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Novel genetic male sterility developed in (*Capsicum annuum* x *C. chinense*) x *C. pubescens* and induced by HNO_2 showing Mendelian inheritance and aborted at telophase of microspore mother cell stage

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ABSTRACT. A novel genetic male sterile germplasm was developed by successively crossing of (*C. annuum* x *C. chinense*) x *C. pubescens* and by chemical mutagenesis in pepper. The sterile anthers showed morphological abnormalities, but pistils developed normally with fine pollination capability. We investigated fertility segregation through sib-crossing of the same strains and test crossing by male sterile plants with 6 advanced inbred lines. The results showed that male fertility in the pepper was dominant in the F_1 generation and segregated at a rate of 3:1 in the F_2 generation, suggesting that monogenic male sterility

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was recessive and conformed to Mendelian inheritance. Cyto-anatomy analysis revealed that microspore abortion of sterile anthers occurred during telophase in the microspore mother cell stage when tapetal cells showed excessive vacuolation, resulting in occupation of the loculi. The microspore mother cells self-destructed and autolyzed with the tapetum so that meiosis in pollen mother cells could not proceed past the tetrad stage.

Key words: Distant hybridization; Genetic male sterility; Pepper; Microspore abortion

INTRODUCTION

Plant male sterility is a common biological phenomenon that has been observed in 617 species, 43 families, and 162 genera in nature (Kaul, 1988). Since 1940s, male sterility has been employed extensively in hybrid seed production of pepper (Martin and Grawford, 1951), carrot (Thompson, 1961), rape (Ogura, 1968), wheat (Wilson, 1968), sunflower (Leclercq, 1969), maize (Beckett, 1971), rice (Li, 1977), and Chinese cabbage (Zhang et al., 1990). Heterotic crops are developed by plant breeders to increase yield, uniformity, vigor, and resistance to disease and pests. Plant male sterility has become an important method to increase heterosis in many farm crops (Li et al., 1963; Block et al., 1997) and economic crops (Ogura, 1968; Zhang et al., 1990).

The origin of male sterility and its ancestry are not well-understood. It has been reported that cosmic rays and X-rays produce energetic electrons by ionization, which can induce different male sterile types such as T type in maize (Fauron and Havlik, 1989), and wild abortion in rice (Li, 1977). In cross-pollinated or often-pollinated plants, male sterility arises along with recessive homozygosis because hybrid progenies are allowed to self-copulate naturally. Some types of male sterility are also observed in the progeny of pollinated plants because of gene reconstruction caused by hybridization (Li et al., 1963). The nap cytoplasmic male sterile lines were developed in F_4 segregation progeny derived using an artificial hybridization system in *Brassica napus* (Shiga,1971). Male sterility trait in pepper was first identified in *Capsicum frutescens* (Martin and Grawford, 1951). Shifriss and Frankel (1969) obtained stable male sterile lines in the BC_2F_2 generation through interspecific crossing of *Capsicum annuum* x *Capsicum sinensis*. Furthermore, nuclear or cytoplasmic gene mutation typically causes male sterility in plants that occurs in the progeny strains treated with the mutagen. A recessive genetic male sterile mutant was observed in the M_2 generation after treatment of dry seeds of red pepper with X-rays (Daskaloff, 1968). In addition, male sterility in a plant can be effectively achieved using genetic engineering and directional selection. Expression of chimeric ribonuclease genes *RNaseT1* and *Barnase* in the anthers of transformed tobacco and oilseed rape plants selectively destroyed the tapetal cell layer surrounding the pollen sac (Mariani et al., 1990).

Following extensive exploration of the genetic mechanism of male sterility traits in plants, the inheritance of male sterility was classified into genetic male sterility, cytoplasmic male sterility, and cytoplasmic-genetic male sterility according to their genetic pattern (Sears, 1947). Inheritance of male sterility in pepper is comprised of genetic male sterility and cytoplasmic-genetic male sterility. Nuclear gene involves in phenotype of pollen abortion associated with genetic male sterility that exhibits Mendelian inheritance (Daskaloff, 1968).

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The cytoplasmic-genetic male sterility is a typical maternal inheritance causing hybrid seed production to require a holonomic system of sterility line, maintainer line, and restoration line (Peterson, 1958). However, the existing male sterile materials still have a narrow origin and low degree of restoration. Thus, the development and breeding new male sterile germplasm resources of pepper have become extremely important.

Pollen abortion occurred through microspore mother cell stage and binucleus microspore stage, but the pattern and stage varied in different plants (Laser and Lersten, 1972). The chromosome bridge and fragments appearing in the later stage of meiosis caused irregular division during meiosis (Zenkteler, 1962). Pollen abortion of pepper occurred during a later stage of microspore development, whereas meiosis of microspores generated normal tetrads (Harry et al., 1974); in contrast, Geng et al. (1994) found malfunction of pollen during the tetrad stage resulted from failed meiosis. Additionally, the male sterility of higher plants was found to be related to the abnormal expansion of tapetal cells in close proximity to sporophyte cells in the pollen sac (Yuan and Li, 2000). It is difficult for microscopes to obtain adequate nourishment from the tapetum because of its early degradation caused by problems in programmed cell death, resulting in pollen abortion (Lu et al., 2006). Although the nature of the plant male sterility has been examined in cytological studies, the processes of microspore abortion emphasize the complicated dissimilarity between male sterility and abnormal anther wall development.

Currently, male sterility has been successfully applied for heterosis breeding and hybrid seed production in pepper. This has greatly improved seed purity and decreased seed cost by avoiding artificial emasculation. In this study, a novel male sterile germplasm of pepper was developed by using successive crossing and artificial mutation. We also studied the morphological and anatomical mechanism of pollen development between male sterile and fertile anthers based on genetic factors for the male sterile trait, and further illustrated the critical stage and characteristics of microspore abortion, allaying a foundation for the enrichment and utilization of male sterile germplasm resources in pepper.

MATERIAL AND METHODS

Plant materials

The pepper lines used in this study included R35 (*C. annuum*), R30 (*Capsicum chinense*), R31 (*Capsicum pubescens*), and 6 advanced inbred lines: B16, B18, B21, B24, B27, and B32. All plants were grown under protection at Northwest A&F University.

Successive cross and chemical mutagenesis

The 3 species of *Capsicum* were hybridized by successive crossing. Offspring derived from a cross of R35 x R30 were pollinated by R31, after which the hybrid seeds were harvested. These dry seeds were treated with 0.05M, pH 4.5 HNO_2 at 28°C for 3 h, followed by flushing with water for 2 h to obtain the M_0 seeds. After germination of M_0 seeds, all seedlings were cultured in a nursery bed and transplanted to a greenhouse when they had 10 true leaves. The M_2 seeds were obtained from each fruit of the M_1 generation separately, and then agronomic characteristics of M_2 and succeeding progeny were measured under natural conditions.

Crossing test

The male sterile plants were pollinated by 10 fertile plants within the same stock, and the 10 fertile individuals were self-pollinated simultaneously. Additionally, the male sterile plants were pollinated by 6 advanced inbred lines: B16, B18, B21, B24, B27, and B32. The F_1 hybrid seeds were harvested and cultivated under protection in accordance with the hybrid combinations to investigate pollen fertility of the population at full-bloom stage. Sib-crossing and test crossing of F_2 progeny were conducted to focus on the segregation of the male sterile trait.

Pollen assay

Investigation of pollen fertility in field

The fertility of pollen grains in various progenies was investigated at full-bloom stage according to the method of Gulyas et al. (2006).

Viability analysis of pollen grains

Pollen grains germinated *in vitro*, after which germination rate and the length of the pollen tube were measured according to the method described by Ma et al. (2013).

Microscopic observation of microspore development

The flower buds at various developmental stages were obtained in early June and fixed in Navashin's fluid. Dehydration, embedding, and staining were carried out as described by Zhao et al. (2012). The paraffin-embedded samples were observed under a Motic B5 digital microscope (Xiamen, China).

Data analysis

The heredity of male sterility was examined using the F_1, F_2, sib-cross, and test-cross generations. An example is shown below for the Chi-square test:

$$\chi^2 = \sum \frac{(|A-T|-0.5)^2}{T} \quad \text{Equation 1}$$

where A is the actual value of observation and T is the theoretical value of observation.

RESULTS

Morphological features of the novel male sterility

Examination of approximately 6000 M_2 plants revealed 5 different families exhibiting male-sterile characteristics such as shrinking anther, long stigma, and malfunctioning pollen

during the bloom stage (Lu et al., 2006; Peng et al., 2010). The sterile anthers of strain HW58 were too shriveled to disseminate mature pollens (Figure 1A), but the male fertile plants had full anthers covered by thousands of viable pollen that were released after anther dehiscence (Figure 1B). Additionally, all opening flowers of male sterile plants fell off the plant during full-bloom stage under artificial isolation conditions, resulting in low fruit setting and seed formation by self-pollination. The style of male sterile plants was outside the petals or twisted inner flower buds. Although the male sterile anther was devoid of pollen (Figure 1C), it showed normal compatibility to the pistil, ovary, placenta, and seeds produced by artificial pollination in male sterile plants. In contrast, the pollen germination rate and tube length were 79.8% and 46.3 µm (Figure 1D), respectively, *in vitro*, indicating that the pollens of male fertile plants were viable and that they could pollinate to other plants of the same strain. Therefore, the strain shows typical male sterile trait, which is useful for hybrid seed production and studies examining the pepper.

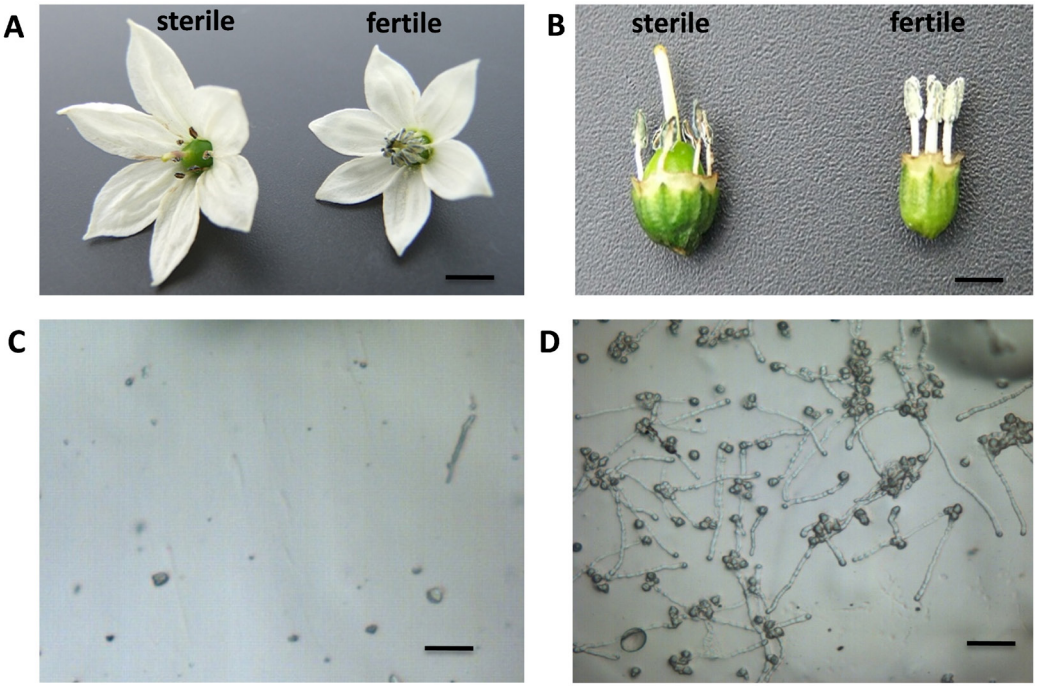

Figure 1. Comparison of morphological structure and pollen grain germination between male sterile and fertile flower organs. **A.** Sterile anthers were thin, shriveled, and disseminated little pollen, but fertile anthers were full and densely covered with pollen. **B.** The flower of the male sterile plant has longer stigma and smaller anthers than the fertile plants. **C.** No pollen was released from sterile anthers. **D.** Pollen of fertile anthers geminated normally and were viable. Bars for A = 6 mm, for B = 2 mm, for C, D = 20 µm.

Inheritance of novel male sterility

Male sterility of pepper was categorized as genetic male sterility (Martin and Grawford, 1951), genetic-cytoplasmic male sterility (Peterson, 1958), and functional male sterility

(Yuan and Li, 2000). Sib-crosses and selfing were conducted in the greenhouse to determine the inheritance of male sterility and allelic relationships in the novel male sterile material of pepper. Ten fertile plants MFP-1-MFP-10 were used to pollinate the male sterile plants in the strain HW58, as well as fertilized with their own pollen. Fertility segregation occurred in the combinations of MSP x MFP-2, MSP x MFP-5, and MSP x MFP-9 at a rate of 1:1 according to the Chi-square test ($P < 0.05$), while the fertile plants were heterozygous. All plants of other 7 combinations showed viable pollen, and fertility segregated at the rate of 3:1 according to the Chi-square test ($P < 0.05$) in the F_2 population (Table 1). Therefore, the novel monogenic male sterility was recessive and conformed to Mendelian inheritance, indicating that genotype of male sterile plant was *msms*, while the male fertile plant was *MsMs* or *Msms*.

To determine the existence of inbred lines that can maintain the male sterility, we used the novel male sterile plants as female parent pollinated by 6 advanced inbred lines through biparental cross. All plants in the F_1 generation were fertile in all combinations, and fertility segregated at a rate of 3:1 according to the Chi-square test ($P < 0.05$) in the F_2 population. In addition, the testcross revealed that the segregation ratio of 2 genotypes, *msms* and *Msms*, was 1:1 based on Chi-square test results ($P < 0.05$) as expected by Mendelian segregation (Table 2). This indicated that the novel male sterile trait in pepper is controlled only by a mononuclear gene with no relationship to the cytoplasm.

Table 1. Segregation and test for allelism with the novel male-sterile material of pepper.

Cross	Progeny	Male fertile	Male sterile	Expected ratio	χ^2*
MSPxMFP-1	F_1	52	0		
	F_2	66	26	3:1	0.362
	MSC	79	0		
MSPxMFP-2	F_1	24	32	1:1	0.875
	MSC	52	20	3:1	0.167
MSPxMFP-3	F_1	82	0		
	F_2	72	32	3:1	1.551
	MSC	85	0		
MSPxMFP-4	F_1	63	0		
	F_2	100	28	3:1	0.510
	MSC	84	0		
MSPxMFP-5	F_1	34	28	1:1	0.403
	MSC	71	29	3:1	0.653
MSPxMFP-6	F_1	63	0		
	F_2	87	25	3:1	0.298
	MSC	48	0		
MSPxMFP-7	F_1	50	0		
	F_2	57	23	3:1	0.417
	MSC	98	0		
MSPxMFP-8	F_1	70	0		
	F_2	50	22	3:1	0.907
	MSC	54	0		
MSPxMFP-9	F_1	20	24	1:1	0.205
	MSC	42	18	3:1	0.556
MSPxMFP-10	F_1	76	0		
	F_2	63	25	3:1	0.379
	MSC	94	0		

MSP = male sterile plant; MFP = male fertile plant; MSC = male self-cross; *Significant at the 0.05 level of probability $\chi^2_{0.05\ (1)} = 3.84$.

Table 2. Fertility performance of filial generation of the novel male-sterile material pollinated by 6 advanced inbred lines of pepper.

Cross	Progeny	Male fertile	Male sterile	Expected ratio	χ^2*
MSPxB16	F_1	58	0		
	F_2	59	25	3:1	0.778
	Test cross	43	49	1:1	0.272
MSPxB18	F_1	42	0		
	F_2	79	21	3:1	0.653
	Test cross	45	39	1:1	0.298
MSPxB21	F_1	63	0		
	F_2	85	23	3:1	0.605
	Test cross	41	37	1:1	0.115
MSPxB24	F_1	50	0		
	F_2	70	26	3:1	0.125
	Test cross	37	45	1:1	0.598
MSPxB27	F_1	47	0		
	F_2	70	30	3:1	1.080
	Test cross	48	42	1:1	0.278
MSPxB32	F_1	57	0		
	F_2	68	20	3:1	0.136
	Test cross	33	29	1:1	0.145

MSP = male sterile plant; *Significant at the 0.05 level of probability $\chi^2_{0.05\,(1)} = 3.84$.

Pollen development in fertile and sterile anther

Cell division in the inner epidermis of the anther formed a saucer-shaped protrusion under which the archesporium immediately differentiated during the sporogenous cell stage when the corolla was strapped tightly by sepal with curly heart leaves (Figure 2A). Periclinal division of the sporogenous cells developed into primary wall cells and primary sporogenous cells that generated secondary sporogenous cells located on the central pollen sacs to grow larger, while cells of the procambium produced the endothecium and tapetal layer (Figure 2B), as well as parenchyma cells.

Sporogenous cells developed into microspore mother cells located in the middle of clinandrium with hyperchromatic nuclei. The anther wall consisted of 4 layers: the tapetal layer, the middle layer, the endothecium, and the epidermis (Figure 2C). Among these, the tapetal layer was primarily composed of very regular binucleate cells, and mostly derived from the primary wall layer.

The nucleus in the shrinking cytoplasm of the microspore mother cell entered the diakinesis stage. The tapetum began to vary during the tetrad stage with the cytoplasm of the anther cell. Most tapetal cells extended radially and dissolved around the tetrad after vacuolization during the first meiotic metaphase when the middle layer cells began disintegrating (Figure 2D). Microspores descend from tetrads dispersed in the anther sac after disintegration of callose at the uninucleate eccentric stage when the nucleus was extruded by the swollen vacuole (Figure 2E). After full dissolution of tapetum cells, the mature pollen grains scattered in the cracking anther sac (Figure 2F).

In the sterile anther, each lobe contained 2 pollen sacs that produced very large quantities of sporogenous cells (Figure 3A). The primary parietal cells developed into the endothecium, middle layer, and tapetum through periclinal division and anticlinal division during the sporogenous cell stage (Figure 3B). The sporogenous cells neatly arranged on the inner side of tapetum, which was very similar to the cytological characteristics of the fertile anther.

Autolysis of the microspore mother cell in the sterile anther occurred occasionally because of extrusion of excessive in tumescence and vacuolization of partial tapetum (Figure 3C). In addition, the microspore mother cell disintegrated and randomly fused into abnormal tapetum (Figure 3D). This aberrant behavior of the tapetum actually exacerbated the extensive necrosis of the microspore mother cell (Figure 3E). When the tapetum later gradually disintegrated, the microspore mother cell had still not entered meiosis and finally disintegrated (Figure 3F).

Therefore, compared with pollen development in the fertile anther, the abortion of microspore mother cells occurred during telophase of meiosis II so that they could not develop into normal tetrads because the pollen sac was filled with necrotic residue. Finally, no mature pollen grains formed in anther chamber.

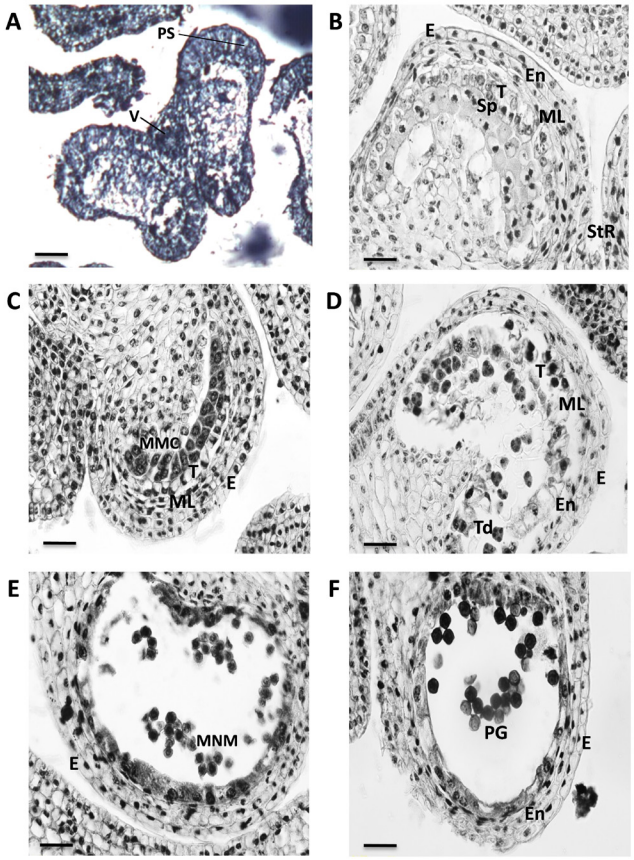

Figure 2. Microspore developmental process of male fertile plant in the novel genetic male sterile material of pepper. **A.** Transverse section of fertile anther. **B.** Sporogenous cells were differentiated from sporule after mitosis. Parenchymas and procambia cells developed into epidermis and tapetum. **C.** Microspore mother cells were present in inner pollen sac. Binucleate tapetum evolved from connective cells and primary epidermis. **D.** Microspore mother cells developed into tetrads after meiosis and degradation of tapetum. **E.** Mononucleus microspores were released from tetrads and disperse in pollen sac. **F.** Mature pollen grains formed in pollen sac. V = vascular bundle; PS = pollen sac; StR = schizocoelom; E = epidermis; En = endothecium; ML = middle layer; T = tapetum; Sp = sporogenous cells; MMC = microspore mother cells; Td = tetrad; MNM = mononucleus microspore; PG = pollen grain. Bars for A = 60 µm, for B, C, D, E, F = 20 µm.

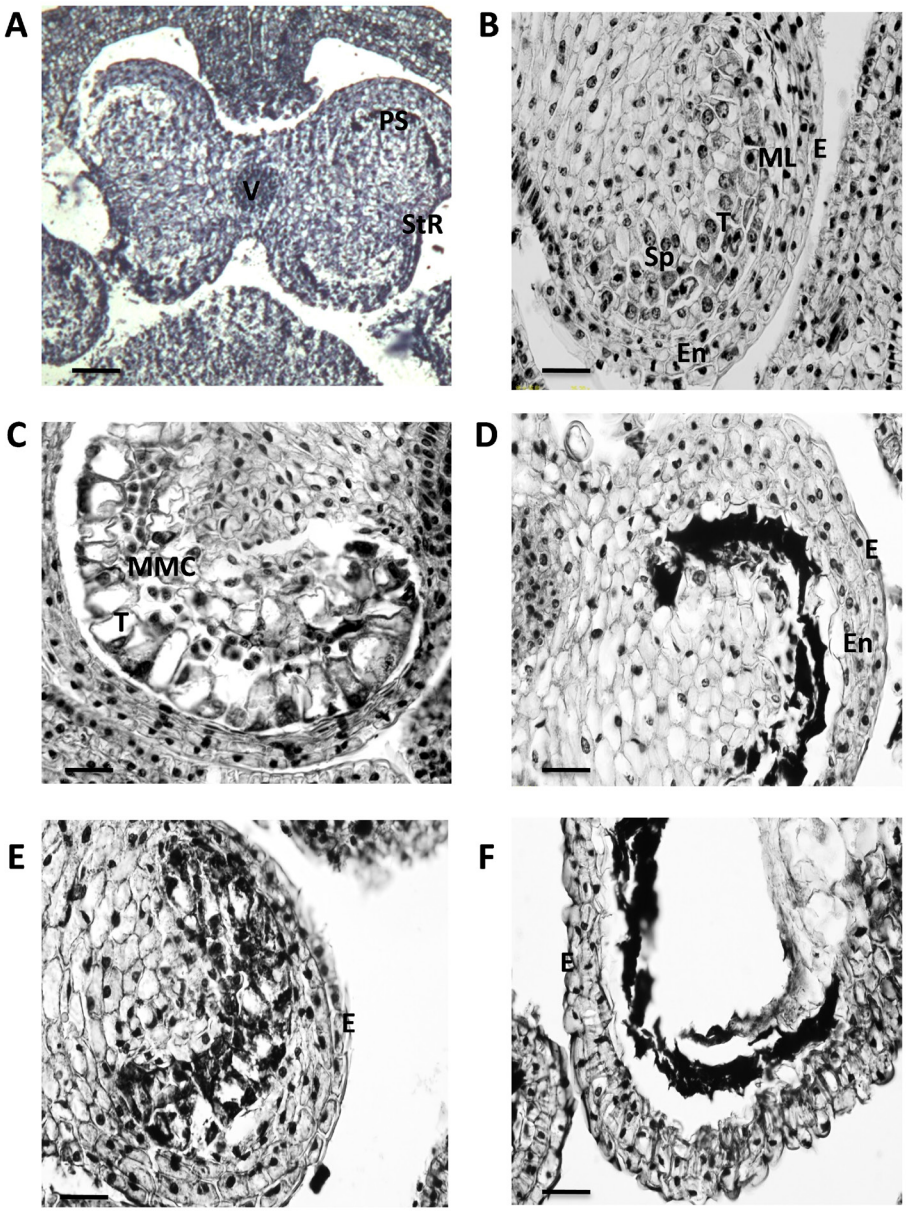

Figure 3. Process of microspore abortion of male sterile plant in the novel genetic male sterile material of pepper. **A.** Transverse section of sterile anther. **B.** Sporule initially occurred and differentiated during sporogenous cell stage. **C.** Tapetal cells excessively expended with considerable vacuolation at the anther loculi during the microspore mother cell stage. **D.** Microspore mother cells autolyzed together with the tapetum layer. **E.** Anther chamber was obstructed by remnant of degradable tapetum and microspore mother cells. **F.** No pollen grains formed in anther chamber. V = vascular bundle; PS = pollen sac; StR = schizocoelom; E = epidermis; En = endothecium; ML = middle layer; T = tapetum; Sp = sporogenous cells; MMC = microspore mother cells. Bars for A = 60 µm, for B, C, D, E, F = 20 µm.

DISCUSSION

A novel male sterile germplasm was bred using a successive cross of (*C. annuum* x *C. chinense*) x *C. pubescens* and artificial mutation in pepper. The male sterile plants had malfunctioned anthers that could not produce viable pollen grains, but the pistil developed normally and showed normal fertilization ability. We also studied fertility inheritance using sib-crosses, biparental crosses, and test crosses. The result showed that the fertility segregation of progeny complied with Mendel's single gene inheritance model, indicating that the novel germplasm was related to genetic male sterility without finding maintainer lines among all tested materials. In addition, microspore abortion occurred during telophase of the microspore mother cell stage when tapetal cells excessively expended with considerable vacuolation, resulting in the occupation of the pollen sac, and then the microspore mother cells began to autolyze and merged with the tapetum layer so that meiosis of pollen mother cells could not be processed to form the tetrad.

The plant male sterility came from a variety of sources including hybridization (Kihara, 1951), artificial mutation (Ko and Yamagata, 1980), sporadic mutation (Pradhan et al., 1991), and biotechnology (Worrall et al., 1992). Sporadic mutation induced male sterility less frequently than hybridization and artificial mutation. Shiga (1971) isolated nap cytoplasmic male sterile lines in F_4 progeny derived from hybrids of different lines in *B. napus*. Grini et al. (1999) screened male sterile mutants of *Arabidopsis thaliana* in the M_2 generation by using seeds treated with ethyl methanesulfonate. In pepper, cytoplasmic male sterility was observed for the BC_2F_2 generation of the wide cross by *C. annuum*, *C. pendulum*, and *C. pubescens* (Rusenova-Kondareva, 1968). A recessive nucleus genetic male sterile mutant was identified in the M_2 generation after treatment of dry seeds of red pepper with X-rays (Daskaloff, 1968). Inherence of genetic male sterility controlled by a single recessive nuclear gene was first observed by Martin and Grawford (1951). In China, genetic male sterile lines, which were bred in hot pepper (Yang, 1981) and sweet pepper (Fan et al., 2004), were successfully applied for the production of hybrid seeds. In this study, we developed a novel male sterile germplasm of pepper by crossing (*C. annuum* x *C. chinense*) x *C. pubescens* and artificial mutation. The sterile anthers were completely abortive to disseminate mature pollens so that the plant had not produced to seeds by selfing. This showed that the novel male sterile trait was consistent with Mendel's recessive monogenic inheritance model.

The mechanism of pollen abortion varies greatly according to the plants because of the complexity of the genotypic milieu in highly diversified male sterility. Microspore abortion occurred during meiosis (Kaul, 1988).The microspores collapsed mainly during late metaphase in monocotyledons, while it occurred during early metaphase in dicotyledons (Ling et al., 2000). Archesporial cells mature into viable pollen through 5 stages of the sporogenous cell, microspore mother cell, tetrad, mononucleus microspore, and mature pollen. Physiological disorder of the tapetum, microspore mother cell, and microspore occurring at any stage can induce male sterility. This indicates that microspores were squeezed by the highly vacuolated tapetal cells (Shifriss and Frankel, 1969; Geng et al., 1994). Tetrad cells could not be released because of degradation of the tapetum (Harry et al., 1974; Lu et al., 2006), or the tetrad was compressed and degraded (Luo et al., 2006). In this study, we demonstrated that pollen abortion of genetic male sterility occurred during telophase in the microspore mother cell stage when the microspores were squeezed by excessive vacuolation of tapetal cells, slowing meiosis.

Heterosis plays an important role in improving crop yield, quality, and tolerance to disease and stress, demonstrating the importance of the development and utilization of male sterility. Recessive genetic male sterility shows advantages of many fertility restorer lines as well as successful crosses available for utilization of heterosis, but it is difficult to breed a specific line that can completely maintain male sterility. In addition, it is difficult to cultivate female parents with desirable qualities because of the limited number of male sterile germplasm resources available. The novel genetic male sterile germplasm bred in this study produced non-functional pollen; moreover, its pistil develops normally and showed normal fertilization with other inbred lines. Thus, the novel genetic male sterile trait can be transferred to pepper lines and can combine with agronomic characters through backcrossing, which can be used to breed male sterile AB lines for efficient hybrid seed production.

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