1 Cytological abnormalities and pollen abortion in interspecific hybrids of *Nicotiana*

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11 **Abstract** Interspecific cross breeding introduces superior agronomic traits into cultivated species;
12 however, problematic pollen sterility occurs in the hybrids. Our previous study obtained interspecific
13 hybrids from the cross between a cytoplasmic male sterility line of *Nicotiana tabacum* and *Nicotiana*
14 *alata*, and some of the hybrids were pollen sterile. Here, we conducted an in-depth cellular study to
15 understand the cytological mechanism of pollen abortion in these hybrids (F1-D sterile) compared with
16 pollen development in the fertile hybrids (F1-S sterile) from the same cross. The ultrastructure
17 observation showed that the membrane of microspore in F1-D sterile hybrid was deficient at all
18 represented developmental stages. Chromosome behavior during meiosis was studied by carbol fuchsin
19 staining, which indicated that cytomixis, chromosome leakage and asymmetric callose wall deposition
20 occurred with high frequency in the microsporocyte of F1-D sterile. The results of the ultrastructure and
21 6-diamidino-2-phenylindole (DAPI) analyses also showed that the cytoplasm and nucleus were unstable
22 and extruded from F1-D sterile microspore during the developmental process, leading to mature pollen

1 grains that were vacuous and collapsed in the aperture region. In addition, delayed tapetum degradation
2 was also detected in the anther of F1-D sterile, and might be associated with irregular sporopollenin
3 deposition in the aperture region of F1-D sterile pollen. Genetic unbalance and cytomembrane deficiency
4 might both be responsible for the instability of the chromosome, nuclear and cytoplasm, and resulted in
5 pollen abortion in F1-D sterile hybrids, and irregular tapetum degradation might also be related with
6 pollen sterility.

7 **Key words:** *Nicotiana*, hybrid pollen abortion, cytological abnormalities, cytomembrane deficiency,
8 delayed tapetum degradation

9 **Introduction**

10 Hybrid sterility is a widespread postzygotic reproductive barrier between species or subspecies. This
11 effect provides the initial force of genetic differentiation and speciation, and it represents a major barrier
12 to the effective use of inter-subspecific genetic resources in many crops, including tobacco [1-3]. Pollen
13 sterility is one of the most important reasons for interspecific hybrid sterility, along with embryo sac
14 sterility[4, 5], and severely prevents the utilization of genetic diversity in hybrids; therefore, it is of great
15 significance to understand the genetic or cytological mechanism of pollen abortion in hybrids.

16 The anther locule is where the pollen grain develops, and the developing anther consists of
17 microsporocytes in the center, surrounded by the tapetum and the anther wall [6, 7]. The anther wall of
18 *Nicotiana* belongs to the basic type and is composed of the endothecium, two middle layers and the
19 tapetum [8, 9]. The tapetum, which is in direct contact with the microspores, serves as a nutritive and
20 secretory tissue to supply the nutrients and other components required for pollen wall development, and
21 it regulates the development of microspores during pollen mother cell (PMC) meiosis and the subsequent
22 microspore maturation [9-11]. Pollen development in most of the species includes the following key

1 stages: meiosis, tetrad and primexine development, microspore release, sporopollenin deposition, mitosis,
2 accumulation of starch or lipids, addition of pollenkit or tryphine to the exine, and dehydration of the
3 cytoplasm [12-14]. Before the tetrad stage, the microsporocyte is encased in the callose wall, which is
4 synthesized by the meiocyte and deposited between the plasmalemma and the original cellulosic wall
5 during prophase I of meiosis [7, 15]. This callose layer is vital for preventing cell cohesion and fusion
6 and useful as a template for the formation of species-specific exine-sculpting patterns; then, upon its
7 dissolution, the free microspores were released [7, 16-20].

8 Male gametophyte development is prone to influence by the external environment and by inherent
9 genetic and physiological conditions; therefore, the coordination between the anther sporophytic tissue
10 and the microspore is crucial for pollen development and fertility. Pollen abortion may occur at various
11 stages of pollen development. Primarily, chromosome behavior during meiosis must be under strict
12 genetic regulation to facilitate the correct segregation of chromosomes into daughter cells, and abnormal
13 meiosis inevitably induces pollen abortion [21, 22]. Scott reported that the absence of a callose wall is
14 not necessarily fatal, and indeed, some species lack callose; however, irregular callose accumulation and
15 dissolution are associated with male sterility in taxa where a callose wall is normally present [6, 21, 23].
16 Moreover, abnormality in the cell membrane might interfere with the movement of substances in and out
17 of cells and with the development of the pollen wall structure [24]. Cell membrane expansion at the
18 unicellular microspore stage is vital for pollen cell enlargement, polarization, mitosis, and finally pollen
19 viability [1]. In addition, the timing of tapetum formation and degradation is critical for microspore
20 development and pollen viability; premature or delayed tapetum degeneration causes male sterility [25,
21 26]. Aberrant pollen wall formation might also lead to pollen abortion. For example, impaired primexine
22 formation often results in abnormal exine and aborted pollen [11, 27, 28].

1 Cytoplasmic male sterility (CMS) lines are a convenient and efficient tool for plant cross breeding,
2 and the nuclear fertility restoration gene is necessary for fertility restoration in offspring obtained using
3 a CMS plant as the maternal parent [29-32]. In a previous study, the CMS line of *Nicotiana tabacum* L.
4 cv. (*gla.*) S ‘K326’ (with degenerated stamens, n=24) was hybridized with *Nicotiana alata* (n=9) ,
5 which overcame the pre-fertilization cross-incompatibility encountered when using the corresponding
6 male fertile line as the maternal parent, and F1 offspring with different morphology and fertility were
7 obtained. Offspring named F1-S was morphologically similar to the maternal parent, with well-
8 developed stamens and fertile pollen grains. However, hybrids F1-D was morphologically different from
9 the female parent, with the stamens (anthers and filaments) developed, but the pollen grains were
10 completely sterile [33]. We deduced that a fertility restorer from the paternal parent (*N. alata*) was
11 responsible for male fertility restoration in F1-S fertile, and this hypothesis is undergoing further
12 validation. However, another interesting question is why the F1-D sterile hybrids showed aborted pollen.
13 To reveal the cause of pollen abortion in F1-D sterile, we studied the cytological mechanism of pollen
14 abortion through analysis of pollen viability and fertility, microsporogenesis, callose wall deposition,
15 cell ultrastructure, cytomembrane integrity, cytoplasm and nucleus stability, and tapetum development
16 and degradation, which were compared with that in the F1-S fertile hybrids.

17 **Materials and Methods**

18 The CMS line *Nicotiana tabacum* L. cv. (*gla.*) S ‘K326’ (n=24) was hybridized with *N. alata* (PI
19 42334, n=9) in June 2015. The materials were all provided by the Yunnan Academy of Tobacco
20 Agricultural Sciences (Nanxiang Road 14, Yuxi, Yunnan, China). Offspring seeds were obtained in July
21 and then germinated in January 2016 and grown for the next 5 months in the greenhouse of the Southern
22 Tobacco Breeding Center, Yuxi, Yunnan. The plants were manually watered once a week and fertilized

1 with water-soluble fertilizer (N: P: K 20:20:20+0.5% microelements) (Demei, Sichuan, China) once a
2 month. Pollen grains from hybrids morphologically different from the maternal parent (F1-D-2 sterile)
3 were utilized to explore the cytological mechanism of male sterility, and pollen from F1-S fertile (F1-
4 S-2) was also studied as a control. The genome size of these two hybrids was previously reported [33].
5 Flowers were categorized into nine developmental stages. Stages 1 and 9 represented the smallest flower
6 bud and the blooming flower, respectively. Information with images of each stage was available in Fig.
7 1.

8 **Pollen viability and germination capacity estimated by FDA-PI and germination**

9 Fluorescein diacetate (FDA) (Solarbio, Beijing, China) shows cell viability by detecting the presence
10 of active esterases in the cytoplasm. Propidium iodide (PI) (Solarbio) could only permeate the membrane
11 of dead cells, embed into double-stranded DNA in the nucleus and exhibit red fluorescence. FDA (10
12 mg) was dissolved in 20 μ L of acetone and diluted to 5 ml with laboratory-pure water, and then PI was
13 added to a final concentration of 1 mg mL⁻¹. Microspores or pollen grains were collected from anthers at
14 different developmental stages and stained with the above FDA-PI solution for 5 min. Signals were
15 detected with a fluorescence microscope (Olympus BX 51, Olympus, Japan) by monitoring emission at
16 520 nm with an excitation wavelength of 485 nm. Only cells exhibiting bright green fluorescence were
17 considered to be viable [23, 25, 34].

18 A basic in vitro germination medium (containing 10% sucrose, 0.01% boric acid and 0.01% Ca²⁺) was
19 used to culture pollen at 28 °C for 3 h. A pollen grain was considered as germination when the pollen
20 tube length equaled or exceeded the pollen grain diameter. Pollen germination capacity was also assayed
21 by pollination. Pollen grains from the sterile and fertile hybrids were collected and deposited on the
22 stigmas of the maternal parent line. Pistils were picked at 24 h after pollination and observed with

1 fluorescence microscopy after aniline blue staining to detect pollen germination [35].

2 **Meiosis and callose wall observed by carbol fuchsin staining**

3 Microsporocytes or microspores were collected from flower buds at stages 1 to stage 3, and anthers
4 from each bud were placed on a glass slide. The microspores were released with the help of a needle and
5 then stained with carbol fuchsin for 15 min at room temperature (Solarbio). Coverslips were added, and
6 the slides were observed with the light microscope (Olympus BX51, Olympus, Japan). Photographs were
7 taken by the DP-73.

8 **Ultrastructure analysis by TEM**

9 The microspore or pollen cell structure was studied by transmission electron microscopy (TEM).
10 Anthers were fixed in 5% glutaraldehyde in phosphate-buffered saline (PBS) at 4 °C overnight, rinsed
11 in 0.1 M PBS for three times, and post-fixed in 1% OsO₄ (dissolved in 0.1 M PBS) for 3 h. After washing
12 three times with PBS, the materials were then dehydrated with 50%, 70%, and 90% ethyl alcohol, 1/2 90%
13 ethyl alcohol:1/2 acetone (V: V) and pure acetone for 15 min respectively. Anthers were embedded in
14 Spurr's resin for the cross-sectioning. Ultrathin sections (70 nm) were cut using a diamond knife on a
15 Leica Ultracut ultramicrotome (Leica EM UC6). Sections were stained by saturated uranyl acetate and
16 lead citrate and then examined with a transmission electron microscope (JEM1230, JEOL, Japan).

17 **Cytomembrane analysis by DiI staining**

18 Pollen grains at stage 5 were collected in a 1.5 mL Eppendorf tube, hydrolyzed with enzyme solution
19 containing 3% cellulose, 1% pectinase and 0.6% D-mannitol, and then by incubating at 35 °C for 3 h
20 [36]. The hydrolyzed pollen grains were further stained with 10 μM 1'-dioctadecyl-3,3,3',3'-
21 tetramethylindocarbocyanine perchlorate (DiI, Sigma, USA) dissolved in acetate buffer (pH 5.5) for 10

1 min and then observed with the Olympus BX51 under an excitation wavelength of 549 nm [37].

2 Nuclear analysis by DAPI

3 The integrity and stability of the nuclei were analyzed by 4', 6-diamidino-2-phenylindole (DAPI)
4 (Beyotime, Shanghai, China). Microspores or pollen grains were squeezed from the anther and stained
5 with DAPI (10 $\mu\text{g mL}^{-1}$) solution prepared in Tris-HCl (0.1 mol L^{-1} pH 9.0). The DAPI-stained
6 microspores were observed with a fluorescence microscope (Olympus BX51, Olympus) and
7 photographed with a DP-73 (Olympus, Japan) [38].

8 Anther wall observation by paraffin sectioning

9 The anther wall was studied by paraffin sectioning. Anthers at different developmental stages were
10 collected and fixed in FAA (5 mL of formaldehyde and 5 mL of glacial acetic acid in 90 mL of 70%
11 (V/V) ethyl alcohol) at 4 °C overnight, and then dehydrated for 2 h in an ethyl alcohol gradient of 80%,
12 85%, 90%, and 95%, followed by dehydration in 100% ethyl alcohol twice, each time for 1 h. The anther
13 was then hyalinized in 1/2 xylene and 1/2 ethyl alcohol overnight, saturated gradually with paraffin at 37
14 °C until saturation, and then immersed in pure paraffin overnight. The saturated materials were embedded,
15 sectioned at 5 μm , dewaxed and then dyed with safranin-Fast Green or toluidine blue.

16 Results

17 Flower developmental stages

18 In convenience of research and description, the flower buds of the hybrids were classified into nine
19 developmental stages according to the development of the male gametophyte, as shown in Fig. 1. The
20 microsporocyte at stages 1 to stage 2 were undergoing the process of meiosis, and microspore release

happened before stage 3 (Fig. 1), followed by the cell wall deposition and microspore mitosis at stage 4. The microspores developed further at stages 5 to 7 and matured into bicellular pollen grains at stages 8 and 9 (Fig. 1).

Pollen viability and germination capacity difference between F1-S and F1-D

Mature pollen of F1-D was proved to be sterile by in vitro culture and seeds set assay [33]. FDA-PI staining was used to further explore the microspore/pollen viability at the process of development. Viable cells exhibited green fluorescence due to the effect of FDA, while DNA of dead cells showed red fluorescence of PI staining. The results revealed that microspores/pollen grains at all developmental stages of F1-S fertile were stained green by FDA, which was typically exemplified as that in Fig. 2. A. Thus the pollen of F1-S fertile were viable. In F1-D sterile, microspores at stage 2 to stage 4 exhibited green fluorescence (Fig. 2. B, C), which weakened during the lateral stages 5 to 7 (Fig. 2. D, E), and was totally absent in mature pollen (Fig. 2. F). The above results showed that the microspore/pollen of F1-D sterile was gradually inactivated at the developmental process.

Furthermore, microspore/pollen germination capacity was also analyzed at the developmental process (Fig. 1) by pollination and in vitro culture. In F1-S fertile, pollen grains at stages 7 to 9 germinated on the stigma (Fig. 2. G, H, I), and the germination ratio increased from stages 7 to 9, while pollen grains at stage 6 could not germinate on the stigma (Fig. 2. J). In F1-D sterile, microspores or pollen of all developmental stages failed to germinate on the stigma (Fig. 2. K). Pollen in vitro culture assay showed that pollen of F1-S fertile at stage 9 germinated well on the in vitro medium (Fig. 2. L), and the germination ratio was higher than 85%. However, the germination ratio was much lower for microspore at stages 7 and 8 (Fig. 2. M, N), and pollen at stage 6 could not germinate at all (Fig. 2. O).

1 In F1-D sterile, pollen at all developmental stages was unable to germinate on in vitro medium.

2 The results of the FDA-PI staining, in vitro germination and pollination assays showed that
3 microspores/pollen grains of F1-S fertile were viable at all developmental stages and fertile at maturation,
4 which could be confirmed by seed set in F1-S fertile. While F1-D sterile pollen was gradually inactivated
5 with development and incapable of germination. FDA-PI staining and the germination assay revealed
6 that the pollen viability and germination capacity were not always consistent. Pollen with germination
7 ability was inevitably positive for FDA-PI staining and viable, while viable pollen could only germinate
8 at maturation.

9 **Abnormality in the process of microsporogenesis**

10 Chromosome behavior during meiosis was vital for pollen fertility, and abnormal meiosis inevitably
11 induces pollen abortion. Therefore, microsporogenesis at stages 1 to stage 2 was investigated by carbol
12 fuchsin staining, to explore its causality with pollen abortion of F1-D sterile. The results showed that
13 PMCs at stage 2 were undergoing meiosis I and meiosis II. Cytomixis (the migration of nuclear material
14 from one PMC to another) was observed at zygotene of meiosis I in both F1-D sterile (Fig. 3. A, B) and
15 F1-S fertile. A total of 120 PMCs at zygotene were analyzed for both F1-S fertile and F1-D sterile, and
16 cells with cytomixis accounted for 7.0% and 18.57% of F1-S fertile and F1-D PMCs, respectively (Table
17 1). Furthermore, the chromosomes in the meiocytes of F1-D sterile were prone to leak at diakinesis of
18 meiosis I (Table 1), implying that the chromosomes escaped out of the cytoplasm and the callose wall
19 (Fig. 3. C, D). Based on an analysis of 140 PMCs at diakinesis in both the F1-S fertile and F1-D sterile,
20 chromosome leakage accounted for 75.54% of the meiocytes of F1-D sterile, while only 2.00% meiocytes
21 showed chromosome leakage in F1-S fertile (Table 1). Chromosome leakage even resulted in the

1 occurrence of cytoplasts-cells that were completely devoid of nuclear material (Fig. 3. C, D). At the
2 tetrad stage, abnormal and vestigial microspores were observed in the anther locule of F1-D sterile, and
3 four microspores within the same callose wall were different in size and shape (Fig. 3. E), unlike those
4 in F1-S fertile. Obviously, these results indicated that cytomixis and chromosome leakage occurred with
5 higher frequency in the meiocytes of F1-D sterile.

6 Table 1. Chromosome and callose wall abnormalities during meiosis

	zygotene		diakinesis	
	Meiocytes with asymmetrical callose wall (%)	Meiocytes with cytomixis (%)	Meiocytes with asymmetrical callose wall (%)	Meiocytes with chromosome extrusion (%)
F1-S-Fertile	53.90±4.50	7.00±0.20	25.24±2.63	2.00±0.01
F1-D-Sterile	55.71±6.45	18.57±2.10	79.09±7.58	74.54±7.89

7
8 Regular callose accumulation and dissolution are vital for male fertility, and the callose wall
9 deposition in F1-S fertile and F1-D sterile was accordingly studied. The results revealed that
10 asymmetrical callose wall deposition, resulting in thinned or perforated areas of the callose wall, was
11 observed in both F1-S fertile and F1-D sterile (Fig. 3. F, G)) at zygotene of meiosis I, and occurred with
12 high frequency in both types of meiocytes at the rates of 53.90% and 55.71%, respectively. However, at
13 diakinesis, the percentage of asymmetrical callose wall deposition dropped to 25.24% in F1-S fertile,
14 while increased to 79.09% in F1-D sterile (Table 1). In F1-D sterile, asymmetrical callose wall deposition
15 occurred at a high percentage at all developmental stages before microspore release (Fig. 3. A-H), while
16 the callose wall was deposited relatively symmetrically after diakinesis in F1-S fertile (Fig. 3. I, J, K, L).
17 Asymmetrical callose deposition was also observed via TEM in the PMCs of F1-D sterile (Fig. 5. C).
18 These results indicated that asymmetrical callose wall deposition occurred with higher frequency in F1-
19 D sterile. Nuclear material translocation and leakage occurred in the areas of thinned or perforated callose

1 wall deposition in the meiocytes (Fig. 3. A, B, C).

2 **Cytomembrane deficiency and pollen wall deposition abnormality in microspore/pollen of F1-D**

3 Abnormality in the cell membrane might interfere with the movement of substances in and out of
4 cells and with the development of the pollen wall structure [24], and aberrant pollen wall formation might
5 also lead to pollen abortion. Microspore/pollen was further studied with TEM, to reveal the cell structure
6 differences between F1-S fertile and F1-D sterile. The most significant difference was that the cell
7 membrane was well developed in the microspores or pollen of F1-S fertile at all represented
8 developmental stages, and became electron-translucent from stage 3 to maturity (Fig. 4. A, B, C);
9 however, the cytomembrane was undetectable in F1-D sterile, as shown in microspores at stage 3, 5 and
10 9 (Fig. 4. D, E, F). The cell membrane deficiency was further validated by DiI staining, which revealed
11 that the protoplasts of the F1-D sterile pollen grains ruptured immediately after hydrolysis, and the
12 cytomembrane was indiscernible after DiI staining (Fig. 4. K, L, M), which might reduce the stability of
13 protoplast and be responsible for the above pollen rupture. In contrast, intact and rounded protoplasts of
14 various sizes were hydrolyzed from F1-S fertile pollen grains, and the cytomembrane was positively
15 dyed red (Fig. 4. G, H, I, J).

16 The cell wall of fertile and sterile pollen was both composed of the exine (nexine and sexine) and
17 intine. In F1-D sterile pollen, the exine did not develop in the aperture region at stage 3 (Fig. 5. G, H),
18 and sporopollenin gradually deposited in this region at stage 5 (Fig. 5. K, L). The aperture of the mature
19 pollen was totally covered by the exine (Fig. 5. O, P). In fertile pollen, only intine was deposited in the
20 aperture region at the above three developmental stage (Fig. 5. F, J, N).

21 **Nuclear and cytoplasmic instability in pollen of F1-D**

1 As cytomembrane deficiency was observed in the microspore/pollen of F1-D sterile, the cell inclusion
2 stability was further studied. TEM observation revealed that the cytoplasm of PMCs in F1-S fertile were
3 covered tightly by the callose cell wall (Fig. 5. A, B), while there was gap between the cytoplasm and
4 callose wall of PMCs in F1-D sterile (Fig. 5. C, D). PMCs of F1-D sterile had abundant cell inclusions
5 (Fig. 5. C), which were extruded gradually at later developmental stages (Fig. 5. G, K), and the cytoplasm
6 was totally absent from mature pollen grains (Fig. 5. O). The pollen of F1-D sterile also collapsed
7 gradually around the apertures, resulting in three apertures close to each other at the cell center of the
8 mature pollen (Fig. 5. O). In F1-S fertile, the cytoplasm, organelles and amyloid became richer from
9 stage 1 to the mature pollen stage, and the mature pollen was plump, regular, and filled with abundant
10 cell inclusions (Fig. 5. A, E, I, M).

11 DAPI staining was used to further examine the microspore/pollen nuclei stability at stage 2 to stage 9
12 (Fig. 1), which indicated that micronuclei were present in the tetrad phase and the following
13 monokaryotic phase in F1-D sterile (Fig. 6. A, B). The released microspores underwent one round of
14 mitosis and generated binucleate pollen grains at stage 4 (Fig. 6. C). In F1-D sterile, two nuclei were
15 present in the microspore at stage 4, absent from some pollen grains at stage 6 (Fig. 6. D), and absent
16 from all pollen grains at stages 7 to 9 (Fig. 6. E, F). In F1-S fertile, two nuclei also arose at stage 4, but
17 they were always present at all the subsequent stages; therefore, mature F1-S fertile pollen had two nuclei
18 (Fig. 6. G), while none was present in F1-D sterile pollen. DAPI analysis further provided evidence that
19 the microspore/pollen nuclei of F1-D sterile were not stable during development.

20 **Abnormal tapetum development in F1-D**

21 The tapetum plays major roles in pollen wall development and pollen viability. Observation of the
22 anther wall structures in the hybrids showed that the anthers developed normally in F1-S fertile, with

1 normal connectivum and four anther locules of equal size (Fig. 7. A). In F1-D sterile, about 66% of the
2 anthers were normal, as described above (Fig. 7. B), while others had enlarged connectivum and reduced
3 anther locules (Fig. 7. C, D), which might morphologically affect microsporogenesis and pollen
4 development. The anther wall was composed of epidermis, fibrous endothecium, one middle layer and
5 the tapetum, which formed normally in both the sterile and fertile hybrids (Fig. 7. E, F) at the beginning
6 of PMC differentiation. For F1-S fertile hybrids, the tapetum started to degenerate at the early tetrad
7 stage (Fig. 7. G) and mostly disappeared at the release of the microspores (Fig. 7. H). However, the
8 tapetum in F1-D sterile degenerated later than that in F1-S fertile. The tapetum was intact at the tetrad
9 stage (Fig. 7. I), mostly remained after the release of the free microspores (Fig. 7. J, K), and ultimately
10 degenerated at stage 5 (Fig. 7. L). Therefore, the tapetum degradation was delayed in the anther of F1-D
11 sterile.

12 Discussion

13 Sterility in interspecific hybrids constrains their application in breeding improvement and has long
14 been an interesting biological topic [39, 40]. Microsporogenesis and microspore development are under
15 strict control by the surrounding sporophytic tissue and the gametophytic intracellular environment. In
16 this study, we investigated the cytological basis of pollen sterility of hybrid obtained from a cross
17 between a *Nicotiana* CMS maternal parent and *N. alata* [33]. The results showed that cytomixis,
18 chromosome leakage, irregular callose wall deposition occurred with higher frequency in the male
19 gametophyte of F1-D sterile hybrid, the cytomembrane was deficient, and the tapetum degradation was
20 delayed.

21 Cytomixis has been reported to occur along cytotoxic channels [26, 27, 41, 42], and cytomixis during
22 the prophase of the first meiotic division is widespread in plant species and is evolutionarily important

[43-46]. Our study showed that cytomixis occurred at zygotene of meiosis I in both the PMCs of F1-D sterile and that of F1-S fertile. However, the cytomixis ratio in the PMCs of F1-D sterile was 11.57% higher than that in the PMCs of F1-S fertile. It is reported that irregular cytomixis may be caused by various disturbances such as the structure of the cytotoxic channels, synthesis of the callose coat, cell wall formation, the structure of the cell cytoskeleton and genetic unbalance [27, 42]. As has been reviewed, the microtubular apparatus does not play an obvious role in the process of cytomixis in tobacco [26, 42, 47, 48]. Furthermore, asymmetrical deposition of the callose wall occurred at similar ratio at the zygotene of meiosis I in the PMCs of both the F1-S fertile and F1-D sterile hybrids. Therefore, the high percentage of asymmetric callose wall deposition at the beginning of meiosis might not result in higher cytomixis in F1-D sterile, but only provide convenience or a prerequisite for the transfer of nuclear material between PMCs during meiosis. Genetic unbalance might be partially responsible for higher cytomixis in F1-D sterile. Furthermore, cytomembrane deficiency observed in this research might also be related with higher cytomixis in the PMCs of F1-D sterile.

The asymmetrical callose wall deposition percentage declined at diakinesis of meiosis I in F1-S fertile, while it increased in F1-D sterile, revealing that callose wall rearrangement happened during the process of meiosis. This result was consistent with the report that callose wall deposition occurred almost continuously throughout meiosis, ceasing only with the final compartmentalization of microspores after telophase II [24]. The results also showed that chromosome leakage occurred 75% more frequently in F1-D sterile than that in F1-S fertile and was always linked with thinned or perforated areas of the callose wall. The high percentage of chromosome leakage in the sterile pollen might be due to a cell membrane deficiency. Moreover, it has also been reported that callose wall deposition at the microspore surface is mediated by the plasma membrane [49]. We deduced that this cell membrane deficiency might be

responsible for the asymmetrical callose wall deposition at diakinesis in the PMCs of F1-D sterile, which then provided a convenient path for chromosome leakage. Chromosome leakage inevitably intensified genetic material distribution abnormalities during meiosis, as reflected by the presence of deformed microspores and micronuclei at the tetrad stage and the monocyte stage.

In this study, microspore and pollen instability in the F1-D sterile was also found. DAPI staining and TEM analysis showed that the cytoplasm and nucleus were extruded from the pollen grain during the processes of microspore maturation. This phenomenon could also be attributed to the plasma membrane deficiency and genetic unbalance of the sterile pollen. In addition, the results also indicated that sporopollenin was irregularly deposited in the aperture region of F1-D sterile pollen, which might be induced by delayed tapetum degeneration after microspore release.

The timely degradation of the tapetum is necessary for pollen development and fertility, and the timing of tapetum degeneration varies among species. For example, degeneration commenced at the tetrad stage and was completed at the bicellular pollen stage in *Brachypodium* and rice [11, 50]. However, in wheat, barley and *Actinidia deliciosa*, the breakdown of tapetum cells began during the vacuolated microspore stage [11, 34, 51]. In this study, tapetum degradation in F1-D sterile was delayed compared with that in F1-S fertile, which might partially cause the observed pollen incapacity.

The genetic basis of hybrid sterility has been studied in some plants, including *Solanum*, rice (*Oryza*), *Mimulus*, *Arabidopsis lyrata*, and *A. thaliana*, revealing that male sterility in these species was either induced by large-scale chromosome organization changes (including chromosome rearrangement) or merely by genic loci (including two loci with Bateson–Dobzhansky–Muller interactions and one locus representing a male sterility factor) [36-38, 52]. In this research, pollen sterility in F1-D sterile might be both genetically and morphologically regulated. Higher cytomixis and chromosome leakage might be

related with the genetic unbalance, and the cytomembrane deficiency might also be responsible for the instability of the nuclear and cytoplasm material. Furthermore, abnormal tapetum degradation might also result in the pollen abortion in F1-D sterile. The cytomembrane deficiency observed in F1-D sterile might be the result of lower expression level of the putative nuclear fertility restoration genes [33], which worked insufficiently in restore pollen fertility in F1-D hybrids.

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Conflict of interest

The authors declare that they have no competing interests.

Reference

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12 **Figure legends**

13 **Fig. 1.** The flower developmental stages in F1-D sterile and F1-S fertile hybrid

14 The flower stage was corresponding to the development stages of PMC, microspore or pollen grains.

15 **Fig. 2.** Microspore or pollen viability assayed by FDA staining and its fertility estimated by pollination
16 and in vitro cultivation

17 A-F. Pollen viability assayed by FDA staining. A. Mature pollen grains of F1-S fertile; B.
18 Microspore at stage 2; C. Pollen grains at stage 4; D. Pollen grains at stage 5; E. Pollen grains at
19 stage 7; F. Pollen grains at stage 9; G-K. Pollen grains viability estimated by pollination; G-J show
20 that the F1-S fertile pollen germination ratio was highest at stage 9; G. Pollen grains at stage 9; H.
21 Pollen grains at stage 8; I. Pollen grains at stage 7; J. Pollen grains at stage 6; K. Pollen grains of
22 F1-D sterile at stage 9; L. Pollen grains at stage 9 germinated well on in vitro medium; M, N. Pollen
23 grains at stages 8 and 7 barely germinated on in vitro medium; O. Mature pollen grains (at stage 9)
24 of F1-D sterile failed to germinate on in vitro medium.

Fig. 3. Chromosome behavior of F1-S fertile and F1-D sterile

A, B. Cytomixis at zygotene of meiosis I, with arrow showing nuclear material translocation between cells; C, D. Chromosome leakage at diakinesis of meiosis I, with arrow showing a chromosome escaping a meiocyte and arrowhead showing a PMC without chromosome; E. Asymmetrical callose wall deposition at the tetrad stage, with arrow indicating microspores of unequal size and morphology; F, G, H. Callose wall deposited asymmetrically at different developmental stages, with arrow and arrowhead showing thinner and thickened callose deposition spots around the meiocytes, respectively; I, J, K, and L show the callose wall deposited symmetrically at diakinesis and metaphase of meiosis I, at telophase of meiosis II, and at the tetrad stage.

Fig. 4. TEM observation and DiI staining of pollen cell ultrastructure

A, B, C. Detailed cell wall structure of F1-S fertile, with arrow indicating the cytomembrane; D, E, F. Detailed cell wall structure of F1-D sterile pollen deficient in cytomembrane; A, D. Pollen cell wall structure at stage 3; B, E. Pollen cell wall structure at stage 5; C, F. Pollen cell wall structure at stage 9; G-M. Observation of cytomembrane by DiI staining; G, H. Mature pollen of F1-S fertile hydrolyzed with cellulase and pectinase, with arrow showing the protoplast; I, J. Mature pollen of F1-S fertile stained with DiI, with arrow showing the cytomembrane; K, L. Mature pollen of F1-D sterile hydrolyzed with cellulase and pectinase, with arrow showing the ruptured protoplast; M. Sterile pollen negative for DiI staining. al, anther locule; cm, cell membrane; cy, cytoplasm; cw, cell wall.

Fig. 5. Cytoplasmic stability analyzed by TEM

A. The PMC was full of cytoplasm; B. The cytoplasm of PMC was tightly covered by the callose layer; C. The arrow indicated the perforated area resulted from asymmetric callose wall deposition;

1 D. The gap between the cytoplasm and callose wall of PMC; E. The microspore was full of
2 cytoplasm; F. The arrow indicated that there was no sporopollenin deposition in the aperture region;
3 G. The cytoplasm was partially extruded; H. The arrow showed the sporopollenin was not deposited
4 in the aperture region; I. The microspore was full of cytoplasm; J. The arrow indicated that
5 sporopollenin did not deposited in the aperture region; K. The pollen gradually collapsed in the
6 aperture region; L. The arrow showed that the sporopollenin was gradually deposited in the aperture
7 region; M. The pollen was full of cytoplasm; N. The arrow showed that there was no sporopollenin
8 deposition in the aperture region; O. The pollen grain was totally absent of cytoplasm, and collapsed
9 at the aperture region; P. The arrow showed that the aperture region was totally blocked by
10 sporopollenin. ap, aperture; al, anther locule; ca, callose; cm, cell membrane; cy, cytoplasm; cw,
11 cell wall.

12

13 **Fig. 6.** Nuclear stability analyzed by DAPI staining

14 A, B. Micronucleus at the tetrad phase and the following monokaryotic phase, with arrow indicating
15 the micronucleus; C. The arrow indicated two nuclei at stage 4; D. Nuclei disappeared in some
16 pollen grains at stage 6; E, F. Nuclei disappeared in all pollen grains at stages 7 and 9; G. Two
17 nuclei in the mature pollen of F1-S fertile.

18 **Fig. 7.** Tapetum development in the F1-D sterile and F1-S fertile hybrids

19 A. Anther of F1-S fertile at stage 3; B, C, D. Anther of F1-D sterile; B. Anther with normal
20 connectivum and four locules at stage 3; C. Anther with enlarged connectivum and three locules at
21 stage 4; D. Anther with inflated connectivum and only two locules at stage 4; E. Tapetum formation
22 in F1-S fertile; F. Tapetum formation in F1-D sterile; G. The tapetum of F1-S fertile began to

1 degrade at the tetrad stage; H. The tapetum of F1-S fertile was almost entirely degraded by the
 2 release of the microspores; I. The tapetum of F1-D sterile was almost intact at the tetrad stage; J, K.
 3 The tapetum of F1-D sterile partially presented at the release of the microspore; L. The tapetum of
 4 F1-D sterile was degraded at stage 5. co, connectivum; en, endothecium; mi, microspore; pmc,
 5 pollen mother cell; ta, tapetum; te, tetrad; va, vascular bundle.

6

A

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1cm

B

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8

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5

4

3

2

1

1cm

F1-D

F1-S

The flower developmental stages in F1-D sterile and F1-S fertile

Microspore or pollen viability assayed by FDA staining and its fer

F1-D

F1-D

F1-S

Chromosome behavior of F1-S fertile and F1-D sterile

F1-S

F1-D

F1-S

F1-D

TEM observation and Dil staining of pollen cell ultrastructure

Cytoplasmic stability analyzed by TEM

Nuclear stability analyzed by DAPI staining

Tapetum development in the F1-D sterile and F1-S fertile