He et al. 2020). The use of male sterile lines for hybrid

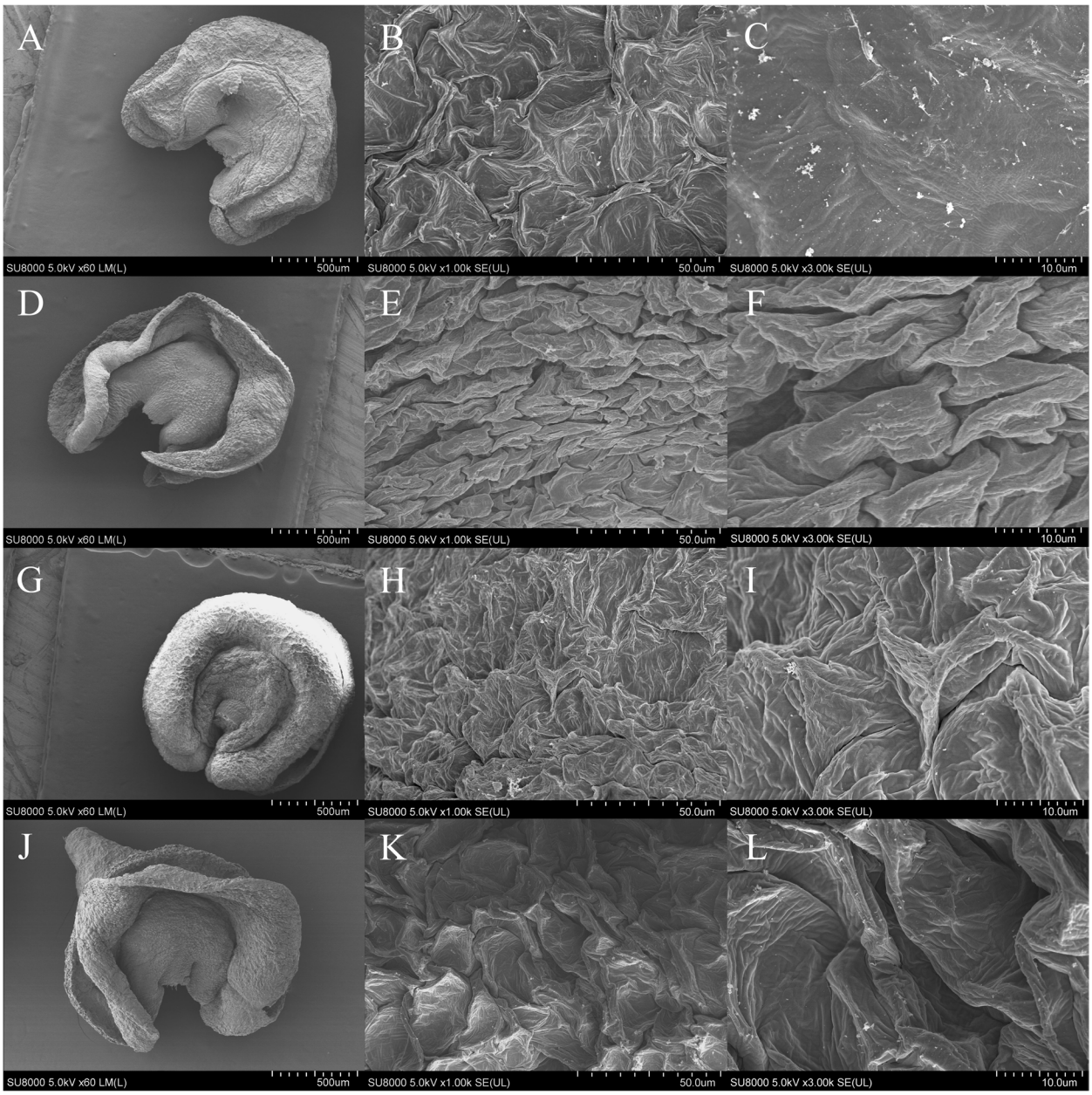


**Fig. 2** SEM observations of the anther epidermis in Y66 and Y73 plants on the flowering day. Anthers and epidermis of Y66 sterile plants (**A**-**C**), Y66 fertile plants (**D**-**F**), Y73 sterile plants (**G**-**I**), and Y73 fertile plants (**J**-**L**). Magnification factor: × 60 (A, D, G, J); × 1 000 (B, E, H, K); × 3 000 (C, F, I, L). Scale bars: 500 μm (A, D, G, J); 50 μm (B, E, H, K); 10 μm (C, F, I, L)

seed production not only optimizes heterosis but also underpins the industrialization of germplasm resources. Thus, many researchers are performing in-depth exploration of excellent germplasm resources or the improvement of existing sterile and restorer lines through genetic engineering technology to promote the application of sterile lines in hybrid production.

### Genetic mechanisms of CMS fertility recovery in cotton

The fertility of the most reported cytoplasmic male sterile lines is controlled by single or multiple genes in plants (Jaqueth et al. 2020; Melonek et al. 2021; Cheng et al. 2023). Meyer (1975) has reported that fertility recovery in *G. harknessii* CMS is controlled by two pairs of independently inherited genes: a dominant gene and a recessive gene. Weaver et al. (1977) and Sheetz et al. (1980) have reported that the fertility recovery was

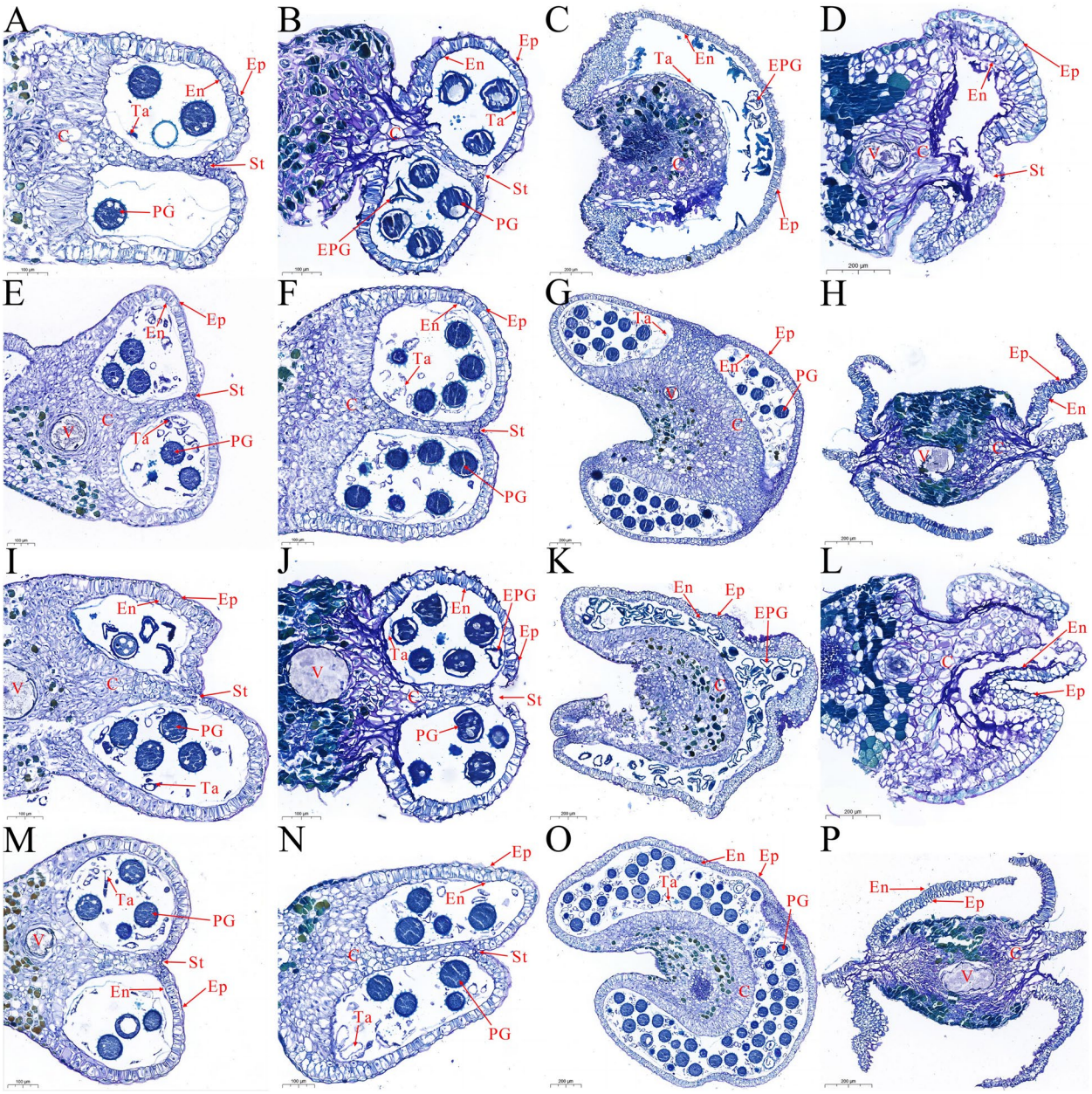


**Fig. 3** Observation of paraffin sections of anther of Y66 and Y73 plants at different developmental stages. Y66 sterile plant anther section at pollen maturation stage (**A**), pre-flowering stage (**B** and **C**), flowering stage (**D**). Y66 fertile plant anther section at pollen maturation stage (E), pre-flowering stage (**F** and **G**), flowering stage (**H**). Y73 sterile plant anther section at pollen maturation stage (**I**), pre-flowering stage (**J** and **K**), flowering stage (**L**). Y73 fertile plant anther section at pollen maturation stage (**M**), pre-flowering stage (**N** and **O**), and flowering stage (**P**). Ep: epidermis; En: endothecium; Ta: tapetum; PG: pollen grain; EPG: empty pollen grain; St: stomium; V: vascular bundle; C: connective. Scale bar: A, B, E, F, I, J, M, and N: 100 μm; C, D, G, H, K, L, O, and P: 200 μm

controlled by a partially dominant gene (*Rf*). Dasilva et al. (1981) have proposed that fertility recovery is controlled by at least three dominant genes. Despite these studies, there is still no consensus regarding the genetic mechanisms underlying fertility recovery in *G. harknessii* CMS. Wang (2019) has reported that fertility recovery in sterile lines is controlled by two independent dominant genes ($Rf_1$ and $Rf_2$), of which $Rf_1$ is completely dominant and $Rf_2$ is partially dominant. The genetic effect of $Rf_1$ on fertility recovery is greater than that of $Rf_2$. In a study by Liu et al. (2018) on the ZBA line, it has been found that the fertility restoration gene in *G. harknessii* is controlled by a single

dominant gene. Further, Gao et al. (2022) have identified 6001A and Cheng et al. (2023) have identified 2074A as genes that restore sterility, suggesting that fertility is controlled by multiple genes. In summary, the complex regulatory mechanisms of CMS leads to inconsistent results across studies. In our study, we have analyzed the fertility of the CMS $F_2$ segregating population genetically and have found that the fertility traits of Y66 and Y73 are controlled by a single dominant gene.

### Morphological characteristics of flower organs in sterile plants of cotton

Flower organs of male sterile cotton exhibit various phenotypes, with different fertility levels. Features such as stamen atrophy, abnormal anther development and pollen sacs, and microspores degeneration are found in abortive flower organs (Zhang et al. 2014; Jiang et al. 2020). Many studies have reported no obvious difference in flower organ composition and developmental processes between sterile and fertile cotton line. However, morphological differences exist between the corresponding fertile plants and the maintainer lines of different sterile lines (Wu et al. 2015; Kong et al. 2017; Yang et al. 2018; Cheng et al. 2020; Li et al. 2021). Our investigations on the flower organ development have shown that the developmental processes of sterile and fertile flowers are generally similar, progressing through seven stages: flower bud differentiation, formation, growth, expansion, whitening, corolla development, and shedding. On the day of flowering, we have noticed no difference in flower organ composition and petal color between the sterile and fertile plants. During the initial phase of flower bud development, the stamens of sterile male flowers have developed normally, and the shape and size of the flower organs are not significantly different from those of fertile flowers. However, differences in filament length, anther morphology, and anther color have been observed between the Y66 and Y73 sterile plants and the fertile plants (Fig. 1). SEM observation has shown that the anthers of sterile plants are smaller than those of fertile plants, and the morphology of the folds in the anther epidermis differs (Fig. 2). In summary, our analysis indicates that the abortive floral organs of Y66 and Y73 plants exhibit stamen atrophy and anther abnormalities.

### Pollen abortion period, characteristics, and causes of sterile plants in cotton

Pollen development is closely linked to anther development (Scott et al. 2004). de Moura et al. (2020) classified cotton (*G. hirsutum*) flower development into 11 stages, with initiation of anther development at stage two. Laser et al. (1972) believe that pollen abortion could occur at any stage of pollen development and that the stage and characteristics of abortion vary among different male sterile lines. Mutations in genes responsible for stamen development, sporogonium differentiation, meiosis, mitosis, microspore development, or flower differentiation can induce male sterility in plants (Glover et al. 1998; Niu et al. 2013; Ko et al. 2014; Han et al. 2018). Xie et al. (2006) have reported that various factors contribute to plant male sterility and the process of anther abortion is intricate, including abnormal tapetal development, ATPase, $Ca^{2+}$ concentration, cytoskeleton, and programmed cell death. Pressman et al. (2012) show that plant anthers accumulate a variety of carbohydrates, such as soluble sugars, starches, and proteins to provide adequate nutrition for normal pollen development. Changes in nutrient metabolism during plant development can lead to male sterility. The tapetum, which is the innermost anther wall and directly connects to the pollen mother cells, plays a crucial role in pollen development (Falasca et al. 2013; Singh et al. 2015; Sun et al. 2019). Precise regulation of tapetum degradation is crucial, as it provides essential enzymes and nutrients for microspore development. Premature or delayed tapetum degradation can lead to microspore abortion (Balk et al. 2001; Ariizumi et al. 2011; Wan et al. 2011). Our observation of anther tissue sections (Fig. 3) has showed no significant difference in pollen development between the sterile and fertile plants at the pollen maturity stage. However, pollen from sterile plants showed abnormal development. Further examination indicated that the degree of tapetum degradation differed between sterile and fertile plants. Prior to flowering, the sterile plants have shown almost no tapetum degradation residue in the anther chamber, whereas fertile plants have displayed more tapetum degradation residue and a even distribution. Over time, pollen grains in the sterile plants have been degraded, resulting in empty pollen grains, while cells in the connecting area between the vascular bundles and the anther chamber have underwent degradation. In summary, pollen abortion in Y66 and Y73 occur from pollen maturity to flowering. Premature degradation of the tapetum and insufficient nutrient supply to maintain pollen activity in the anther chamber likely result in pollen grain degradation and eventually male sterility.

## Conclusion

Based on morphological investigation of flower organs, cytological observation of anthers, and genetic analysis, we analyzed the phenotypes of the two $F_2$ segregating populations established from hybrid of sterile lines and restorer lines and revealed possible causes of the abortion of fertility. Our genetic analysis suggests that the fertility restoring gene is controlled by a single dominant gene. Morphologically, sterile plants exhibit stamen

atrophy and abnormal anthers. Pollen abortion has been observed during pollen maturity and flowering. The formation of empty pollen grains may have been caused by the premature degradation of the tapetum. Overall, this study provides valuable information on the CMS resources of cotton and establishes a foundation for hybrid breeding.

**Abbreviations**

| | |
|---|---|
| C | Connective |
| CMS | Cytoplasmic male sterility |
| EGMS | Environment-sensitive genic male sterility |
| En | Endothecium |
| Ep | Epidermis |
| EPG | Empty pollen grain |
| GMS | Genic male sterility |
| PGMS | Photoperiod-sensitive genic male sterility |
| PG | Pollen grain |
| PTGMS | Photoperiod and temperature-sensitive genic male sterility |
| Rf | Restorer of fertility |
| SEM | Scanning electron microscopy |
| St | Stomium |
| Ta | Tapetum |
| TGMS | Temperature-sensitive genic male sterility |
| V | Vascular bundle |

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**Authors' contributions**
Pei QY and Liu JS contributed equally to this work. Nie XH, Pan ZY, Guo CP, and Liu JS designed and supervised the experiments. Nie XH provided the funding. You CY and Zhu B provided the cytoplasmic male sterility lines. Guo CP, Liu JS, Ma XM, and Liu XY constructed the segregating population, investigated the fertility and phenotype observation. Lin HR, Li ZB, Zhao RH, Zhu B, Pan ZY, and Pei QY performed the experiments. Pei QY sorted out the results, wrote the main manuscript and prepared all figures. Wu YL, Nie XH, Pan ZY, and Guo CP revised and polished the manuscript. All authors contributed to the article and approved the final manuscript.

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**Availability of data and materials**
The datasets used during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics approval and consent to participate**
Not applicable

**Consent for publication**
Not applicable

**Competing interests**
The authors declare that they have no competing interests. Author Nie XH is a member of the Editorial Board of Journal of Cotton Research. Author Nie XH was not involved in the journal's review of, or decision related to this manuscript.

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