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Ultrastructural Study of Rice Tapetum under Low-Temperature Stress

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The development of male reproductive organs in rice is very sensitive to various environmental stresses. For example, exposing plants to low temperatures during the heading stage leads to a reduction in grain yield. Here, we grew rice under normal conditions and also at three different temperatures -- 16, 18, and 20°C. Treatment at a low temperature significantly decreased pollen viability and grain production. Cytological observations of the anther showed that the tapetum was the most sensitive to low-temperature stress, resulting in male sterility due to functional loss of the tissue. Detailed observations by transmission electron microscopy suggested that this abnormality was restricted primarily to the ER structures. The endoplasmic reticulum, a highly vulnerable sub-cellular organelle, showed two typical morphological aberrations, one in its pattern of arrangement, the other in the formation of ER bodies. Of our three experimental chilling temperatures, the most severe abnormalities were observed in tapetal cells exposed to 16°C.

Keywords: anther development, low temperature, male sterility, rice, tapetum

Rice, an important food crop, is grown in tropical regions as well as in temperate climate zones, where cooler temperatures at the end of the growing season can reduce pollen fertility and grain harvests (Nishiyama, 1984). Exposure to cold weather at the reproductive stage limits rice yields in all temperate areas, especially Australia, Korea, Japan, and USA. It is estimated that, worldwide, 7 million hectares of rice are prone to chilling damage (Oliver et al., 2005). Cold-induced pollen sterility is the major factor (Oliver et al., 2005; Mamun et al., 2006), with crop yields being decreased by an average of about 5 to 10%. However, unpredictable periods of low temperatures that occur every 3 to 4 years can result in losses as high as 20 to 40% (Angus and Lewin, 1991; Jacobs and Pearson, 1994). The reproductive stage is particularly sensitive to abiotic stress (e.g., from cold, drought, or heat) not only in rice but also in other cereal crops (Saini and Westgate, 2000; Boyer and Westgate, 2004).

During microgametogenesis, microspores develop into mature pollen via two mitotic divisions. The tapetum, the innermost cell layer of the anther wall, plays a crucial role in supplying nutrients to these microspores and in regulating their release. Abiotic stress and mutations during tapetal development (such as early degeneration, hypertrophy, or mutations in the archesporial cell) lead to aborted microgametogenesis and male sterility (Chaudhury, 1993; Wilson et al., 2001; Kapoor et al., 2002; Sorensen et al., 2002, 2003; Higginson et al., 2003; Jung et al., 2005; Oliver et al., 2005; Wi et al., 2005). Morphological and histological examinations have shown that the transition of the tetrad to a uninucleate stage is particularly sensitive to chilling (Ito et al., 1970; Satake and Hayase, 1974; Wada et al., 1990). Cold-treated rice plants exhibit greater abnormalities in the anthers than in their pistils or any other floral organs (Satake and Hayase, 1974; Nishiyama, 1995).

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Previously, we have reported on thermosensitive genic male sterile (TGMS) rice, in which fertility is controlled by thermovariation, and male sterility is associated with premature programmed cell death of the tapetum (Ku et al., 2001, 2003). Here, we document the ultrastructural variations found in the tapetum after exposure to different low-temperature conditions.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

'Dongjin' rice plants were grown, after the tillering stage, under four different sets of conditions: 1) optimum, at 26/20°C (day/night) with 10 h daylight and 50% humidity; 2) 16°C, 10 h daylight, 50% humidity; 3) 18°C, 10 h daylight, 50% humidity; or 4) 20°C, 10 h daylight, 50% humidity.

Sample Fixation and Infiltration

Flowers harvested from each of the four treatments were fixed overnight at 4°C in solutions containing 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M phosphate buffer (PBS) (pH 7.4). They were then rinsed in 0.1 M PBS (pH 7.4) and further fixed in 1% (w/v) osmium tetroxide (OsO_4) at 4°C overnight. After rinsing again in PBS buffer, the samples were dehydrated through an ethanol series (10 to 100%) and embedded in LR white resin (London Resin, UK). The resin polymerization reaction was processed at 60°C in a dry oven overnight.

Light Microscopy

The resin-embedded flower samples were sliced into 1-mm sections with an ultramicrotome (2088; LKB Bromma, Sweden), and stained with 0.5% toluidine blue containing 0.1% sodium carbonate. The sections were then observed under a light microscope (Axiovert 100 M; Karl Zeiss, Germany).

Transmission Electron Microscopy

Thin sections (40 to 50 nm thick) were prepared with an ultramicrotome (2088; LKB Bromma), then collected on nickel grids (1-GN, 150 mesh) and stained with 2.5% uranyl acetate for 20 min. After the grids were washed with pure water, the specimens were stained with lead citrate for 7 min at room temperature. Following a double staining, observations were made under a transmission electron microscope (JEM 100 CX-I, 80 kV; JEOL, USA).

Pollen Viability

Tetrazolium staining was done in a solution containing 1% (w/v) aqueous 2,3,5-triphenyltetrazolium chloride in 50% sucrose at 28°C under darkness for 1 h.

RESULTS

Influence of Low Temperature in Seed Formation and Pollen Viability

To study the influence of low temperature during rice development, plants were grown under either optimum conditions (control; 26°C/20°C day/night) or at 16, 18, or 20°C. At the lower temperatures, grain yield was less than from the control plants (90.4 ± 6.2%). These reductions were very severe (2.4 ± 1.4%) at 16°C, whereas yields from plants grown at 18 and 20°C were 40% (± 3.7%) and 70% (± 4.2%) of the control amount, respectively (Fig. 1A). To examine pollen viability, rice anthers were stained with tetrazolium (Fig. 1B-E). Grains from the control plants were completely stained, indicating viable pollen (Fig. 1B), whereas most of the mature anthers from plants grown at 16°C were completely devoid of pollen grains (Fig. 1C). In contrast, the plants grown at 18°C had a reduced number of pollen grains, many of which were non-viable, suggesting that some development was able to occur at that temperature (Fig. 1D). Anthers from plants grown at 20°C contained a large number of pollen grains, but many of them also were non-viable (Fig. 1E). Thus, we may conclude that low-temperature-stressed anthers fail to develop pollen to maturity, thereby causing reduced rice yields.

Light Microscope Observations of Low-Temperature-Stressed Anthers

To study in more detail the effect of low temperatures on pollen development, we prepared anther sections from numerous spikelets of plants grown at 26/20, 16, 18, or

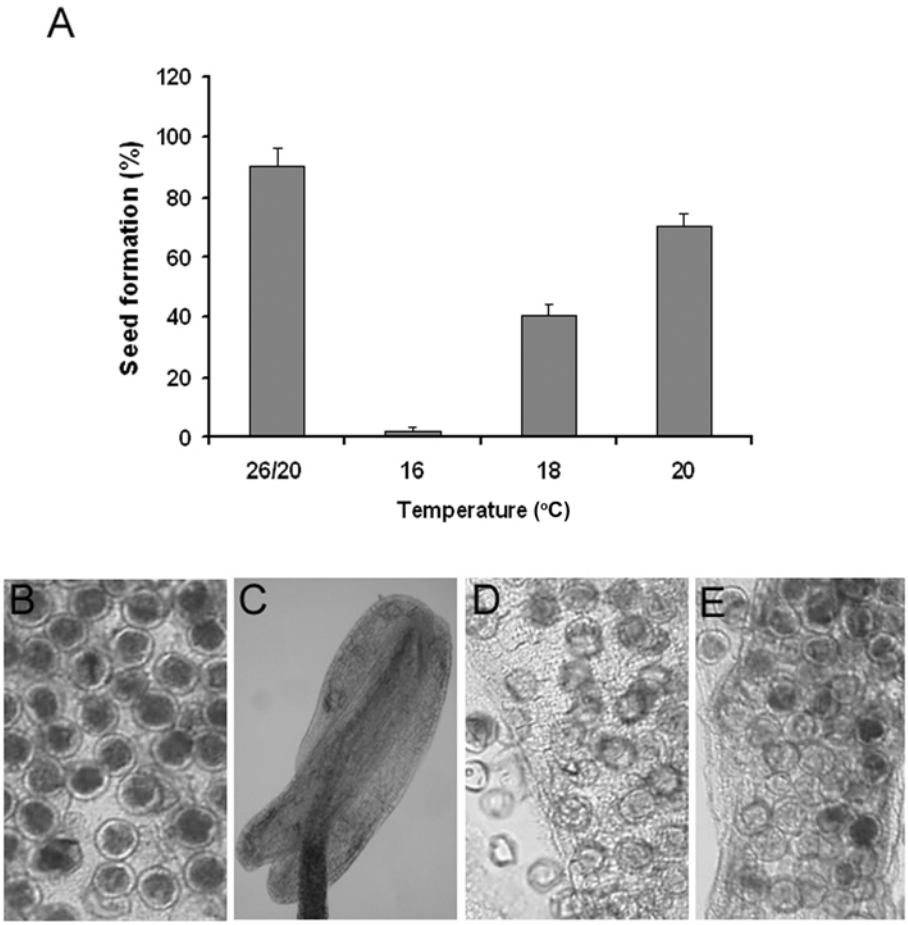

Figure 1. (**A**) Seed set ratio of control and cold-treated plants, 45 per treatment. (**B**-**E**) Tetrazolium staining of control and cold-treated anthers: (**B**) anther from plants under optimum conditions, showing viable pollens; (**C**) anther from plant exposed to 16°C, with empty locule and no pollen grains; (**D**) anther from plant at 18°C, with very few viable but many distorted pollens; (**E**) anther from plant treated at 20°C, having equal numbers of viable and non-viable pollen.

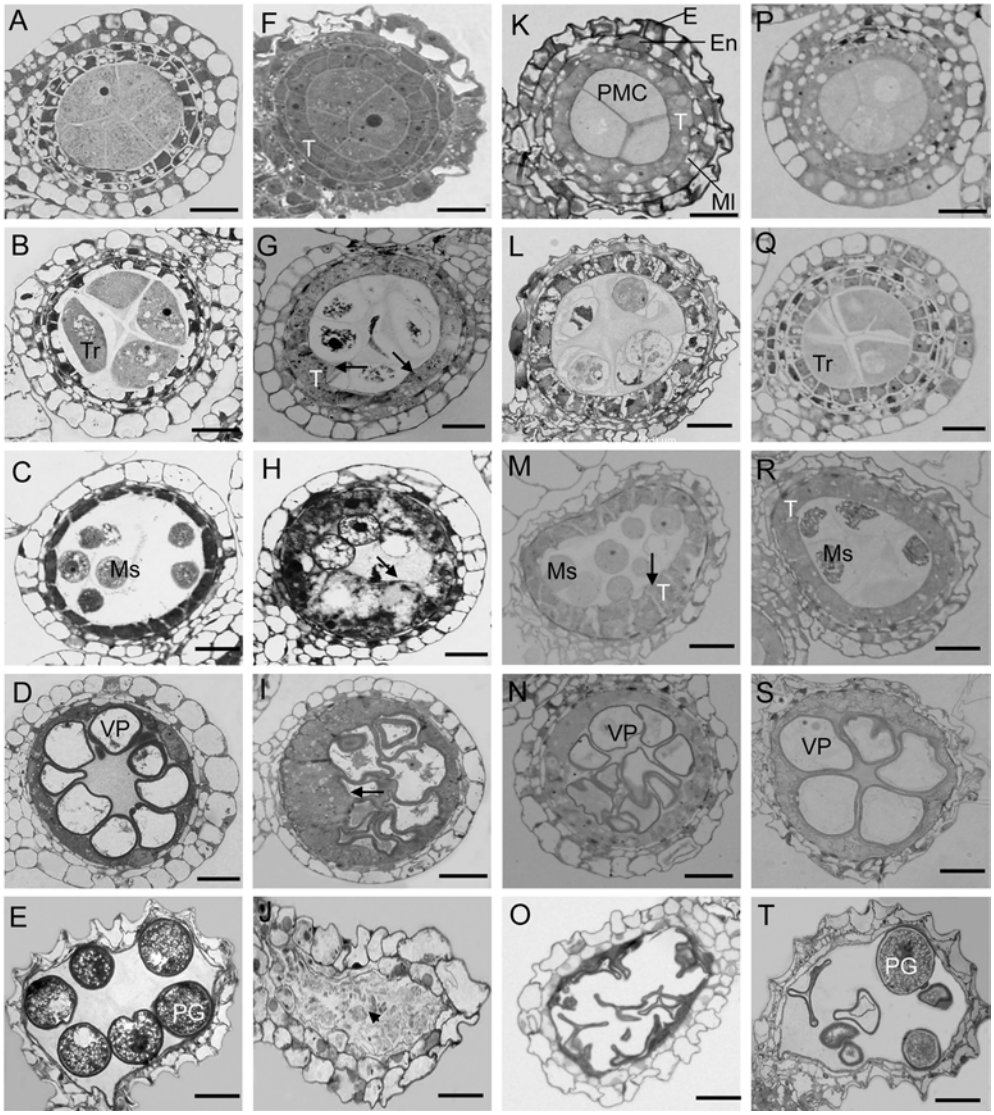

Figure 2. Cytological analysis of anther development in plants from control (**A**-**E**), 16°C treatment (**F**-**J**), 18°C treatment (**K**-**O**), and 20°C treatment (**P**-**T**). Pollen mother cell stage (**A**, **F**, **K**, and **P**); tetrad stage (**B**, **G**, **L**, and **Q**); uni-nucleated pollen stage (**C**, **H**, **M**, and **R**); vacuolated pollen stage (**D**, **I**, **N**, and **S**); and mature pollen stage (**E**, **J**, **O**, and **T**). E, epidermis; En, endothecium; MI, middle layer, PG, pollen grain; PMC, pollen mother cell; T, tapetum; Tr, tetrad; Ms, Microspore; VP, vacuolated pollen. Arrow indicates swelling of tapetum. Arrowhead shows abnormal materials in locule. Scale bar = 20 μm.

20°C, then examined them under a light microscope to compare their cytological features. We investigated their development during five different periods: pollen mother cell stage, tetrad stage, uni-nucleated pollen stage, vacuolated pollen stage, and mature pollen stage (Fig. 2). At the pollen mother cell stage, no significant structural abnormalities were observed in anthers from any of the control or cold-treated plants. For example, the control plant clearly showed distinct sporophytic tissue layers that included the tapetum, middle layer, endothecium, and epidermis (Fig. 2A). Tetrads in a tetrahedral arrangement surrounded by a callosic wall were formed in the anther locule after meiosis (Fig. 2B). Afterward, the tetrads were further developed into uni-nucleated pollen (Fig. 2C) and then into vacuolated pollen (Fig. 2D). Normal degeneration of the entire tapetal layer was also observed during pollen maturation (Fig. 2E). In contrast, at those four later stages, the anthers from plants grown at lower temperatures exhibited abnormal pollen development (Fig. 2F-T), with the most severe aberrations, and complete male sterility, occurring in the 16°C-treated plants (Fig. 2J). At that temperature, the first sign of abnormality was detected in the tetrad stage, when the tapetum began to swell (Fig. 2G, arrows). In the later stages (uni-nucleated and vacuolated pollen), this abnormal swelling further dilated into the locular space (Fig. 2H, I). In the mature stage, the locule contained cellular debris, but without any organized structures (including pollen grains), probably because of cellular degradation of the tapetum (Fig. 2J).

For plants grown at 18°C, this abnormality was comparatively less severe, with the first sign of an abnormality being observed in the uni-nucleated pollen stage when the tapetal cells became swollen (Fig. 2M, arrow). However, this swell-

ing did not progress further as had been observed with the 16°C-treated plants (Fig. 2N). In the mature stage, most of the pollen from the 18°C-treated plants was severely distorted, suggesting the importance of temperature to rice pollen development (Fig. 2O). Similarly, plants grown at 20°C responded to low-temperature stress by producing numerous, but primarily distorted, pollen grains (Fig. 2R-T). Thus, our light-microscopic observations suggested that male sterility is mainly due to malfunctioning of the tapetum under cold stress, resulting in abnormal pollen development.

Transmission Electron Microscope Observations

Although our light-microscopic examination demonstrated the negative effect of low temperatures on tapetum development, such a technique could not adequately elucidate structural alterations in the tapetum at the sub-cellular level. Therefore, we performed TEM analysis (Fig. 3). Under optimum (control) conditions, the tapetal cells were regular, showing proper cell wall separations and connections between each other and with the middle layer via numerous plasmodesmata (Fig. 3A). However, for plants grown at 16°C, the tapetal cells were swollen and lacked proper cellular divisions. In all cases, however, the tapetal cells were rich in cytoplasmic contents and contained cellular organelles, such as ribosomes, mitochondria, endoplasmic reticulum (ER), lipid bodies, and plastids, as well as specialized organelles with electron-opaque spots known as amyloplasts. Interestingly, under conditions of 16°C, many oval-shaped ER-derived compartments, i.e., the ER bodies (ERB), were present (Fig. 3B). These bodies were surrounded by ribosomes and their inner matrix was filled with fibrous material. For plants exposed to 18 or 20°C, the disorganization of cellular division and sub-cellular organelles gradually normalized as the growing temperature was increased (Fig. 3C, D). These TEM results, therefore, suggest that it is more likely that temperature stress caused these tapetal abnormalities.

Structural Changes in the ER Network Are Influenced by Low Temperature

TEM analysis at the sub-cellular level showed no significant structural changes in any cellular organelles except the ER. Stacks of ER that normally appeared under optimum conditions (Fig. 4A) were not observed in tapetal cells from plants treated at 16 or 18°C (Fig. 4B, C). We observed various ER arrangements, including linear, wavy, looped, or cir-

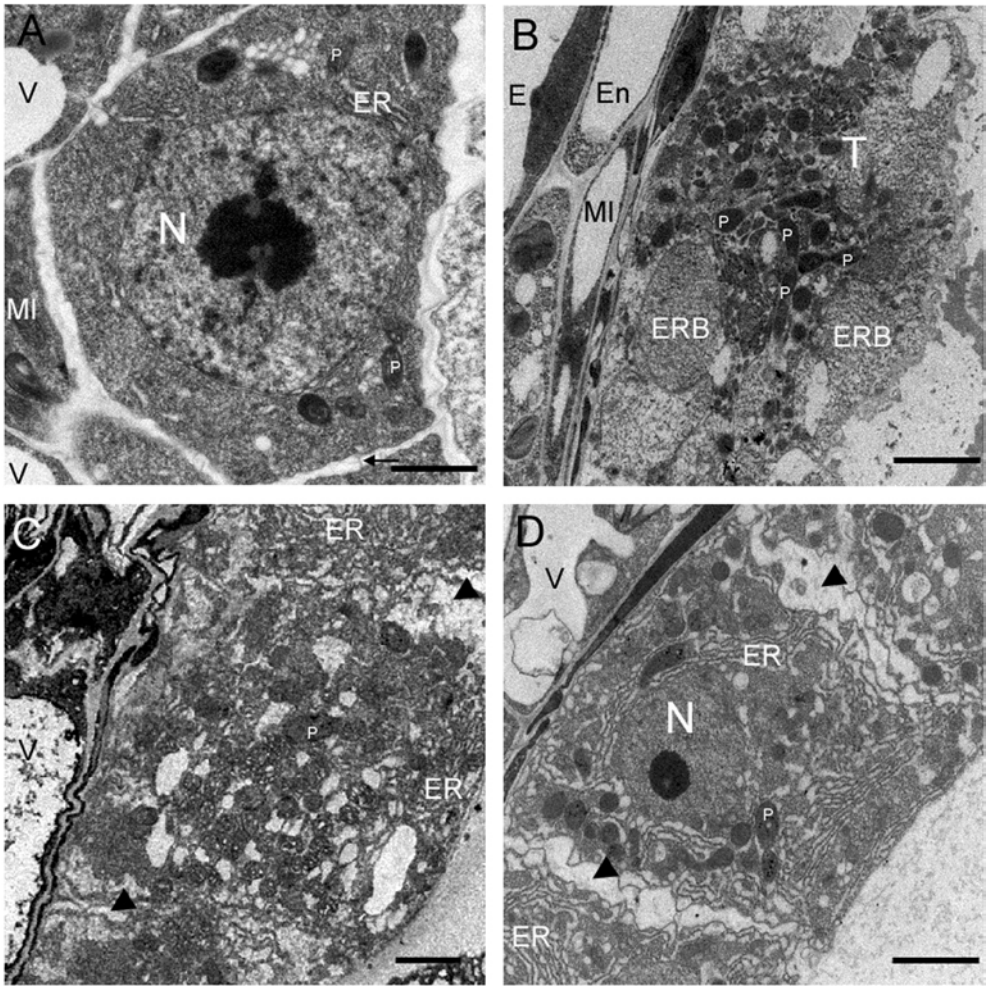

Figure 3. Transmission electron micrographs of rice anthers at tetrad stage from (**A**) control, (**B**) 16°C treatment, (**C**) 18°C treatment, and (**D**) 20°C treatment. N, nucleus; P, plastid; V, vacuole; ER, endoplasmic reticulum; ERB, ER bodies; Ml, middle layer; En, endothecium; T, tapetum. Arrow indicates plasmodesma between tapetal cells. Arrow head shows disorganized cellular division. Scale bar = 1 μm.

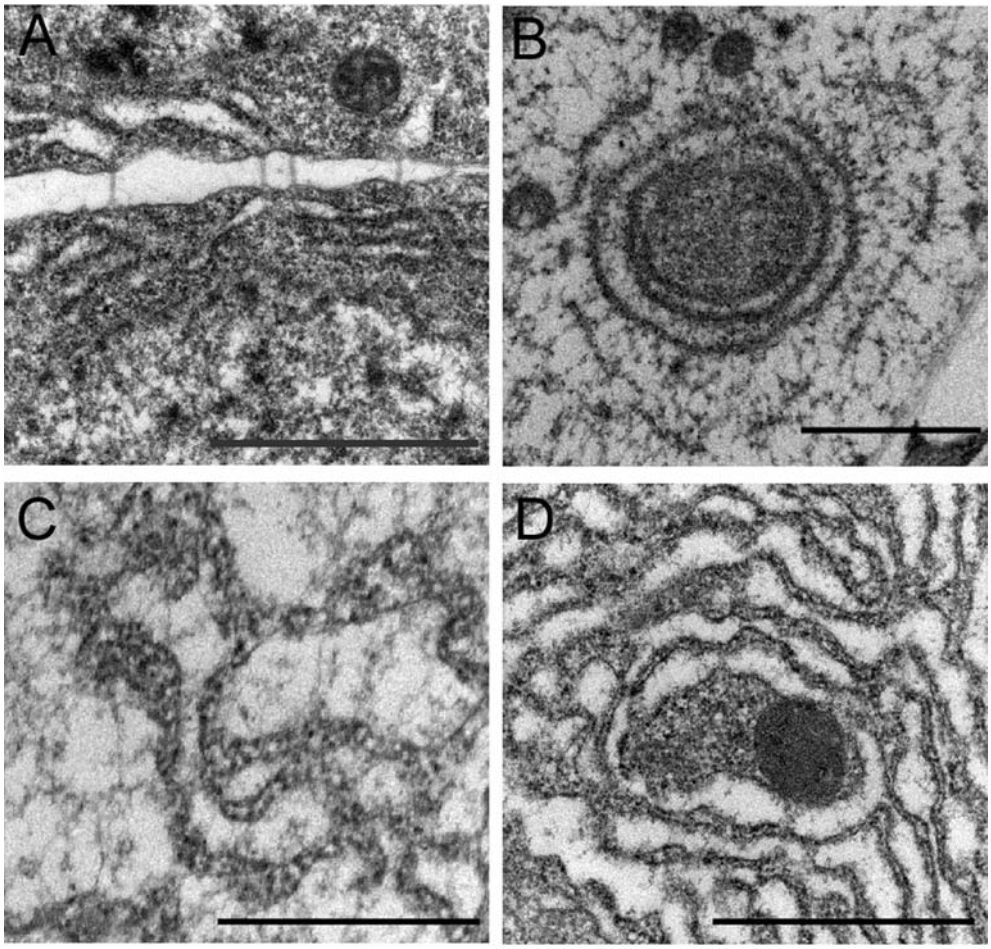

Figure 4. Transmission electron micrographs showing ER arrangements in tapetal cells from control and cold-treated anthers at tetrad stage (**A**-**D**). (**A**) Stacks of RER in cytoplasm of tapetum cell from control. (**B**) Circular form of RER and lipid bodies in cytoplasm of tapetum cell from 16°C-treated anthers. (**C**, **D**) Wavy and loop-like RER in 18- and 20°C-treated anther tapetum. Scale bar = 1 μm.

cular forms. Concentric rings of ER surrounding the chloroplasts, mitochondria, and microbodies were frequently noted in the 16°C-treated tapetal cells (Fig. 4B). Electron density in the ER lumen also was higher at that temperature. Wavy and looped structures were found in cells exposed to 18 and 20°C (Fig. 4C, D). These observations indicate that the ER is the organelle most sensitive to low-temperature stress in tapetal cells.

DISCUSSION

When rice plants incur cold damage at the reproductive stage, the result is male sterility and reduced grain yields (Satake and Hayase, 1970; Nishiyama, 1995). Likewise, our observations of seed formation and pollen viability demonstrated that such sterility was very severe when plants were grown at 16°C, whereas yields increased gradually as the growing temperature rose (Fig. 1A). Hayase et al. (1969) also have concluded that the absence of grain development in cold-stressed florets is due to pollen sterility and the inability to fertilize female gametophytes.

The tapetum is a highly specialized secretory cell layer responsible both for pollen cell wall deposition and for the production of locular fluid that supplies the nutrients required for pollen maturation (Steer, 1977; Pacini et al., 1985; Clement et al., 1994, 1998). Plasmodesmata between the tapetum and the outer cell layers of the anther wall disappear at meiosis, and callose deposition isolates the meiocytes from the rest of the anther (Steer, 1977; Pacini and Franchi, 1983; Pacini et al., 1985; Raghavan, 1988; Clement and Audran, 1995). After the tetrad breaks up, the young microspore attaches to the tapetum and the pollen cell wall is deposited (Steer, 1977; Raghavan, 1988). Our cytological observations of anthers under different temperature conditions revealed that the tapetum swelled severely and the cellular divisions between tapetal cells were greatly altered. Cold treatment at the peak of tapetal functioning can result in reduced activity of cell wall-bound invertase, thereby causing an accumulation of sucrose that leads to tapetal swelling in rice anthers (Nishiyama, 1995; Kawaguchi et al., 1996; Sheoran and Saini, 1996; Oliver et al., 2005). Imin et al. (2006) also have determined that cold treatment down-regulates the T42 protein in rice anthers, possibly affecting the transport of lipids or other substances from the tapetal cells to the developing microspores (Imin et al., 2006). Thus, the tapetal layer plays a crucial role in controlling pollen development and, ultimately, plant fertility.

We have previously studied a thermosensitive genic male sterile (TGMS) rice strain in which fertility is controlled by thermovariation (Ku et al., 2001); there, the process of male sterility is associated with premature programmed cell death (PCD) of the tapetum. Distinct features of PCD, such as cytoplasmic shrinkage, vacuole rupturing, and DNA fragmentation, indicate that this sterility is due to premature PCD of the tapetum (Ku et al., 2003). To examine whether our low temperature-treated tapetal cells also might have undergone premature PCD, we performed a TUNEL (TdT-mediated dUTP nick-end labeling) assay in order to identify any nuclear DNA fragmentation. Fluorescence microscopic observations did not indicate a nuclear DNA fragmentation signal in the chilled anthers (data not shown). Moreover, we did not find other hallmarks of apoptosis, e.g., shrinkage of the cytoplasm and nuclei, condensation of the chromatin, or vacuolation. This suggests, therefore, that male sterility is not caused by premature PCD of the tapetum.

Our sub-cellular observations of the low temperature-treated tapetum suggest that changes in the ER arrangement are associated with tapetal malfunctioning. The ER is the primary organelle of the endomembrane system, and is responsible for the synthesis and maturation of proteins used for secretion, for the plasma membrane, and for transport to various organelles in the endocytic and exocytic pathways (Vitale et al., 1993). The ER is also fundamentally important for cellular metabolism, e.g., callase and cellulase synthesis (Fei and Sawhney, 1999), lipid synthesis, and calcium regulation (Harris, 1986). Stacks of ER are frequently observed in the tapetal cells of *Arabidopsis thaliana* (Owen and Makaroff, 1995), *Brassica napus* (Platt et al., 1998), *Phaseolus vulgaris* (Suzuki et al., 2001), and *Oryza sativa* (Mamun et al., 2005). Thus, the different patterns of ER arrangement in our low temperature-treated tapetal cells may have affected its physiological role in pollen development. Structural changes in the ER network also are reportedly associated with heat stress in the barley aleurone layer (Belanger et al., 1986), common bean cotyledons (Chrispeels and Greenwood, 1987), tobacco pollen tubes (Kandasamy and Kristen, 1989), tobacco pollen (Ciampolini et al., 1991), and the snap bean tapetum (Suzuki et al., 2001). Concentric rings of ER are generally considered to be the result of a stress response (Morisset, 1983; Hauser et al., 2005). Moreover, we observed many ER-derived compartments (ERB) in the 16°C-treated tapetal cells. These bodies are generally formed in response to environmental stress, especially in the most sensitive cell types (Hara-Nishimura and Matsushima, 2003; Matsushima et al., 2003)

In summary, our study has revealed that, during its peak activity, the rice tapetum is very sensitive to low temperatures. For example, the concentric ER rings found at 16°C, which indicated a highly abnormal structure, failed to support the secretory functioning of the tapetal cells, leading to hypertrophy and, eventually, to the abortion of microspores. At both 18 and 20°C, the ER network somehow was sustained, supporting the survival of the microspore. Thus, we can conclude that changes in the ER network of the rice tapetum are associated with tapetal malfunctioning and, consequently, male sterility.

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