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RESEARCH PAPER

Altered tapetal PCD and pollen wall development in the *Arabidopsis ms1* mutant

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Abstract

The *Arabidopsis male sterility1* mutation results in mature anthers that are devoid of pollen. Meiosis and early development progress normally; however, after microspore release, the microspore cytoplasm and tapetum become abnormally granular and vacuolated, and degeneration occurs. Pollen wall development is seriously affected; primexine formation within the callose wall appears to occur normally, however, once the callose is degraded, abnormal deposits of electrodense material are detected which result in irregular spike-shaped structures, rather than the characteristic rod-like shape of the wild-type bacula. The internal intine wall is also reduced compared with wild type. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) staining and ultrastructural analysis have indicated that programmed cell death (PCD) occurs in the wild-type tapetum after microspore mitosis I. However, no signs of PCD are seen in the *ms1* tapetum, where large autophagic vacuoles and mitochondrial swelling suggest that necrotic-based breakdown of the tapetum is occurring in the *ms1* mutant rather than the normal, regulated PCD process. After the formation of the large, autophagic vacuole in the tapetum, TUNEL staining is detected in the mutant microspores, indicating that they may go through a PCD-based breakdown as a secondary consequence of the observed tapetal aberrations. Based on these observations, two possible roles for MS1 can be hypothesized; MS1 may function by modifying the transcription of tapetal-specific genes implicated in pollen wall development, which then regulate pollen wall material secretion and in turn wall development and tapetal PCD. Alternatively, the *MS1* gene may control tapetal development by directly regulating tapetal PCD and breakdown.

Key words: Anther, exine, intine, male sterility, pollen, programmed cell death (PCD), sporopollenin, tapetal.

Introduction

One of the most distinctive features of the pollen grain is the pollen wall. It consists of two different layers: exine and intine. The intine is the innermost layer that is located adjacent to the pollen plasma membrane; it has a relatively simple composition of cellulose, pectin, and various proteins, and is secreted by the microspore (gametophytic origin) around the ring-vacuolated microspore stage (Owen and Makaroff, 1995). It consists of two layers: a granular exintine towards the exterior, and a microfibrillar endintine towards the interior. The exine, facing the exterior, comprises two layers: the innermost featureless nexine and the outer sculpted sexine, which presents multiple pores and furrows. This outermost exine wall is ornamented in such a specific fashion that it serves as a diagnostic tool for taxonomists and paleobotanists (Shukla *et al*., 1998). The sexine can have separate sublayers: an outer roof, or tectum; and a central segment formed by columns, or baculae. The exine is composed mostly of sporopollenin, a widespread substance present in algae, fungi, moss, and fern spore walls. It is extremely resistant and it serves as a protective barrier against excess dehydration, and fungal and bacterial attack, while the male gametophyte is free from the sporophyte prior to fertilization (Bedinger, 1992). The sporopollenin is secreted by the tapetum (sporophytic

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; PCD, programmed cell death; PI, propidium iodide; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

origin) and polymerizes onto the primexine, a microfibillar polysaccharide matrix that serves as the anchoring point for sporopollenin deposition, to form the baculae. The exact composition of the sporopollenin is still not known, but evidence indicates that it may be composed of aliphatic polymers containing aromatic or conjugated side chains (Domínguez and Mercado, 1999). Filling the gaps between baculae, is the pollen coat (sporophytic origin) consisting of two different tapetally synthesized coat materials, pollenkitt and tryphine, mainly of lipidic origin (Owen and Makaroff, 1995). The pollen coat has numerous functions, for example, allowing pollen grains to stick to pollinator vectors or to the dry surface of the stigmas. It also carries proteins involved in self-incompatibility responses (Piffanelli *et al*., 1998).

Aberrations in exine sculpturing have been found in several *Arabidopsis* mutants, for example, *ms2*, *flp1*, *nef1*, and *dex1*. In the *ms2* mutant, a complete lack of exine layer was reported, suggesting a defect in normal sporopollenin deposition. The MS2 protein is a fatty acyl reductase and has been proposed to be involved in the sporopollenin polymerization pathway (Aarts *et al*., 1997). In the *dex1* mutant, sporopollenin synthesis takes place, but primexine development is abnormal, which alters the conformation of the membrane and, therefore, sporopollenin deposition. Different roles have been hypothesized for the DEX1 protein in pollen wall formation: DEX1 could be a linker protein participating in the attachment of the primexine or sporopollenin to the plasma membrane, or it could be a component of the primexine itself and play a role in its polymerization, or be part of the rough endoplasmic reticulum and be involved in the processing or transport of primexine precursors to the plasma membrane (Paxson-Sowders *et al*., 1997, 2001). The *flp1* mutant was reported to show an excess of tryphine covering the exine baculae. In this mutant, although baculae are formed and sporopollenin polymerizes, the sculpturing of the exine is irregular with patchy architecture, and some parts of the exine are broken apart. Also, an excess of tryphine covering the interstices of the exine gives the inviable pollen grain a smooth surface in contrast to the reticulate aspect of the pollen in the wild type. The FLP1 protein is therefore likely to be involved in the synthesis of the tryphine and sporopollenin (Ariizumi *et al*., 2003). Defects at an earlier stage of wall development were found in the *nef1* mutant, in which the primexine is poorly developed and although sporopollenin is produced it does not polymerize. NEF1 has been proposed to be a plastid inner envelope membrane protein that maintains plastid envelope integrity (Ariizumi *et al*., 2004). In all of these mutants, either the sporopollenin synthesis is altered (*nef1*, *ms2*, *flp1*) or the exine pattern is defective (*dex1*).

The *male sterility1* (*ms1*) mutant in *Arabidopsis* was isolated from an EMS- (ethyl methane sulphonate) mutagenized population of *Arabidopsis thaliana* Landsberg *erecta* (L*er*) seed (Van der Veen, 1968). The *ms1* mutation is recessive and causes the abortion of immature pollen after microspores are released from the tetrads (Dawson *et al*., 1993). Preliminary light microscopy studies revealed that in the *ms1* mutant the process of pollen development begins normally, with meiosis of the pollen mother cells and tetrad formation progressing as in the wild type. However, pollen development is affected soon after the microspores are released from the tetrad. At this point, the microspore cytoplasm and tapetum become abnormally granular and vacuolated. The microspores and tapetum degenerate, resulting in a male-sterile plant, which does not produce pollen but is otherwise phenotypically normal (Wilson *et al*., 2001). To understand further the role of *MS1*, a comparative ultrastructural study of pollen development in the wild type and the mutant was conducted, paying special attention to the tapetal tissue and the formation of the pollen wall. This has shown that in the *ms1* mutant, pollen wall formation is aberrant, with intine and deposition of exine incomplete, leading to the total degeneration of the microspores and subsequently to empty anther locules. A similar phenotype has been described in the *hackly microspore* (*hkm*) mutant which has been found to be allelic to *ms1* (Ariizumi *et al.*, 2005).

Further analyses were conducted in order to address the defects observed in the *ms1* mutant tapetum. There are a large number of reports of male sterility associated with disturbances in tapetal development and degeneration. Tapetal degeneration is not an uncontrolled event, but a process of programmed cell death (PCD) (Bedinger, 1992; Papini *et al*., 1999; Wu and Cheun, 2000). In normal development, soon after microspores are released from the tetrad and before mitosis I, the tapetum cells starts to degenerate. This is marked by cytoplasm shrinkage and subsequent separation from the cell wall. Tapetal protoplasts become vacuolated as the wall degrades, and multiple vesicles containing exine precursors (sporopollenin) fuse with the plasma membrane, releasing their contents into the anther locule. As the tapetum undergoes cell death, tapetal cells produce tryphine and pollenkitt, which will coat the mature pollen grains. Ultimately, the cells rupture and degradation of the entire tapetum occurs, releasing all the cell remnants, which are essential for completion of the extracellular sculpting of the pollen grains. In this manner, tapetal cells, prior to their death, manage to secrete all the tapetal-derived pollen coat components necessary for pollen maturation. It is clear that the degeneration of the tapetum is a very tightly controlled process, which must occur in a synchronized manner; failing to do so frequently results in sterility (Kaul, 1988). Therefore, PCD in the tapetum is a physiological process in which timing is crucial for pollen maturation. In order to address the abnormal degeneration observed in the *ms1* mutant, a comparative TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) assay was conducted in wild-type and mutant anther

tissues. The results of this experiment along with the data obtained from the ultrastructural analysis are discussed.

Materials and methods

Plant material

Seeds of *A. thaliana* (L). Heynh. var. L*er* and *ms1.1-ttg* mutant were sown onto a compost mix of Levington M3:vermiculite (ratio 3:1) and grown in a greenhouse at 21/17 °C (day/night) and with a 22/2 h photoperiod. Whole inflorescences from *ms1* and wild-type plants were collected when plants were 28–30-d-old, and all open flowers were discarded. Plant tissue was first fixed in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS; 1.3 mM NaCl, 0.03 M Na_2HPO_4, 0.03 M NaH_2PO_4, pH 7.2), 0.1% (v/v) Triton X-100 and 0.1% (v/v) Tween-20 for 1 h at room temperature and under a very gentle vacuum, and then transferred to fresh fixative and incubated overnight at 4 °C, to decrease the mobility of cellular components.

Microscopy

For transmission electron microscopy (TEM) analysis, samples were washed with PBS (pH 7.2) at room temperature and post-fixed in 2% (w/v) OsO_4 in PBS (pH 7.2) for 3 h, and then rinsed twice in PBS. Prior to dissection of individual buds, the tissue was dehydrated in a graded ethanol series (10% increments) and infiltrated with propylene oxide. Individual buds were finally embedded in Spurr's resin. Ultra-thin (50–70 nm, silver grey to pale gold) cross-sections through the buds were cut with a diamond knife (DIATOME, Switzerland) on a Reichert-Jung Ultracut ultramicrotome, floated off onto filtered distilled water, and collected on uncoated 200 hexagonal mesh copper grids (Gilder grids, TAAB). Sections were stained with a saturated solution of uranyl acetate in 50% (v/v) ethanol and Reynold's lead citrate. After double staining, observations were made under a Jeol JEM, 1010 electron microscope operating at 100 kV, and images were photographed with a Kodak Megaplus Camera, Model 1.6i.

TUNEL assay

For the TUNEL assay, fixed samples were washed with PBS (pH 7.2) at room temperature and dehydrated in a graded ethanol series (10% increments), cleared by dipping into 2:1, 1:1, and 1:2 (v/v) ethanol/histoclear for 1 h each, and then three times in 100% histoclear for 30 min each at room temperature. Tissue was then embedded in paraffin wax for sectioning. Sections of 6 μm were cut using a Microm HM315 microtome attached to polylysine-coated slides, deparaffinized with histoclear, and hydrated in a graded ethanol series (10% increments). *In situ* nick end labelling of nuclear DNA fragmentation was performed in a humid chamber for 1 h in the dark at 37 °C with a TUNEL apoptosis detection kit (DeadEnd™ Fluorometric TUNEL System, Promega) following the manufacturer's instructions. For each experiment, a positive control was prepared by treating the sections with 1 U μl^{-1} DNase I for 10 min at 37 °C before labelling as above. The negative controls were labelled in parallel, except for the absence of the enzyme terminal deoxynucleotidyl transferase (TdT). The labelling reaction (TUNEL) was stopped with 2× SSC and then slides were rinsed with PBS (pH 7.2). Slides were counterstained with 1 μg ml^{-1} of propidium iodide (PI) and 2 μg ml^{-1} of 4′,6-diamidino-2-phenylindole (DAPI), for fluorescence microscopy. Slides were mounted temporally with citifluor (antifading agent) (Citifluor Ltd., London) and kept overnight (if necessary) at 4 °C in the dark until microscopic observation. Samples were analysed under a fluorescence confocal scanner microscope (Leica TCS SP2 Confocal, Leica Microsystems, Heidelberg, Germany). The fluorescent filter was set to view the green fluorescence of fluorescein at 520±20 nm, the red fluorescence of PI at >620 nm, and the blue of DAPI at 460 nm.

Results

Early stages of pollen development are not affected by the ms1 *mutation*

Ultrastructural studies demonstrate that the early stages of pollen development are not affected in the *ms1* mutant (data not presented). After meiosis whilst the microspores are still within the callose wall, normal primexine development is seen in the mutant, although more electrodense material appears to be present on the surface of the callose wall (Fig. 2B). However, it is only after the dissolution of the callose wall, when the microspores are free in the anther locule, that the effects of the mutation are clearly visible. The cytoplasm of the tapetum cells and microspores becomes granular and abnormally vacuolated, then the microspores and tapetum degenerate and collapse, leaving only cell remnants in the anther locule. In the *ms1* mutant, some of the microspores manage to develop further and go through mitosis, although they do not reach maturation since no pollen is seen in the anthers (Fig. 1).

Exine and intine formation

At the tetrad stage in the wild type, a preliminary cell wall is laid down by the microspores (primexine) (Fig. 2A); the membrane of the microspores shows smooth undulations which serve as specific sites for further deposition of

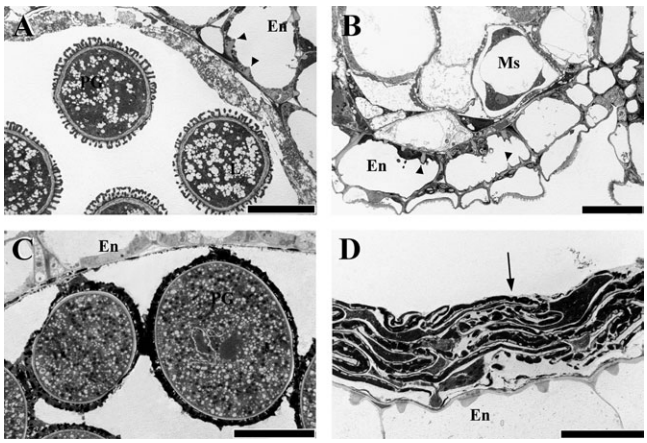

Fig. 1. Transmission electron micrographs of cross-sections through anthers of wild type and the *ms1* mutant at the pollen mitosis II stage (A, C, wild type; B, D, *ms1*). (A) Wild-type microspores go through a second mitosis; the generative cells divides to form two small sperm cells. The cytoplasm of the microspore contains lots of small vacuoles. Tapetal cells are degenerating. In the mutant (B), most of the microspores have degenerated completely although some reach mitosis I (asterisk). Lignin thickenings are observed in the endothecial cells (arrowhead). (A, B) Bar=10 μm. (C) Wild type anther locule. (D) The contents of the anther locule (arrow) have collapsed in the mutant. Bar in (C)=10 μm; bar in (D)=5 μm. En, endothecium; PG, mature pollen grain; Ms, microspore.

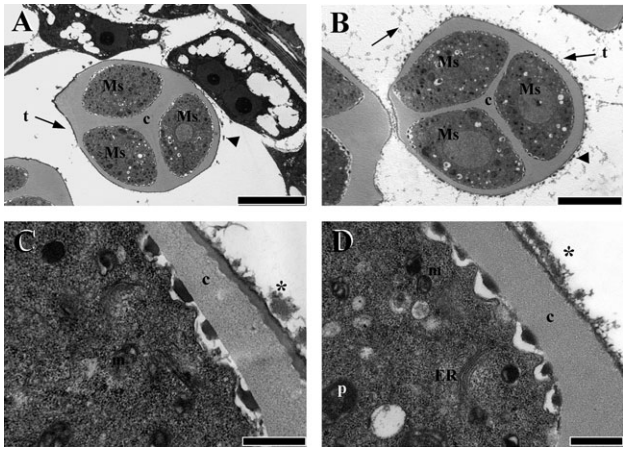

Fig. 2. Transmission electron micrographs of cross-sections through tetrads of wild type and the *ms1* mutant (A, C, wild type; B, D, *ms1*). (A, B) Tetrads of microspores in the wild type and mutant, respectively. The four haploid microspores are surrounded by a callose wall. Aggregation of electrodense material can be seen at the surface of the callosic wall (arrowhead). Excess fibrillar material is observed in the *ms1* locules (arrow). (A, B) Bar=5 μm. (C, D) Higher magnification view of the microspore undulations in the wild type and the mutant, respectively. Deposits of sporopollenin are visible on the surface of the outer callose wall that surrounds the microspores (asterisk). (C, D) Bar=0.5 μm. Ms, microspore; c, callose; m, mitochondria; p, plastid; ER, endoplasmic reticulum.

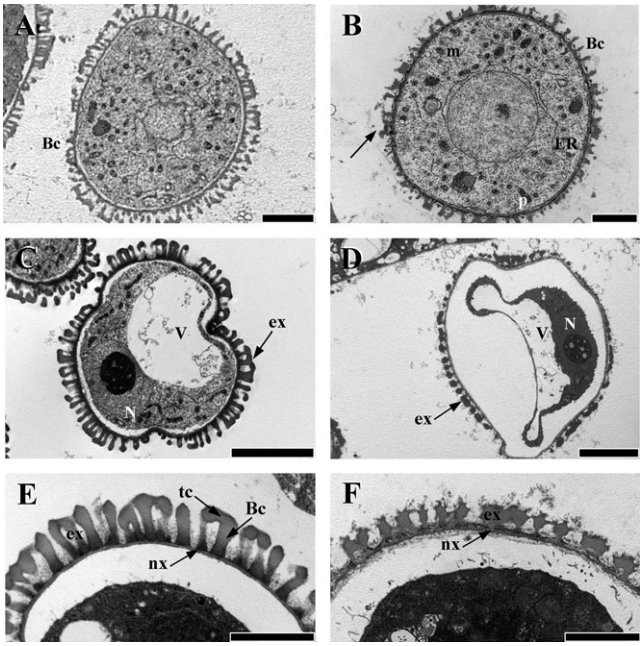

Fig. 3. Transmission electron micrographs of cross-sections through anthers of wild type and the *ms1* mutant at the ring-vacuolated stage (A, C, E, wild type; B, D, F, *ms1*). (A, B) Just after the microspores are released, differences can be observed between the wild-type (A) and mutant (B) microspore wall. In the mutant, the baculae are not well resolved (arrow). Deposition of sporopollenin has taken place but it does not seem to polymerize as in the wild type. (A, B) Bar=2 μm. (C, D) At the 'ring' stage, the nucleus is displaced to the side of the microspore in the wild type (C). In the mutant (D), the vacuole occupies the microspore cytoplasm almost in its totality; only the nucleus is visible. Also, in the mutant, the microspores are larger than in the wild type. (C, D) Bar=5 μm. (E, F) Higher magnification view of the maturing exine wall in the wild type (E) and the aberrant exine wall of the mutant (F). (E, F) Bar=2 μm. Bc, bacula; m mitochondria; p, plastid; ER, endoplasmic reticulum; ex, exine; V, vacuole; N, nucleus; tc, tectum; nx, nexine.

electrodense material (sporopollenin) (Fig. 2C). This deposition (probacula) serves as the anchoring point for the formation of the exine baculae. Before the callose wall that surrounds the microspores is degraded, by means of a tapetally secreted callase, the sculpturing of the *ms1* mutant microspore wall is already visible (Fig. 2D). As the microspores are released from the tetrad, the baculae of the mutant microspores wall appear poorly resolved, with patches of electrodense material rather than the distinctive baculae structure observed in the wild type. The microspores then round up and the exine structure becomes more prominent (Fig. 3A, B). In the wild type by the ring-vacuolated stage, the sporopollenin has polymerized, forming the characteristic bacula and tectum; in the mutant, the structure of the exine is abnormal, the deposited sporopollenin forms irregular-shaped spike-like structures that do not resemble the characteristic rod-like shape of the wild-type bacula, and mutant microspores appear to stick together and are bigger (~1.5-fold) than the wild type (Fig. 3C, D). Although the deposition of sporopollenin does not occur at random, indicated by the distribution/presence of the bacula-like structures observed in *ms1*, the mutant exine appears disorganized and translucent, presenting an irregular nexine or foot layer and on some occasions even tectum (Fig. 3E, F). Secretion of the inner intine wall starts around the ring-vacuolated stage, when lots of small vesicles can be seen adding material to the intine (Fig. 4A). At this point, in the wild type, the exine is completely mature. However, in the mutant, intine formation is limited and no vesicles are seen adding material to the wall (Fig. 4B). By the time the microspores go through pollen mitosis II (Fig. 4C), the intine wall is completely established in the wild type, presenting a wavy appearance, while in the mutant a very limited intine wall is observed (Fig. 4D).

Ultrastructural features of apoptosis are observed in ms1 *microspores*

Further TEM observations revealed morphological features characteristic of PCD in the mutant microspores (Fig. 5), such as nuclear DNA condensation, cytoplasm degeneration, and disintegration of the nuclear membrane and the vacuole tonoplast. The microspores also appear leaky, and autophagic processes were seen in the *ms1* tapetal vacuole.

TUNEL assay:apoptosis

Degeneration of the tapetum is known to occur via PCD. In order to investigate the abnormal degeneration observed in the *ms1* mutant tapetum, cleavage of nuclear DNA was followed using the TUNEL assay on wild-type and *ms1*

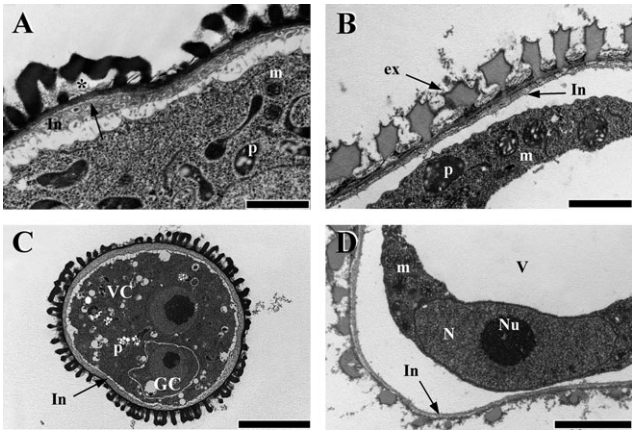

Fig. 4. Transmission electron micrographs of cross-sections through pollen of wild type and the *ms1* mutant (A, C, wild type; B, D, *ms1*). (A, B) In the wild type (A), deposits of tryphine (asterisk) fill the gaps between the baculae; lots of little vesicles can be seen adding material to the intine layer that is forming at this stage. In the mutant (B), no further deposits of tryphine or any other substances are observed. The spaces between the unresolved baculae contain very little material. (A, B) Bar=1 μm. (C, D) After pollen mitosis I, in the wild type (C) the generative cell is located at one side of the cell and is surrounded by a continuous wall of intine. Vesicles fuse to the plasma membrane contributing to intine formation (arrow). Plastids are also present in the cytoplasm of the vegetative cell. A small vacuole is present in the cytoplasm of the generative cell. In the cytoplasm of the mutant microspore (D), the only visible organelles are a few mitochondria. Mutant microspore cytoplasm is reduced and pushed to the periphery of the cell by the vacuole. Bar in (C)=5 μm; bar in (D)=2 μm. In, intine; m, mitochondria; p, plastid; ex, exine; VC, vegetative cell; GC, generative cell; V, vacuole; N, nucleus; Nu, nucleolus.

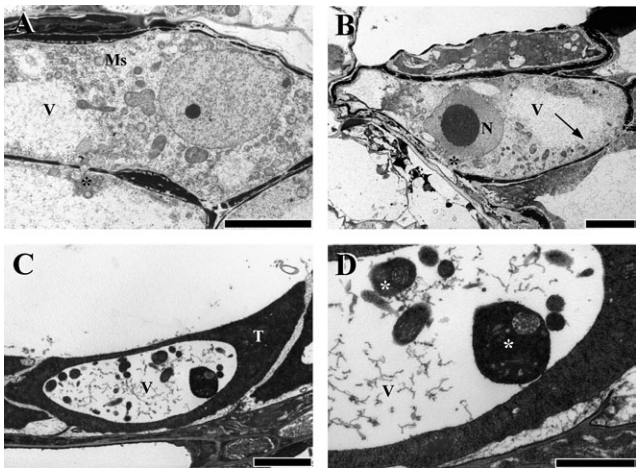

Fig. 5. Transmission electron micrographs of cross-sections through anthers at the ring-vacuolated stage of the *ms1* mutant. (A, B) At later stages, as the mutant microspores degenerate, signs of cytoplasm degeneration are obvious (A); the microspores become leaky (asterisk in A and B). The vacuole membrane (tonoplast) has lost integrity (arrow in B) and the nuclear membrane degrades after the vacuole has lysed (asterisk in B). (A, B) Bar=5 μm. (C, D) *ms1* tapetal cell. Most of the cell cytoplasm is occupied by an autophagocytic vacuole (C), which contains entire sections of cytoplasm and organelles (asterisks) (D). Bar in (C)=2 μm; bar in (D)=1 μm. Ms, microspore; N, nucleus; V, vacuole.

anther sections. The TUNEL assay has been designed to detect *in situ*, fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at the 3′-OH DNA ends using the enzyme TdT. The fluorescein-12-dUTP-labelled DNA can then be visualized directly by fluorescence microscopy.

A range of developmental stages were analysed, including microspore release, pollen mitosis I, and pollen mitosis II from the wild type, and the equivalent stages selected from the *ms1* mutant. Just after microspore release from the tetrad, wild type and *ms1* appear very similar; both present tapetal cells with TUNEL-negative nuclei (data not shown), indicating a lack of DNA fragmentation in the tapetum at this stage. TUNEL signal is, however, observed in the outer cell layers of the anther (epidermis, endothecium, and middle cell layer) and in the vascular bundle cells. This is likely to be an artefact of the fixation and embedding processes, since these tissues appear to be particularly prone to damage at this stage. In the wild type during pollen mitosis I, the tapetum starts degenerating, and this is corroborated by a TUNEL-positive signal in the tapetal nuclei at this stage (Fig. 6A). By mitosis II, the wild-type tapetal cells degrade completely and disappear, and no TUNEL signal is observed. TUNEL-positive nuclei are, however, present in the middle layer and the stomium cells,

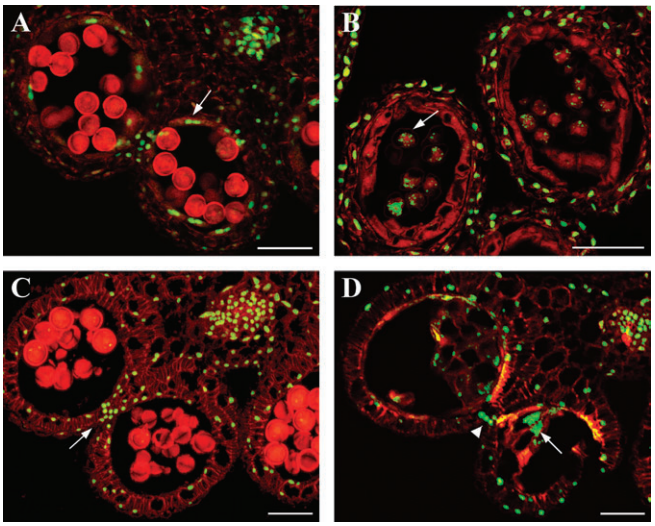

Fig. 6. Confocal microscopy showing DNA cleavage as indicated by the TUNEL assay in wild-type and *ms1* anther tissues. Nuclei have been stained with propidium iodide, indicated by red fluorescence, whilst TUNEL-positive nuclei are indicated by green fluorescence. (A) Wild-type pollen mitosis I. Tapetal degeneration has started, cells have shrunk, and TUNEL-positive signal is detected in the polynucleated tapetal cells (arrow). (B) Ring-vacuolated *ms1* microspores. TUNEL-positive signal is detected in the microspores (arrow), but not in the tapetal cells. (C) Wild-type pollen mitosis II. The tapetum has degenerated completely; no TUNEL signal is detected. TUNEL-positive signal is detected in the stomium area (arrow). (D) *ms1* stage corresponding to that in (C). Tapetum and microspores have degenerated completely. TUNEL-positive signal is detected in the cell remnants filling the anther locule (arrow). A strong signal is also detected in the stomium (arrowhead). All bars=40 μm.

and some cells of the epidermis, endothecium, and vascular bundle as the anther matures and undergoes endothecium secondary thickening and stomium lysis in preparation for dehiscence (Fig. 6C).

However, in the *ms1* mutant, no TUNEL-stained nuclei are observed in the tapetal cells or the microspores immediately after microspore release (Fig. 6B), suggesting a lack of DNA fragmentation in the tapetum, whilst the corresponding wild-type tissue shows TUNEL-positive staining. In the *ms1* mutant, a positive signal is only observed in the microspores at the ring-vacuolated stage (Fig. 6B). No TUNEL-positive signal is observed in the tapetum even during the later stages, when the microspore cytoplasm is occupied almost entirely by the vacuole (data not shown). Only as the microspores collapse is a TUNEL-positive signal detected amongst the collapsed microspores and tapetal cell remnants (Fig. 6D). During the later stages in development, as seen in the wild type, TUNEL-positive nuclei are present in the stomium and the middle layer, and also in the epidermis, and connective and endothecial tissue of the *ms1* mutant anthers (Fig. 6C, D).

Discussion

Exine sculpture is defective in the mutant microspores

The early stages of microsporocyte meiosis and primexine deposition appear to occur normally in the *ms1* mutant. Primexine is deposited on the microspore plasma membrane whilst the microspores are still in the tetrad. It has been suggested that primexine synthesis may occur *de novo* in the microspores, or by using precursors from the tapetum (Ariizumi *et al*., 2004), with the callose wall serving as a molecular filter, allowing the entrance of specific macromolecules (Shukla *et al*., 1998). Coinciding with the start of primexine deposition, the microspore plasma membrane undulates slightly (Paxson-Sowders *et al*., 1997), corresponding to the sites where sporopollenin will form the probaculae. Instead of moderate undulations, both wild-type and *ms1* plasma membranes show little 'pockets' where primexine accumulates (Fig. 2C–F); however, these could be artefacts due to tissue processing or chemical fixation (Ross *et al*., 2000).

In the *ms1* mutant, early deposition of sporopollenin follows the normal pattern, and subsequent sporopollenin anchoring is not compromised, in contrast to the defects in the *dex1* and *nef1* mutants (Paxon-Sowders *et al.*, 2001; Ariizumi *et al*., 2004). However, although sporopollenin deposition occurs, abnormal baculae are formed, with the sporopollenin appearing spiky and more translucent, instead of the characteristic pilum-like appearance; a very limited and sometimes interrupted nexine can also be observed in the *ms1* mutant. A similar phenotype was described in the *ms1* allele *hackly microspore* (*hkm*) in which changes in exine formation were seen during the microspore stage (Ariizumi *et al.*, 2005). The *ms1* tapetum appears to contain highly developed vacuoles, whereas the fertile tapetum presents a more electrodense cytoplasm. Secretion polarity within the tapetum also changes, with no movement of vacuoles towards the inner locule wall. The *ms1* mutant microspores tend to stick together, suggesting an unusual chemical composition of the pollen wall, possibly due to impaired sporopollenin synthesis and/or secretion in the *ms1* mutant. Deficient polymerization of sporopollenin was reported along with the lack of a continuous primexine matrix in the *dex1* mutant (Paxson-Sowders *et al*., 1997); abnormal deposition of sporopollenin was observed in the *ms2* mutant (Aarts *et al*., 1997). Abnormally resolved baculae are also present in the male-sterile flowers of the dioecious species *Actinidia deliciosa* (Biasi *et al*., 2001).

Minimal intine formation is observed in ms1 *microspores*

Intine secretion is regulated by the microspores, but the timing of intine development is highly variable, occurring in lily during the tetrad stage and in maize after microspore mitosis I (Shukla *et al*., 1998). In *Arabidopsis*, intine secretion starts during microspore release and is complete by mitosis II (Owen and Makaroff, 1995). In *ms1* microspores, only a preliminary intine structure is detected and, in contrast to the wild type, no vesicles, which contribute to the formation of this layer, are observed in the mutant microspore. Intine formation is also defective in the *Arabidopsis ms33* mutant, which affects pollen grain desiccation and therefore pollen viability (Fei and Sawhney, 2001). It has been reported that intine and exine growth are regulated by different mechanisms and that exine deposition, in contrast to intine growth, can carry on after spore death (Shukla *et al*., 1998). In *ms1* microspores, viability is compromised, as shown by the TUNEL-positive signal, which could result in the defective intine layer. Alternatively, since the intine layer adapts to the exine layer as the pollen grain volume changes during dehydration in the anther (Fei and Sawhney, 2001), the defective exine may result in intine aberrations. The presence of the large vacuole observed in *ms1* microspores indicates that the normal process of pollen dehydration is not taking place, and this could at least in part be due to the limited formation of the intine in the mutant.

TUNEL analysis failed to identify DNA fragmentation in the ms1 *tapetum*

The final contribution that the tapetum makes to pollen coat formation is during tapetal degeneration when all the cell remnants are released into the anther locule and then incorporated by the maturing pollen grain into the pollen coat (Papini *et al*., 1999). This breakdown is thought to occur via PCD, and is 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 wild type, tapetal PCD, as indicated by DNA fragmentation, could be detected by TUNEL-stained nuclei at the end of pollen mitosis I, coinciding with major shrinkage of the tapetal cytoplasm. No evidence of chromatin condensation was observed in the tapetal nuclei using TEM, although this has been reported in other cases.

However, a lack of TUNEL signal, indicating an absence of DNA fragmentation, was seen in the *ms1* tapetum, suggesting that, in the absence of *MS1* expression, tapetal breakdown does not occur by the normal process of PCD. A large, autophagic vacuole occupies most of the cytoplasm of the *ms1* tapetal cells; autophagic vacuoles have not been associated with plant PCD. The *ms1* tapetal cells swell due to the abnormal growth of the vacuole, resembling the inflammatory response that accompanies necrosis in animal cells (Berninghausen and Leippe, 1997), instead of the shrinkage observed in the degenerating wild-type tapetum. In addition, the mitochondria present in the degenerating *ms1* tapetum appear abnormal and swollen. In the PET1-CMS sterile sunflower, premature PCD was reported in the tapetum; however, despite cell collapse and DNA fragmentation, *in situ* localization of porin showed that mitochondria were not completely degraded (Balk and Leaver, 2001). These features point towards a necrotic breakdown process in *ms1* tapetal cells, which is passive and has no need for active mitochondria. Only during the final stage when microspores and tapetal cells collapse is a very faint TUNEL-positive speckle observed in the tapetum. Although major DNA fragmentation is known to be a late event in apoptosis (Collins *et al*., 1997), the possibility that this TUNEL labelling could be the result of cell death unrelated to a specific apoptosis-related pathway cannot be excluded.

Ultrastructural studies on degeneration of the tapetum of snap bean under heat stress (Suzuki *et al*., 2001) showed degeneration features resembling those observed in *ms1*, such as severe vacuolation, cell swelling, and partial loss of plasma membrane; however, they also reported tapetal residues in the anther locule after degeneration. In TGMS (thermosensitive genic male-sterile) rice, TUNEL signal is detected early in the PMC, the microspores lack primexine and sporopollenin, and they burst, releasing cellular debris into the locule space (Ku *et al*., 2003). Evidence of microspore leakage is observed in the *ms1* mutant; however, the cells do not appear to burst but leave cell remnants as 'empty sacs' with no tapetal residue in the anther locules. This implies that the limited *ms1* pollen wall provides sufficient strength to support the internal pressure of the microspores, in spite of the distorted shape and the cytoplasm leakage. During the final stages of anther development in *ms1-1*, the enlarged microspores and tapetal cells fill the locule; this is distinct from that reported for the *hkm* mutant (Ariizumi *et al*., 2005), where they describe the tapetum expanding and crushing the microspores.

Features of PCD are observed in the degenerating ms1 *mutant microspores*

Although DNA fragmentation was not observed in the *ms1* tapetum, TUNEL signal and DNA condensation, with apparently normal mitochondria, are seen in the *ms1* microspores, suggesting that the degeneration of the mutant microspores could be following a PCD pathway. Lytic vacuoles were also detected within the *ms1* microspores. These have frequently been reported as a feature of PCD; however, it is still not clear whether they have a key role defining the 'point of no return' or whether they have a supporting role by providing the necessary enzymes for recycling the contents of the dying cells (Groover and Jones, 1999; Danon *et al*., 2000; Fukuda, 2000; Obara *et al*., 2001; Gunawardena *et al*., 2004; Lam, 2004).

Possible role of MS1 during tapetum development

In light of the TUNEL results and the ultrastructural observations, it seems likely that *ms1* tapetal cell breakdown does not occur by the normal process of PCD and might be following an alternative cell death process. However, the tapetal dysfunction may subsequently induce PCD in the microspores, blocking the process of pollen development. Two possible roles for MS1 during tapetum development can be hypothesized. First, MS1 may act by modifying transcription of tapetal genes involved in pollen wall development, which regulate wall material secretion and, in turn, wall formation. These processes may then initiate normal tapetal PCD to facilitate complete deposition of tapetal wall materials onto the pollen grains. Alternatively, the *MS1* gene may regulate tapetal development by directly regulating tapetal PCD. PCD is a 'ready-to-be-activated' process in the tapetal cells (Balk and Leaver, 2001). Normally the process of PCD is 'on hold' until the tapetal cells have fully synthesized and secreted the required metabolites, then a signal triggers PCD and the tapetum goes through an irreversible sequence of events that leads to cell death. During the final stages of this degeneration process, the tapetum disintegrates, releasing all cell contents into the anther locule; this tapetal cell debris (pollenkitt and tryphine) will be incorporated into the wall of the maturing pollen grains contributing to the mature pollen coat. A possible role for *MS1* could be as a co-ordinator of these events. The *MS1* gene might regulate tapetal proliferation by inhibiting PCD while it is expressed. PCD suppressor genes involved in microsporogenesis and microgametogenesis have been previously reported, for example, the tapetum-specific zinc finger gene *TAZ1* (Kapoor *et al*., 2002) and the *Arabidopsis MMD1* gene which, like *MS1*, is a transcription factor with a PHD domain (Yang *et al*., 2003). In the *mmd1* mutant,

male meiocytes arrest, but the rest of the sporophytic tissues are not affected, implying that while in many cases of male sterility, aberrations in the tapetum lead to microspore cell death, death of the meiocytes does not necessarily imply degeneration of the tapetum.

However, if lack of *MS1* expression is a trigger for tapetal PCD, why does the *ms1* tapetum still degrade? The morphological changes occurring during the initial tapetal secretion, including cell wall loss, partial degradation of the plasma membranes, and energy expenditure on synthesizing pollen wall components, may mean that the tapetal cells are inviable and thus degeneration is inevitable. An alternative explanation is that the abnormalities in vacuoles in the mutant tapetum could be due to a loss of polar secretion. The cell wall materials may then be taken up by the vacuole, which turns autophagic and ends up engulfing the cell cytoplasm.

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