

The pollen wall and tapetum are altered in the cytoplasmic male sterile line RC₇ of Chinese cabbage (*Brassica campestris* ssp *pekinensis*)

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ABSTRACT. Cytoplasmic male sterile line RC₇ of Chinese cabbage produces mature anthers without pollen. To understand the mechanisms involved, we examined the ultrastructural changes during development of the microspores. Development of microspores was not affected at the early tetrad stage. During the ring-vacuolated period, some large vacuoles appeared in the tapetum cells, making them larger, extending to the anther sac center during the monocyte period. At the same time, the tapetum degenerated as the microspores aborted, resulting in pollen-deficient anthers. As a result, the locules collapsed and the anthers shriveled. The callose was degraded in the pollen walls; abnormal deposits of electron-dense material gave rise to irregular spike-shaped structures, rather than the characteristic rod-like shape of the B7 bacula. The internal intine wall of RC₇ was thinner than that of the B7 type. At the mitosis I microspore stage, the tapetum cells contained multiple plastids, with numerous small spherical plastoglobuli, and lipid bodies. Based on these observations, we suggest that RC₇ abortion may be due

to mutated genes that normally regulate development of the pollen wall and cell walls in the RC₇ line.

Key words: *Brassica campestris*; Pollen development; Tapetum; Cytoplasmic male sterility; Transmission electron microscopy

INTRODUCTION

Genetic male sterility is a common phenomenon in higher plants and has been used widely in breeding programs (Sodhi et al., 2006). Sterile males fail to produce functional anthers or pollen at various stages of microsporogenesis and dehiscence of the anthers owing to the action of nuclear or mitochondrial genes (Ying et al., 2003). Some types of male sterility produce no pollen, whereas others produce small amounts of pollen and set seeds after self-fertilization (Ying et al., 2003). Gynodioecy, the presence of hermaphrodite and male sterile plants in a population, is an outcome of the mutation and survival of androsterility alleles (Frankel and Galun, 1977).

Chinese cabbage (*Brassica campestris* L. ssp *pekinensis*) is widely cultivated in China. Two types of male sterility exist in Chinese cabbage: genic and cytoplasmic (Ying et al., 2003). Genic male sterility is controlled by a pair of recessive genes, whereas cytoplasmic male sterility (CMS) is a maternally inherited characteristic that causes failure to produce functional anthers, pollen grains, or male gametes (Kaul, 1988). Thus, CMS is agronomically important in F₁ hybrid seed production (Duvick, 1959). During the last several decades, considerable progress has been made in understanding the mechanisms of plant male sterility through genetics (Athwal et al., 1967), cytology (Sodhi et al., 2006), physiology (Jiang et al., 2007), biochemistry (Biasi et al., 2001), and molecular biology (Crouzillat et al., 1991), information on tapetal alterations, and pollen wall development, which have been studied in the *Arabidopsis ms1* mutant (Vizcay-Barrena and Wilson, 2006), but CMS in Chinese cabbage has not been examined.

The features of pollen are mainly reflected in the pollen wall structure, pollen outer sculpted exine, pollen size and shape, and germination characteristics. The pollen wall is generally thought to consist of 2 layers: the outer exine and the inner intine (Paxson-Sowders et al., 2001). Two layers of exine are distinguished - namely ectexine and endexine. Ectexine may be solid or contain internal foramina and comprises tectum (tegillum), columella (bacula), and foot layers. The intine, secreted by the microspore, is the innermost layer, is located between plasma membrane and the nexine (Heslop-Harrison, 1971), and contains cellulose, pectin, and various proteins (Owen and Makaroff, 1995). The morphological features of the tectum, bacula, and nexine vary considerably, but they are the most representative structural characteristics of the pollen wall.

Sporopollenin, a biopolymer that makes up the major material of the exine, plays a pivotal role in higher plants (Chaloner, 1976) by protecting them from excessive dehydration and attacks of fungi and bacteria (Bedinger, 1992). Although the exact chemical composition of sporopollenin remains unknown, evidence has indicated that it may be composed of long chain fatty acids, phenylpropanoids, phenolics, and traces of carotenoids (Boavida et al., 2005). Existing studies have shown that phenylalanine is a major precursor, but other carbon sources may also be involved (Guilford et al., 1998; Boavida et al., 2005). Therefore, sporo-

pollenin likely derives from several precursors that are chemically cross-linked to form a rigid structure (Guilford et al., 1998; Boavida et al., 2005).

Programmed cell death (PCD), a physiological cell death process involved in the selective elimination of unwanted cells (Pennell and Lamb, 1997), is defined as death resulting from the activation of a specific biochemical pathway that is under the control of the cell and, therefore, amounts to cellular suicide (Drew et al., 2000). In plants, PCD is correlated with developmental processes, including leaf senescence, the formation of sexual organs, the disappearance of aleurone cells, the removal of root cap cells, and the differentiation of specialized cell types such as tracheary elements (Pennell and Lamb, 1997; Jones, 2000; Kuriyama and Fukuda, 2002). The objective of the present study was to investigate pollen wall abortion and tapetum change in RC₇ male sterile Chinese cabbage and analyze the reasons it occurs, which will become the basis for further complementary molecular studies.

MATERIAL AND METHODS

Plant materials

CMS line RC₇ (Zhao and Ke, 2007) of Chinese cabbage was used in this study. The maintainer line B7 served as a reference. The plants were grown in the experimental fields of Northwest A & F University, Yangling, China. RC₇ plants are sterility stable, seedling yellow-free, and nectary normal and have good power, and the infertility and sterility rates in the strains reached 100%. Two varieties of Chinese cabbage 'Golden 70' and 'Golden 90' were bred for the production of the application using RC₇.

Ultrastructural studies

Flowering buds at various developmental stages were obtained in early April and fixed in 4% (w/v) glutaraldehyde in sodium phosphate buffer, pH 7.2. Sepals and petals were removed to allow the solution to reach the inner parts of the flowers, and dissected buds were then vacuum infiltrated (30 Pa for 30-60 min). The fixation, dehydration, and embedding were carried out according to a method described by Kang (1996), but phosphate buffer, pH 7.2, was used in our study. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate aqueous solutions and viewed with a JEOL-1230 transmission electron microscope (Tokyo, Japan).

RESULTS

Pollen structure at the early tetrad stage in RC₇

Ultrastructural studies demonstrated that pollen structure at early development stages was unaffected in the RC₇ line (Figure 1). The tetrad stage was surrounded by a dense polysaccharide wall, representing a callose wall. A quarter of these lanes was surrounded by a callose wall (Figure 1A and B). Normal primexine development was seen in RC₇, although more electrodeposited materials appeared to be present on the surface of the callose wall (see Figure 1A).

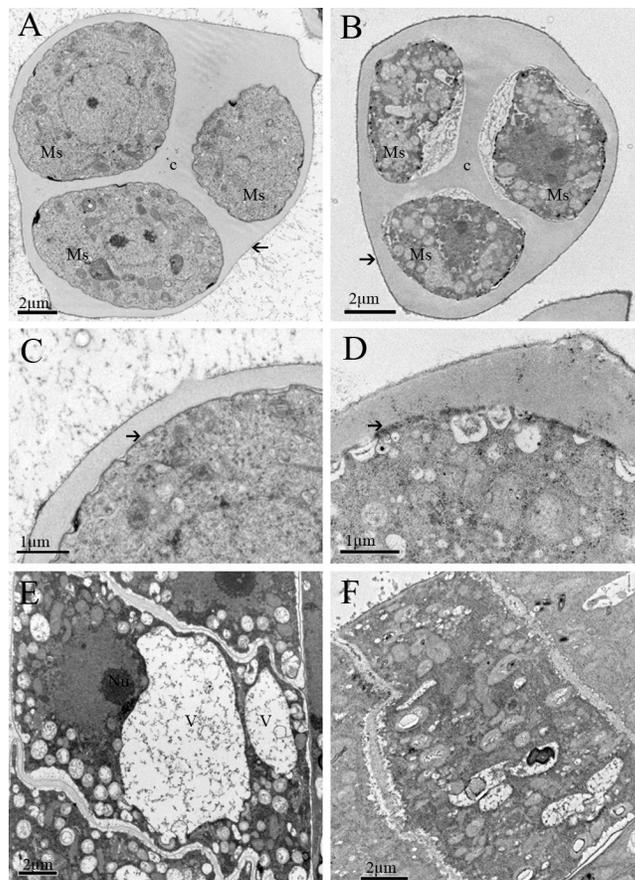


Figure 1. Ultrastructural structure of cross-sections of anthers of RC_7 and B7 at the tetrad stage. Tetrads of microspores in RC_7 (A) and B7 (B). The four haploid microspores are surrounded by a callose wall. Aggregation of electron-dense material can be seen on the surface of the callose wall (arrows). Higher magnification view of the microspore undulations in RC_7 (C) and B7 (D). Deposits of sporopollenin are visible on the surface of the outer callose wall that surrounds the microspores (arrows). Higher magnification view of the tapetum cell in the B7 type (E) and RC_7 (F). In B7 type, tapetum neatly arranged, but vacuoles become larger in the tapetum cell (E). Ms = microspore; c = callose; Nu = nucleolus; V = vacuole. Bars for A, B = 2.0 μm , for C, D = 1.0 μm , and for E, F = 2.0 μm .

Exine and intine formation

In both the B7 and RC_7 types, a preliminary cell wall was laid down by the microspores (primexine) at the tetrad stage (see Figure 1A and B). The membrane of the microspores had smooth undulations that served as specific sites for further deposition of electron-dense materials (sporopollenin) (Figure 1C and D). This deposition (probacula) served, in turn, as the anchoring point for the formation of the exine baculae. Before the callose wall that surrounds the microspores degraded, the sculpturing of the microspore wall of RC_7 had already become visible (see Figure 1C). At the ring-vacuolar stage, the RC_7 exine appeared disorganized and translucent, thus forming a discontinuous nexine (Figure 2A and C). In the B7 type, the sporo-

pollenin had polymerized, forming typical bacula and tectum (Figure 2B and D). The structure of the RC₇ exine was abnormal; the deposited sporopollenin formed irregularly shaped, spike-like structures that differed from the rod-like shape of the fertile type (see Figure 2C). In the B7 type, secretion of the tapetum cell was seen, thus adding materials to the exine (see Figure 2D). However, in RC₇, exine formation was limited owing to little or no secretion (see Figure 2C). By the time the RC₇ microspores underwent pollen mitosis I (Figure 3), a very limited and discontinuous exine wall had formed, creating a seriously discontinuous nexine or foot layer at some points (Figure 3A), whereas the intine wall had a wavy appearance and was completely established, indicating that the exine was completely mature in the B7 type (Figure 3B).

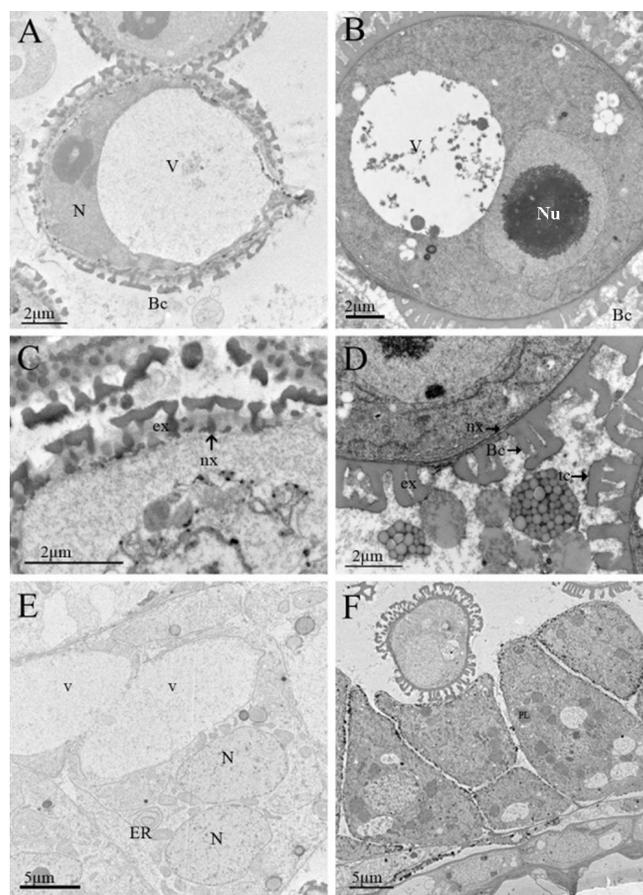


Figure 2. Ultrastructural structure of cross-sections of anthers of RC₇ and B7 at the ring-vacuolated stage (A, C, E = RC₇; B, D, F = B7). **A.** The vacuole occupies the microspore cytoplasm almost in its whole part in the vacuolate microspore stage; the microspores become leaky. Microspore cytoplasm is reduced and pushed to the periphery of the cell by the vacuole. **B.** At the 'ring' stage, the nucleus is displaced to the side of the microspore in the fertile type. Higher magnification view of the aberrant exine wall of RC₇ type (**C**) and the maturing exine wall in B7 type, lots of plastids (secretion of the tapetum cell) can be seen adding material to the extine layer (**D**). **E.** Big vacuolated (v) in RC₇, tapetal cells in the vacuolate microspore stage. **F.** Tapetal at the vacuolated microspore stage; the tapetum cells distort severely and contain plastids (PL). V = vacuole; N = nucleus; Nu = nucleolus; Bc = bacula; ex = exine; tc = tectum; nx = nexine; ER = endoplasmic reticulum. Bars for A, B, C, D, E, F = 2.0 μ m.

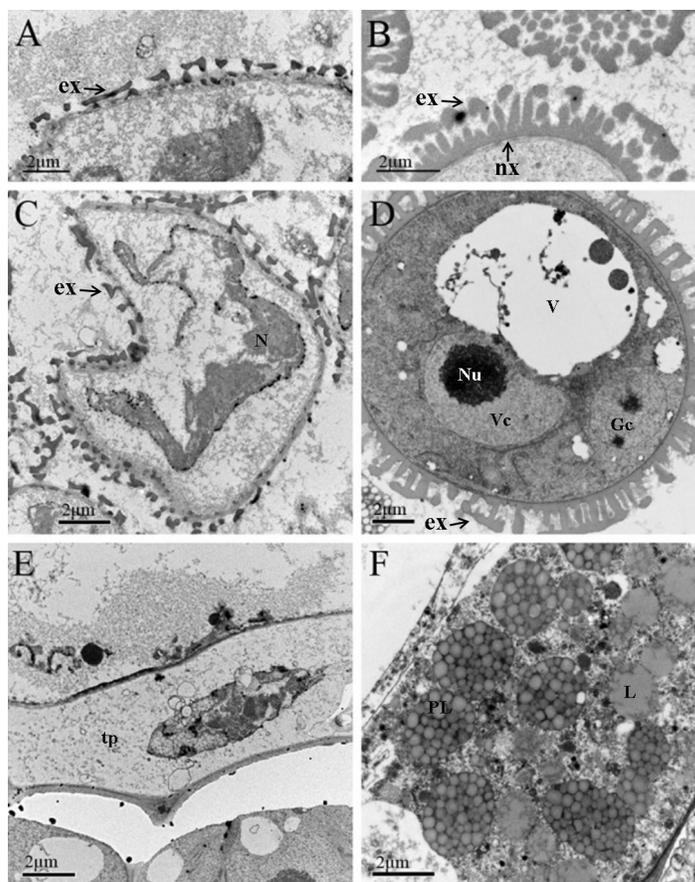


Figure 3. Ultrastructural structure of cross-sections through mitosis I of RC₇ and B7 type (A, C, E = RC₇; B, D = the sterile type). In the RC₇ (A), no further deposits of tryphine or any other substances are observed. The spaces between the unresolved baculae contain very little material. B. In the fertile type, deposits of tryphine fill the gaps between the baculae. After pollen mitosis I, in the RC₇ microspore (C), cytoplasm shrink, the nuclear membrane has lost integrity. In the fertile type (D) the generative cell is located at one side of the cell and is surrounded by a continuous wall of intine, a generative cell (Gc) and a vegetative cell (Vc) are observed. E. In the RC₇, tapetal cells (tp) were plasmolysed and the cell walls of the sterile tapetum have now degenerated completely and collapse. F. In the fertile line of B7; the tapetum cells contain multiple plastids (PL) with numerous small spherical plastoglobuli, and lipid bodies (L), which are released into the anther cavity following lysis of the tapetum cell. ex = exine; nx = nexine; tp = tapetal cells; Vc = vegetative cell; Gc = generative cell; V = vacuole; N = nucleus; Nu = nucleolus. Bars for A, B, C, D, E, F = 2.0 µm.

After fertile microspores underwent pollen mitosis II, lignin thickening and chloroplasts were not observed in the anther epidermis in the RC₇ line (Figure 4A). However, in B7 anthers, many chloroplasts were observed in anther epidermis, and the endothecium of the anther epidermis appeared to have thickened lignin (Figure 4B). In RC₇ anthers, some of the microspores managed to develop further and go through mitosis, although they did not reach maturation because no pollen was seen in the anthers (Figure 4C). Cell remnants of dissolved microspores and tapetal cells were observed as a disorganized mixture of organelles that oc-

cupied a large area in the anther locule (see Figure 4C). However, pollen grains containing vegetative and generative cells formed in the fertile anthers (Figure 4D).

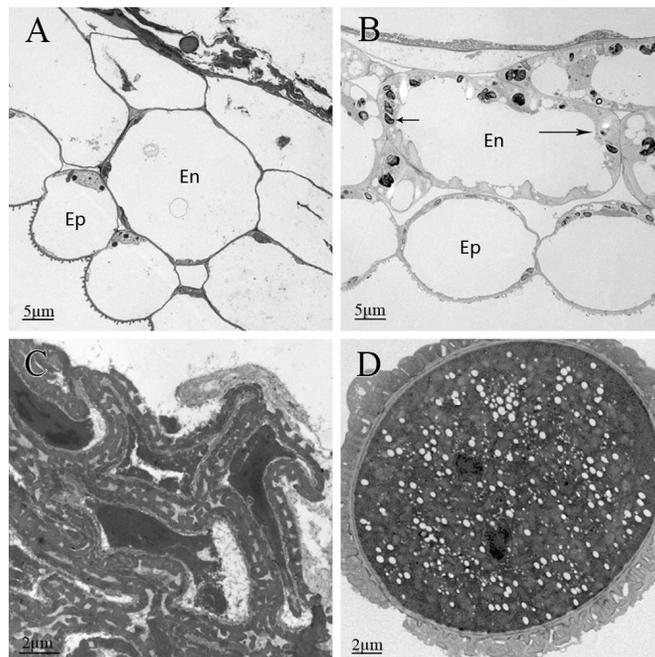


Figure 4. Ultrastructural structure of cross-sections through mitosis I of RC_7 and B7 type at the pollen mitosis II stage (A, C = sterile type; B, D = fertile). **A.** Endothecium (En), and epidermis (Ep) in the sterile type. **B.** En and Ep in the fertile type. Lignin thickenings (long arrow) and chloroplast (short arrow) are observed in the endothecial cells. **C.** Some remnants of abortive microspores are evident, but no pollen grains. **D.** Mature pollen in the fertile type. Bars for A, B = 5.0 μm , for C, D = 2.0 μm .

Tapetum cells

The tapetum cells were neatly arranged in B7, whereas those in the RC_7 type had larger vacuoles in the tetrad stage (see Figure 1E and F). However, only after the dissolution of the callose wall, when the microspores were free in the anther locule, was the change in RC_7 clearly visible. The cytoplasm of the tapetum cells became abnormally vacuolated (see Figure 2E). In B7, the tapetum cells distorted severely and contained multiple plastids (see Figure 2F). At the pollen mitosis I stage, the tapetum cells of B7 contained multiple plastids with numerous small spherical plastoglobuli and lipid bodies, which were released into the anther cavity after lysis of the tapetum cell (see Figure 3F). The tapetum of the RC_7 line degenerated and collapsed, leaving only cell remnants in the anther locule at the pollen mitosis II stage (see Figure 3E).

Ultrastructural features of apoptosis in RC_7 microspores

Transmission electron microscopy further revealed morphological features characteristic of PCD in the RC_7 microspores (Figure 5), such as nuclear membrane invagination

(Figure 5A), cytoplasm condensation, disintegration of the nuclear and vacuole membranes, and an abnormal increase in rough endoplasmic reticulum (RER) (Figure 5C). The vacuole membrane lost integrity (Figure 5B); most of the cell cytoplasm was occupied by an autophagocytic vacuole, and autophagic processes were seen in the RC₇ tapetal vacuole. However, mitochondria in the RC₇ type were normal compared with those in the B7 type.

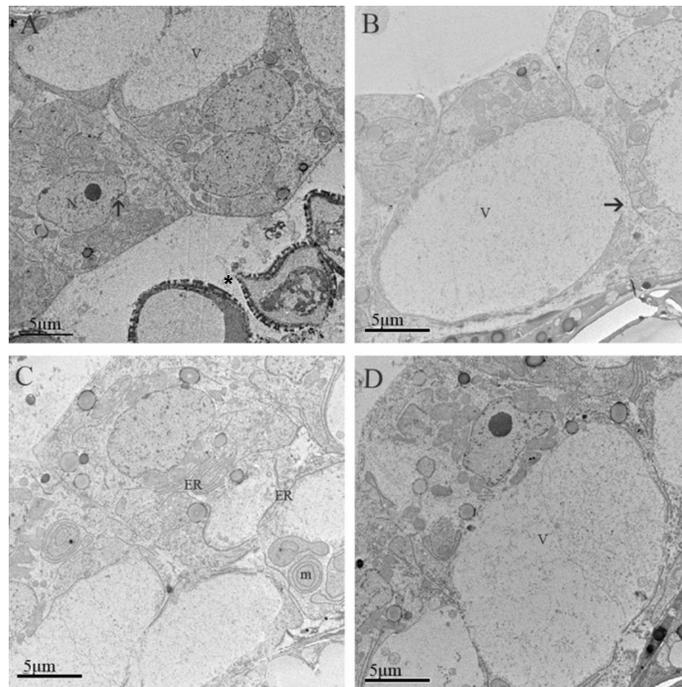


Figure 5. Ultrastructural programmed cell death features of the RC₇ tapetal cell. **A.** Nuclear membrane invagination in the tapetal cell is obvious (arrow in A). **B.** The vacuole membrane (tonoplast) has lost integrity (arrow in B). Mutant microspores degenerate, signs of cytoplasm degeneration in the microspores become leaky (asterisk in A). **C.** Abnormal increasing rough endoplasmic reticulum and normal mitochondria is obvious. **D.** Most of the cell cytoplasm is occupied by an autophagocytic vacuole. N = nucleus; V = vacuole; ER = endoplasmic reticulum; m = mitochondria. Bars for A, B = 5.0 µm, for C, D = 5.0 µm.

DISCUSSION

Defective exine sculpture in RC₇ microspores

The pollen exine plays important roles in protecting pollen grains from various environmental stresses and attacks by pathogens as they move from the anthers to the stigmas and during species-specific adhesion of the female stigma cells (Piffanelli et al., 1998). In the present study, the *ms2*, *dex1*, *flp1*, and *nef1* mutants were characterized as being defective in exine formation. The exine of *nef1* did not form at all. The sporopollenin was randomly deposited on the plasma membrane in the *dex1* mutant (Paxson-Sowders et al., 1997, 2001). The mutant of *ms2* had a very thin exine, whereas the exine of the *flp1* mutant looked normal

but was acetolysis sensitive (Aarts et al., 1997; Ariizumi et al., 2003). The early stages of microsporocyte meiosis and primexine deposition appeared to occur normally in the RC₇ line. At the tetrad stage, primexine appeared to be deposited on the microspores based on the regular undulation of the plasma membrane. Primexine with the callose wall may serve as a molecular filter, allowing the entrance of specific macromolecules (Shukla et al., 1998). The microspore plasma membrane undulates slightly (Paxson-Sowders et al., 1997), corresponding to the sites at which sporopollenin forms the probaculae. During microsporogenesis, the microsporocyte (or microspore) plasma membrane plays multiple roles in pollen wall development, including callose secretion, primexine deposition, and exine pattern determination (Guan et al., 2008).

In RC₇, early deposition of sporopollenin followed the normal pattern, and subsequent sporopollenin anchoring became abnormal, which was similar to that in the *Arabidopsis ms1* mutant (Vizcay-Barrena and Wilson, 2006). Abnormal baculae, with spiky and more translucent appearing sporopollenin and a very limited and sometimes interrupted exine were also observed in the RC₇ line, as reported in the *ms1* mutant (Vizcay-Barrena and Wilson, 2006). The RC₇ tapetum appeared to contain highly developed vacuoles, whereas the fertile tapetum had a more electron-dense cytoplasm. Vacuoles did not move toward the inner locule wall, which changed secretion polarity within the tapetum. Deficient polymerization of sporopollenin without a continuous primexine matrix has also been reported in the *dex1* mutant (Paxson-Sowders et al., 1997), whereas abnormal deposition of sporopollenin has been observed in the *ms2* mutant (Aarts et al., 1997).

Compared with that in the fertile type, intine formation in RC₇ microspores was defective, thus affecting pollen grain desiccation and, in turn, pollen viability, as reported for the *Arabidopsis ms33* mutant (Fei and Sawhney, 2001). Intine and exine growth have been reported to be regulated by various mechanisms (Vizcay-Barrena and Wilson, 2006). Exine deposition, in contrast to intine growth, can carry on after spore death (Shukla et al., 1998), but the defective exine may result in intine aberrations.

Aberrant tapetal cells in the microspores of RC₇

In the RC₇ type, the tapetum cells had some larger vacuoles in the tetrad stage, and the cytoplasm of the tapetum cells became abnormally vacuolated after the dissolution of the callose wall. No lipid materials were observed in tapetum cells at the ring-vacuolated stage, similar to those found in male-sterile *ms1* mutant of *Arabidopsis thaliana* (Vizcay-Barrena and Wilson, 2006) transgenic tobacco plants with the antisense gene of mitochondrial pyruvate dehydrogenase (PDH) (Jan et al., 2006). PDH is indispensable for the operation of the tricarboxylic acid cycle (Sun et al., 2009). Acetyl coenzyme A is produced directly from pyruvate via a PDH complex in the mitochondria. Acetyl coenzyme A released from mitochondria functions as a substrate for *de novo* fatty acid synthesis in plastids. Therefore, the absence of PDH in the mitochondria may fail to produce fatty acids for exine formation (Fischer and Weber, 2002; Yui et al., 2003). The no exine formation 1 gene, a novel male sterile mutant of *A. thaliana*, has apparent defects in exine formation, resulting in a lack of fatty acid or lipid biosynthesis in the plastids. Results from the present study indicated that RC₇ may have a defect in fatty acid or lipid biosynthesis in the plastids, as reported with the no exine formation 1 gene.

Features of PCD in degenerating RC₇ microspores

PCD is a physiological cell death event leading to the selective elimination of cells that are unwanted, unnecessary, or have exhausted their functions (Ellis et al., 1991; Pennell and Lamb, 1997). The genetically based programs for cell death are integral components of plant development (Panavas and Rubinstein, 1998) as well as for cells such as those of the tapetum, which serve temporary functions (Papini et al., 1999). Tapetal degeneration is thought to occur via PCD and be associated with changes such as cytoplasm and nuclear shrinkage, chromatin condensation, endoplasmic reticulum swelling, and rupture of vacuole membrane (tonoplast) (Papini et al., 1999).

In the *Arabidopsis* male sterility 1 mutation, degeneration of the tapetum includes severe vacuolation, cell swelling, and partial loss of the plasma membrane (Vizcay-Barrena and Wilson, 2006). All of these ultrastructural changes were also observed in the RC₇ line in the present study. In addition, microspore leakage was found in RC₇; however, the cells did not appear to collapse. At the pollen mitosis II stage of anther development in RC₇, the locule was filled with enlarged microspores and tapetal cells. These results are inconsistent with those reported in the hackly microspore mutant by Ariizumi et al. (2005), who observed the tapetum expanding and crushing the microspores.

Although invagination of the nuclear membrane and RER was observed, mitochondria were apparently normal in the RC₇ microspores, suggesting that their degeneration might follow a PCD pathway. Some ultrastructural markers of PCD were seen in the vacuolated microspores in the RC₇ CMS line - for example, expansion of the RER cisternae to confine portions of the cytoplasm.

Possible reason for RC₇ abortion

PCD, similar to cell proliferation and differentiation, is an active, controlled phenomenon in developing systems (Sanders and Wride, 1995). The normal process of PCD is "on hold" until the tapetal cells fully synthesize and secrete the required metabolites, and then a signal triggers PCD and the tapetum goes through an irreversible sequence of events that lead to cell death, which is characterized by cellular swelling, rupture of the plasma membrane, and spilling of the cell contents (Vizcay-Barrena and Wilson, 2006). After the tapetum disintegrates, all cell contents are released into the anther locule, and released tapetum cell debris (pollenkitt and tryphine) is incorporated into the wall of the maturing pollen grains, which contribute to the mature pollen coat. Based on ultrastructural observations, RC₇ tapetal cell breakdown likely does not occur in the normal process of PCD but through triggering PCD in control. We presume that tapetal dysfunction may subsequently induce PCD in the microspores, thus blocking the process of pollen development.

Successful pollen wall development, especially exine wall formation, requires precise coordination of the microspore and tapetum (Paxson-Sowders et al., 1997; Piffanelli et al., 1998). During initial tapetal secretion, the partial degradation of the plasma membranes, large vacuolation, and energy expenditure on synthesizing pollen wall components indicate that the tapetal cells may be inviable, making degeneration inevitable. Based on the above observations, we speculate that the genes responsible for regulating pollen wall material secretion and development may be absent in RC₇. The exact mechanisms of PCD signal

transmission and the molecular nature of the apoptotic machinery in RC₇ anthers require further study.

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