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Full Length Article

Cytological Studies of Anther Development of the Double Recessive Genetic Male Sterile Line, ms_5ms_6, of Upland Cotton (*Gossypium hirsutum*)

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Abstract

Although the double recessive genetic male sterility (GMS) line, ms_5ms_6, of upland cotton has been extensively used in breeding programs, possible mechanisms of pollen abortion remain unknown. In this study, the development stages of anther of this line were cytologically investigated. The results showed that the initial abortive development of sterile microspores occurred at meiosis I. Further cytological analyses revealed that deformed tapetal cells due to disintegration of middle layer may accelerate the process of apoptosis, leading to vacuolization. Moreover, pollen mother cells of sterile plants went into abnormal meiosis in which tetraspore, trispore, bispore, and even paraspore were formed. Also, there was no pollen formation possibly, because of anther deformity and narrow microsporangium as well as premature disintegration of tapetum in the GMS line. Taken together, the results indicated that abnormal development of tapetum may be the main reason for male sterility.

Keywords: Upland cotton; Genetic male sterility; Anther development; Cytological study

Introduction

Cotton (*Gossypium spp*.) is the world leading natural fiber crop (Sapkal *et al*., 2011) and also second largest oilseed crop (Dogan *et al*., 2012). To improve cotton yield, various strategies have been developed to utilize heterosis in cotton (Wu *et al*., 2011). As in other plants, male sterility is an effective means for producing hybrid cotton. Based on its inheritance mode, male sterility is totally divided into genetic male sterility (GMS) and cytoplasmic male sterility (CMS). The GMS line is more effective and economical in commercial production of cotton hybrid seeds compared with hand emasculation and pollination. The use of GMS could enhance random mating to develop hybrids since almost all commercial cultivars could be used as restorer lines (Chen *et al*., 2009a). Furthermore, GMS in the *Gossypium arboreum* L. was found to be under the control of recessive gene (Geddam *et al*., 2012). Recently, it has been widely used in breeding programs for F_1 hybrid seed production in cotton. In China, most of the cotton hybrids bred from GMS lines are generally based on the double recessive GMS line, ms_5ms_6, of upland cotton (Jiang *et al*., 2011). The first GMS line based on hybrid cotton "Suguna" was developed from the Central Institute for Cotton Research (CICR) Regional Station, Coimbatore, India, in 1978 (Yuan *et al*., 2000). So far, seventeen different genotypes of cotton GMS lines originated from hybridation have been identified in China and other countries of which 12 GMS lines were from upland cottons and five originated from the sea island cottons (Jiang *et al*., 2011).

While the double recessive GMS line ms_5ms_6 has been extensively used in breeding programs, studies on the double recessive GMS line (ms_5ms_6) mainly focused on the analyses of the amino acid content, peroxidase, and endogenous changes in the developmental stages of fertile and sterile anthers as well as discrepancy between sterility and fertility related gene expression. Moreover, it is reported that abnormal vacuolization of the tapetum during the tetrad stage was possible reason causing recessive GMS (Wan *et al*., 2010). However, when the abortive development occures and what are the cytological mechanisms controlling pollen abortion still remain largely unknown. The objective of this study was to cytologically compare the anther development between the double recessive GMS line, ms_5ms_6 and its normal fertile counterpart in a time-dependent manner, was carried out.

Materials and Methods

Plant Materials

The homozygous seeds of the double recessive GMS line, ms_5ms_6, of upland cotton, obtained from the Anqing Institute of Cotton, Anhui Academy of Agricultural Sciences, China, were sown in pots (5.5 cm diameter × 9 cm depth) outdoors in a large and rainproof net house on 5 April 2010.

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Each pot was filled with 350 g soil containing 80% surface soil from the experimental site and 20% water-logged compost (6.35 g kg^{-1} total N, 2.50 g kg^{-1} total P, 14.66 g kg^{-1} total K). On 18 May 2010, a total of 129 seedlings were transplanted to the experimental site (7 m × 3 m), containing 1.3% organic matter, 0.1% total N (101.5 mg kg^{-1} available N), 8.50 mg kg^{-1} available P, 92.4 mg/kg available K. The distance of row to row and plant to plant is 75 and 44 cm, respectively. Anthers between 1-20 days old were collected every day from both fertile and sterile plants after flower budding.

Pollen Sterility and Fertility Determination

Samples used to determine pollen sterility/ fertility were collected from the first and second pollen of the third branch of stem in all of 129 plants. Pollen sterility/ fertility was determined by staining pollen grains with 1% I-KI solution (1% iodine in 2% potassium iodide) followed by examination under a microscope (COIC-BA2303, Chongqing Optical Electrical Instrument Co., Ltd. China). Pollen grains were classified based on their shape and extent of staining. The unstained, withered or spherical pollen grains and the lightly stained round pollen grains were classified as sterile (Raj and Virmani, 1988). The fertile pollen grains were darkly stained and round (Salgotral *et al*., 2012). One hundred and three fertile plants produced seeds, whereas the other 26 sterile lines did not produce any seeds.

Anatomical Study

Anthers of at least five individuals were taken at different anther development stages from corresponding fertile and sterile plants. Bracts, petals, and calyxes were stripped from flower buds whose diameters were almost 3 mm. Treated flower buds were fixed in FAA (95%Alcohol: Glacial Acetic Acid: 38% Formaldehyde = 18: 1: 1, v:v:v) (Chen *et al*., 2009b), then dehydrated in alcohol gradient, cleared in xylene and embedded in paraffin at 54-56°C. The paraffin chips, 10 µm thick, were stained with safranine fast green and mounted with neutral resin. Cytological analysis was made on a light microscopy and photographs were taken on Leica DW 3000 with the Leica DFC 295 camera. Bright-field photographs of anther development cross-sections in fertile and sterile plants at different anther development stages were taken using a compound microscope.

Electron Microscopic Observation

Electron microscopic studies were performed using the buds of sterile plants at different anther development stages with bracts, petals and calyxes stripped. Samples were fixed in Karnovski's fixative solution (4% paraformaldehyde and 5% glutaraldehyde in 50 mM phosphate buffer, pH= 7.2), then transferred to 2% osmium tetroxide in 50 mM phosphate buffer. The samples were dehydrated in ethanol gradient and propylene oxide, embedded in Araldite epoxy and polymerized at 70°C for 24 h. Ultra-thin sections (70 – 90 µm) were cut with a diamond knife and placed on 150 mesh copper grids. The grids were stained with 2% uranyl acetate for 20 min followed by lead citrate for 5 min. The grid samples were observed under transmission electron microscope (TEM) (TECNAI 20 Philips®, The Netherlands) at 80 kV (Sami *et al*., 2013).

Results

Abnormal Development of Middle Layer and Tapetum

At 4 to 5 days after flower bud formation, no differences in structure and development of anther between the sterile and fertile flower were observed. The anthers in both sterile and fertile flowers developed into archesporial cell (Fig. 1) and connective tissue (Fig. 2). As shown in Fig. 2, 3(I-II), the microsporangium wall of sterile anthers consisted of epidermis, endothecium, middle layer, and tapetum. It is shown that tapetal cells were large with condensed cytoplasm, several organelles, normal single nucleus and double nuclei, as well as large nucleus and conspicuous nucleolus (Fig. 3IIc, Fig. 4).

The epidermis and connective ground tissue in the anther of the sterile plants contained abundant starch (Fig. 2), and there were 4 pollen sacs in both sterile and fertile anthers (Fig. 5), 5 to 6 days after flower bud. It is interesting that abnormal phenomena in the tapetum of sterile plants were observed during metaphase period of the development of the wall of pollen sac.

The middle layer was disintegrated prematurely (Fig. 6), which resulted in the abnormal shape of tapetal cells. However, it was difficult to observe the middle layer in the anthers within 6 to 7 days after flower bud formation and the tapetal cells are deformed (Fig. 7). It could be due to the fact that PMCs might have not yet initiated meiosis. In the meantime, deformed tapetal cells may have accelerated the process of apoptosis, leading to vacuolization and collapse of protoplast (Fig. 4b-c). Thus, deformed tapetal cells resultd in failure to develop pollens.

Premature disintegration of tapetum retarded pollen development.Within 8 to 9 days after flower bud formation, when PMCs has already gone into meiosis stage, the protoplast of tapetal cells began to vacuolize and collapse (Fig. 4d-e). Moreover, 10 days after flower budding, tetrads began to form microspores, the tapetal cells were ruptured, and their cytoplasm dispersed in the pollen chamber (Fig. 8; Fig. 4f). Hence, the failure of pollen development probably was due to accelerated degradation of tapetum, which could not provide enough nutrients for further development of pollen.

However, in fertile plants, the middle layers gradually dissociated and were finally absorbed by the tapetum (Fig. 6II). The generated tapetal cells were large and had single nuclear cells in the initial stage. When PMCs underwent meiosis, a part of tapetal cells were divided without cytokinesis, which led to the generation of double nuclear cells (Fig. 3Ic).

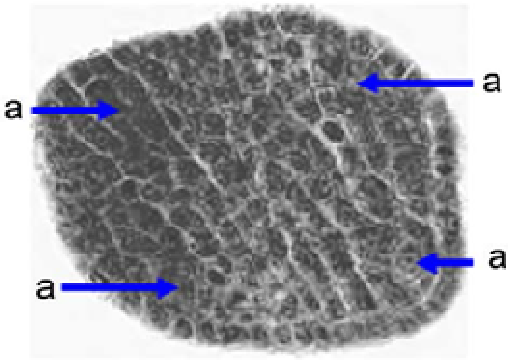

Fig. 1: Micrograph of cross – section of inchote anthers, 1-4 days after flower bud formation. a. archesporial cell

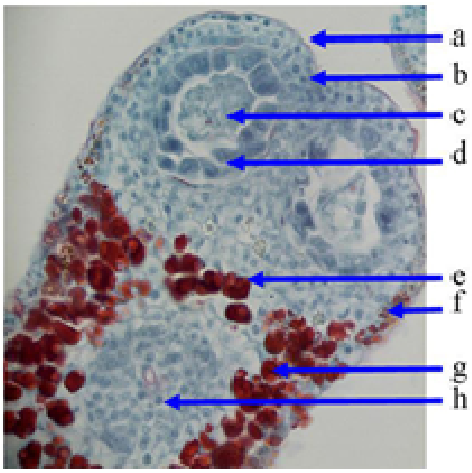

Fig. 2: Micrograph of cross section of the early anthers, 4-5 days after flower bud formation. a. epidermis, b. endothecium, c. pollen mother cell, d. tapetum, e. connective ground tissue, f and g. starch grain in the epidermis & the connective, h. connective vascular bundle

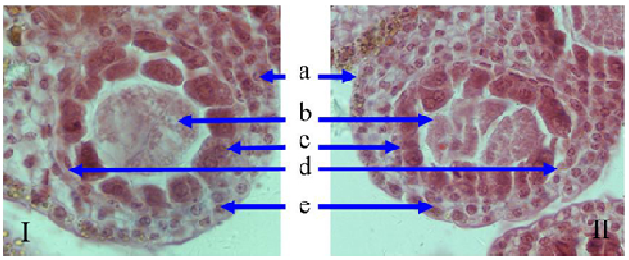

Fig. 3: Micrograph of tapetum and pollen mother cell of the early anthers, 4-5 days after flower bud formation. I. fertile pollen sac, II. sterile pollen sac
a. epidermis, b. pollen mother cell, c. tapetum, d. middle layer, e. fibrous layer

Pollen maturity occurred 20 days after flower bud formation. At the same time, microsporangium wall often disappeared or retained insignificant vestige for the disintegration of the tapetum leaving only epidermis and fibrous layers (Fig. 9).

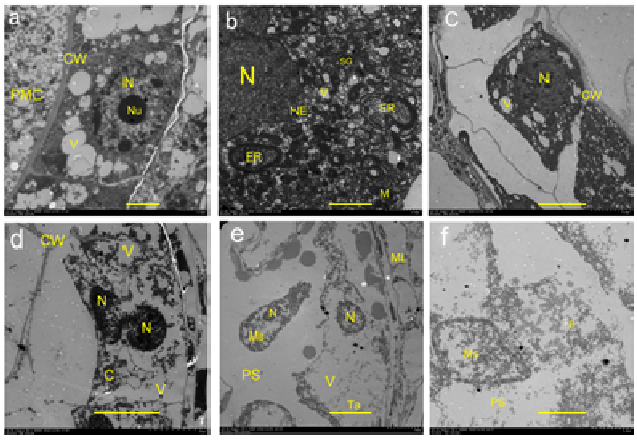

Fig. 4: Ultrastructural features of tapetal cells from sterile anthers
a and b. the tapetal cell which was 4 days after flower bud formation, c. the deformed tapetal cell which was 6 days after flower bud formation, d. the tapetal cell which was 7 days after flower bud formation began to vacuolize and collapse, e. the tapetal cell which was 8 days after flower bud formation was collapsing, f. the collapsed tapetal cell which was 10 days after flower bud formation
CW. cell wall, PMC. pollen mother cell, V. vacuole, N. nucleus, Nu. Nucleolus
ER. endoplasmic recticulum, NE. nuclear envelope, SG. starch grain, M. mitochondrion
C. cytoplasm, PS. pollen sac, Ms. microspore, Ta. tapetum, ML. middle layer
a and b bars=20 µm, c and f bars=50 µm, d and e bars=100 µm

The results showed that pollen abortion in this line may be due to premature disintegration of tapetum.

Meiosis of Sterile PMCs

PMCs were generated from primary sporogenous cells simultaneously with the development of microsporangium wall. The shape, size and structure of PMCs in sterile plant are normal and the same to those in fertile plant (Fig. 3II). The newly formed PMCs were larger and polygon shaped. In particular, it had big nucleus, dense cytoplasm, and no significant vacuoles (Fig. 3IIb). Meiosis began when calyxes protruded from the petals, 7 to 8 days after flower budding. Both normal and abnormal meiosis of PMCs were observed in the development of sterile plants. Normal meiosis of PMCs produced ordinary tetrads. As shown in Fig. 6Ia and 6IIf, cytokinesis occurred in classical simultaneous types, in which tetrads aligned in a plane. However, Fig. 6Ib and 6IIg suggested a rarely occurring non-classical successive type in which the tetrads aligned in tetrahedron.

The number of daughter cells produced in abnormal meiosis of PMCs varied greatly. Fig. 5a-d showed examples of four daughter cells (tetrad), three daughter cells (trispore), two daughter cells (bispore) and multiple ones (paraspore) produced.

Sterile Pollen Grains had Abnormal Developmental Phenotype

The wall of fertile pollen grain started development shortly after tetrad formation. Thick and hard extine with spike and deposit of sporopollenin began to grow from the outside of the thin and soft intine. Twenty days after flower budding, pollen maturated with light yellow color and spherical shape (Fig. 9).

Although the sterile PMCs underwent normal meiosis due to the premature disintegration of the middle layer and tapetum, the lack of nutrition resulted in the failure of pollen grain development (Fig. 7b). Additionally, irregular shaped pollens without extine (Fig. 7b) were also formed. Consequently, there was no mature pollen in the late stage of anther development. In another case, although extine and conspicuous spikes of pollen were formed in the middle or late stages of development, internal vacuolization appeared at the same time and was accompanied by pollen abortion (Fig. 10a). Therefore, only hollow pollens with spikes were produced (Fig. 10d).

In the third case, the sterile PMCs underwent abnormal meiosis (Fig. 5), which caused pollen grains irregularly development, and did not form normal mature pollens, resulting in sterile pollen (Fig. 11(I) d).

Deformed Anther in Sterile Line is Associated with Pollen Abortion

On the other hand, the anthers of sterile plants were much softer than normal ones and were covered with dispersed brown spots. Paraffin section indicated that deformed anthers contained normal epidermis and fibrous layers, but the development of PMC was not observed (Fig. 11(I)). Only dissociated cytoplasm was observed in the narrow microsporangium (Fig. 11II - III). In contrast, deformed anthers were not found in fertile plants.

Observing the development of 129 plants from seedling to ball stage via tagging demonstrated that 26 sterile lines were identified, sterile ratio accounting for 26:129. Four plants from July to August had deformed anthers in the confirmed 26 plants of genetic male sterile line, ms_5ms_6. Deformation ratio reached 2:13 in sterile plants and 4:129 in all the plants. The rest of twenty two plants with deformed anthers were identified from September to October after bolting. Deformation ratio accounted for 11:13 of sterile plants and 22:129 of total plants.

Discussion

It was shown that anther abortion in the double recessive GMS line, ms_5ms_6, of upland cotton occurred during PMC and microspore stage. There were no differences on the tapetum between fertile and sterile anther, which corresponded well with the study of Dou *et al.* (2009).

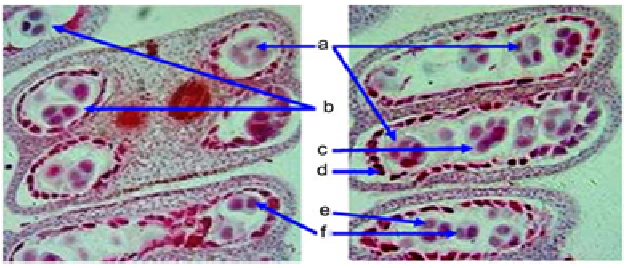

Fig. 5: Micrograph of abnormal meiosis of sterile pollen mother cell, 5-6 days after flower bud formation
a. tetraspore, b and e. terspore, c. paraspore, d. tapetum, f. dyad

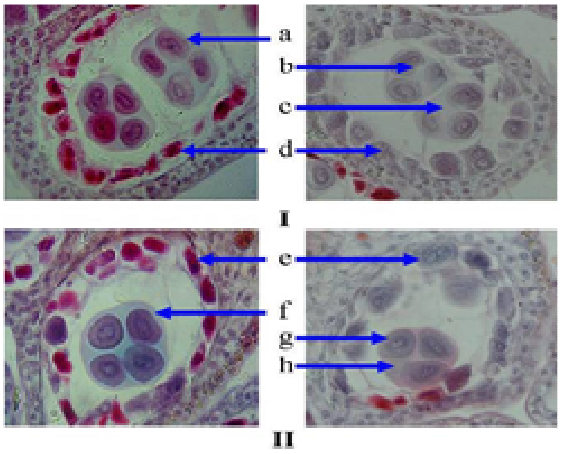

Fig. 6: Micrograph of sterile and fertile tetrad during meiosis stage, 5-6 days after flower bud formation. I. sterile tetrad, II. fertile tetrad. a and f. planar type, b and g. tetrahedral type, c and h. the hidden microspore of tetrad, d and e. tapetum

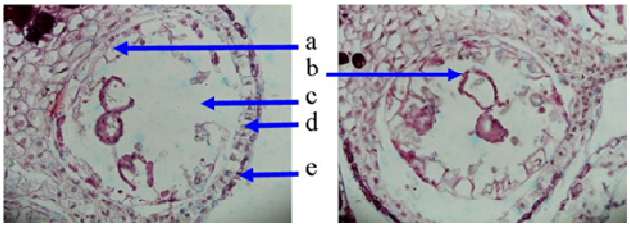

Fig. 7: Micrograph of sterile pollen sac cross section I, 6-7 days after flower bud formation
a. deformed tapetal cell, b. deformed pollen grain, c. chamber, d. fibrous layer, e. epidermis

However, our present study revealed that abortion of sterile microspores occurred at meiosis stage as found in other plants (Larrosa *et al.*, 2012). Moreover, the degradation of tapetum was accelerated in sterile lines, resulting in male sterility.

Tapetum, which is the innermost layer of the microsporangium wall, plays a crucial role in pollen development. This secretory tissue produces numerous nutritious proteins necessary for pollen maturation.

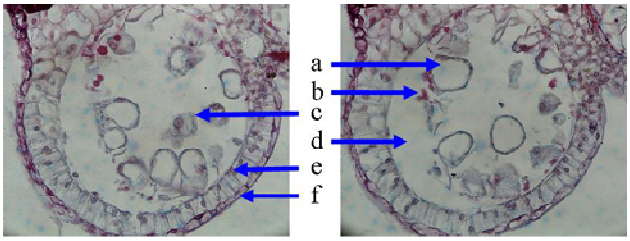

Fig. 8: Micrograph of sterile pollen sac cross section II, 8-10 days after flower bud formation. a. microspore with large vacuoles, b. remnants of tapetum, c. microspore in early development stage, d. chamber, e. fibrous layer, f. epidermis

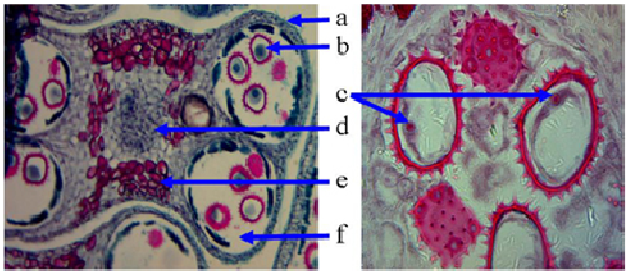

Fig. 9: Micrograph of mature anther connective and pollen grain, 20 days after flower bud formation
a. pollen sac wall, b. pollen grain, c. nucleus, d. connective vascular bundle, e. connective ground tissue and starch grain, f. chamber

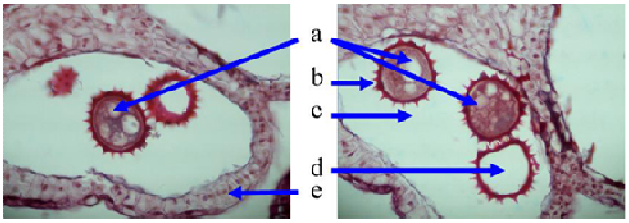

Fig. 10: Micrograph of sterile mature pollen sac cross section III, 15-20 days after flower bud formation
a. pollen grain with multiple vacuoles, b. pollen grain spike, c. chamber, d. hollow pollen, e. fibrous layer

Degradation of the tapetum is closely associated with the abortion of the PMCs (Wang *et al*., 1998; Ku *et al*., 2003). Exuberant metabolism of the tapetum could provide enzymes, hormones, favorable nutrition for developing PMCs and microspores, and regulate the proper release of microspore (Zhao *et al*., 2002; Jung *et al*., 2005; Vinod, 2005).

The results in our study indicated that deformed tapetal cells may lead to vacuolization and collapse of protoplast and premature disintegration of tapetum may be associated with the pollen abortion. This is supported by previous studies (Zhang, 1995; Wang *et al*., 1998; Li *et al*., 2002). It was demonstrated that any mutations of the tapetum may lead to the pollen abortion and male sterility

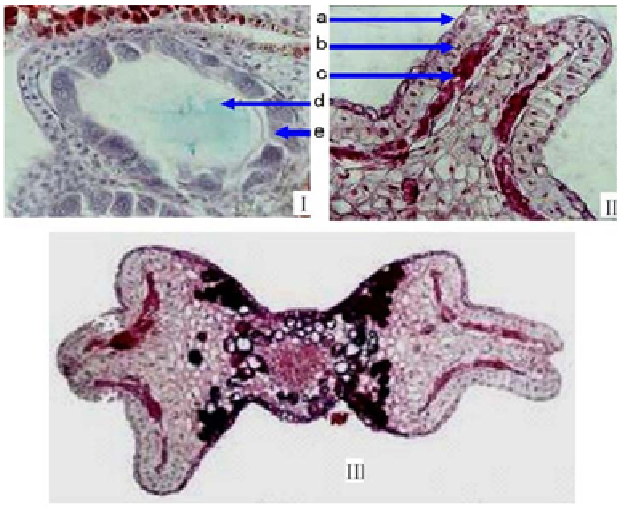

Fig. 11: Micrograph of cross section from deformed sterile anthers, no pollen developed
I. inchote pollen sac with no pollen mother cell, 4-5 days after flower bud formation
II. two locally amplified pollen sac, 15-20 days after flower bud formation
III. complete cross section, 15-20 days after flower bud formation
a. epidermis, b. fibrous layer, c. remnants of chamber, d. vacuolized chamber
e. tapetum

(Wilson, 2001; Sorensen *et al*., 2003; Jung *et al*., 2005; Luo *et al*., 2006). In other species such as tobacco and cabbage, selective destruction of tapetal cells can disrupt the formation of pollen grain and lead to sterility (Cho *et al*., 2001; Lee *et al*., 2003). Hence, it has been suggested that premature disintegration of tapetum would not supply enough nutrients for the development of the pollen grain. Also, the formation of irregular shaped pollens without extine is possibly due to the premature disintegration of the middle layer and tapetum.

Additionally, irregular shaped pollens without extine (Fig. 7b) were also observed. Exine plays an important role in microspore development (Scott *et al*., 2004; Ma, 2005). It is known that growth of exine is closely related to the tapetum. Recently, much attention has been paid on the possible molecular mechanism. For example, the *myb103* mutant in Arabidopsis, which has a mutation in a transcription factor gene, showed a complete absence of exine (Higginson *et al*., 2003; Zhang *et al*., 2007). Thus, it would be interesting to investigate the possible molecular mechanism of exine formation in the GMS line.

Conclusion

The present study revealed that in the offspring there were only 26 sterile plants out of 129 double recessive GMS line of upland cotton. The sterility segregation ratio only reached 26:103. It has been reported that the sterile segregation ratio of offspring is 1:4–3:10, stably (He *et al*., 2003). The following two possibilities may account for the different

results. One is that the cumulative diverse factors such as abnormal development of the tapetum and abnormal meiosis of PMCs may lead to male sterility. The other may be the different planting environment. Hence, in the future work the potential regulation of factors such as day length and temperature in GMS should be investigated.

Acknowledgments

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