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Cytological study and PCD assay on pollen development of photoperiod sensitive genic male sterile rice

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Abstract A systematic cytological comparison of the anther development of photoperiod sensitive genic male sterile (PSGMS) rice with its normal fertility counterpart was conducted. The results showed that pollen abortion in PSGMS rice occurred first no later than the pollen mother cell (PMC) stage and continued during the entire process of pollen development till pollen degradation. This abortive process was closely associated with the abnormal behavior of tapetum. Although tapetum degeneration in the PSGMS rice initiated as early as at the PMC stage, it proceeded slowly and did not complete until the breakdown of the pollen, in sharp contrast to the rapid disintegration of the tapetal layer during the late microspore to the bicellular pollen stage in the fertile rice. Such cytological observation was supported by the results of the TUNEL (TdT2 mediated dU TP Nick End Labeling) assay that detects DNA fragmentation resulting from programmed cell death (PCD), indicating that the tapetum degeneration occurs in the process of PCD.

Keywords Nongken 58S, male-sterility, tapetum, programmed cell death

1 Introduction

The photoperiod sensitive genic male sterile (PSGMS) rice was discovered as a spontaneous mutant from a japonica rice (*Oryza sativa* L. ssp. *japonica*) cultivar Nongken 58 (abbreviated as 58N) (Shi, 1985). A major characteristic of the PSGMS rice (referred to as 58S) is that its pollen fertility is regulated by day-length, which means the pollen is completely sterile under long-day conditions while the fertility varies from partial to fully fertile under short-day conditions (Zhang et al., 1987). This characteristic makes

it a desirable germplasm for developing “two-line” hybrids in rice breeding programs. Currently, hybrids developed using this germplasm to occupy a large area in rice production in China.

The PSGMS rice has also provided very interesting material for investigating male sterility in plants. A number of genetic analyses have established that the male sterility of 58S is controlled by one or two Mendelian loci depending on the genetic backgrounds of the materials used in the crosses (Jin, 1995). Typical single locus segregation of male fertility was consistently observed in crosses between 58S and 58N (Mei et al., 1999a, b). While in progenies of many other crosses, the fertility segregation was controlled by two independent loci (Zhang et al., 1994b; Mei et al., 1999a). Physical mapping localized one of the two loci *Pms1* and *Pms2* to a genomic region of 85 kb in length on chromosome 7 (Liu et al., 2001), and the other locus *Pms3* to a genomic DNA fragment of 28.4 kb on chromosome 12 (Lu et al., 2005).

Several studies have also reported that the cytological characteristics are related to pollen abortion of PSGMS rice. Wang and Tong (1992) ascribed the pollen abortion to abnormal vascular bundle and delayed disintegration of the tapetum in the anthers of PSGMS rice. Li et al. (1993) observed disintegration of mitochondria, endoplasmic reticula and other organelles, as well as delayed tapetum degeneration in anthers of the 58S, which they regarded as the main causes for pollen abortion. Tian et al. (1993) reported that, compared to the normal fertile rice, PSGMS rice showed less endoplasmic reticulum and more polysomes in the tapetum cytoplasm, and delayed degeneration of the tapetum and middle layer. Sun and Zhu (1995) observed failure of the timely collapse of the tapetum and middle layer, which they identified as the cause of male sterility in PSGMS rice. However, it is not yet clear when the abortive development of male fertility in PSGMS rice initiates. Therefore, it is necessary to identify the cytological cause for the abortive development.

Degradation of tapetum cells is a regulated process in programmed cell death (PCD) during anther development

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(Papini et al., 1999). In general, the PCD process is associated with oligonucleosomal cleavage of DNA resulting in DNA fragmentation. The cleaved DNA fragments can be detected by the TUNEL (TdT-mediated dUTP nick-end labeling) assay, an *in situ* technique that displays DNA fragmentation by incorporating fluorescein 12-dUTP at the free 3'-OH ends. This technique has been previously applied in cytological investigation of cytoplasmic male sterility (CMS) and temperature sensitive male sterility in rice (Li et al., 2004; Ku et al., 2003).

In this study, we conducted a systematic cytological observation of the anthers of PSGMS rice 58S in comparison with its fertile counterpart 58N. A TUNEL assay was also performed comparatively to reveal DNA fragmentation in the anthers of both 58S and 58N. Our objective was to identify, at the cytological level, the causal mechanism for male sterility in PSGMS rice.

2 Materials and methods

2.1 Plant materials and preparations

Plants of 58N and 58S were grown in the field under natural long-day conditions in the rice growing season of 2005 on the Experimental Farm of Huazhong Agricultural University in Wuhan, China. Spikelet fertility was examined by scoring forty plants and three panicles per plant that headed before September 3 to ensure that all the examined panicles developed under long-day conditions. Plants with spikelet fertility below 10% were considered to be sterile. Panicles from forty plants were harvested during the stage from pistil and stamen differentiation to one or two days before flowering, and 10–20 florets per panicle for microscopy. The samples were fixed in a FAA fixative solution containing an 18:1:1 mixture of 37% formaldehyde, 70% ethanol and 100% acetic acid. Anthers were collected from the fixed floral tissues and dehydrated through an ethanol series of 70%, 85%, 95% and 100% (v/v). After being embedded in paraffin, transverse sections of 8–10 μm were cut using an LEICA RM 2265 microtome, and stained with 0.05% toluidine blue O. The tissue sections were then observed under a light microscope (Olympus BH2).

2.2 The TUNEL assay

The TUNEL procedure was applied to detect DNA fragmentation, using the *in situ* Cell Death Detection Kit (Promega, USA, G3250), according to the manufacturer's instruction. After dehydration with the ethanol series, the anther was embedded in paraffin, and sliced into 8- μm thick sections. The sections were washed in PBS (0.137 mol·L⁻¹ NaCl, 0.003 mol·L⁻¹ KCl, 0.008 mol·L⁻¹ Na₂HPO₄, and 0.001 mol·L⁻¹ KH₂PO₄) for 5 min,

fixed in 4% paraformaldehyde solution and then incubated in a solution containing 20 $\mu\text{L}\cdot\text{mL}^{-1}$ proteinase K, 100 mmol·L⁻¹ Tris-HCl (pH 8.0) and 50 mmol·L⁻¹ EDTA. After rinsing again in the PBS buffer the samples were fixed in 4% paraformaldehyde solution. The 3'-OH ends of DNA were labeled with fluorescein 12-dUTP using the fluorescein apoptosis detection system. The signal was then detected under a fluorescence microscope (Leica DM 4000B).

3 Results

3.1 Stages and cytological characteristic of anther abnormality in the PSGMS rice

The most sensitive stage of fertility alteration of PSGMS rice to photoperiod length lies between secondary branch differentiation and PMC formation (Yuan et al., 1988). We thus made a systematic cytological comparison of the anthers sampled during this period from 58S and 58N grown under natural long-day conditions. No obvious abnormality was observed in anther and pollen development in 58N during this period (Plate 1 a–f). In contrast, defects were observed in 58S at various stages of the anther and pollen development giving rise to completely empty pollen grains at maturity stage (Plate 1 g–l). The details of the differences at these stages are described below.

3.1.1 PMC stage

In rice, the archesporial cells are the first to differentiate during anther development to form primary parietal cells and sporogenous cells. The sporogenous cells undergo several divisions to form PMC, while the anther wall differentiates into 4 layers: epidermis, endothecium, middle layer and tapetum from outer to inner. No difference was observed between 58N and 58S in these developmental processes (omitted).

However, compared with 58N, abnormality was detected in various tissues of the developing anthers in 58S soon after the sporogenous cell stage (Plate 1 a, g). The abnormality occurred in the tapetal cells as evidenced by condensation and vacuolation of the cytoplasm, and loss of regular cell shape. Abnormality was also observed in some PMCs in 58S, such as condensation and vacuolation of cytoplasm (Plate 1 g). These abnormalities were observed in approximately 65% of anther examined from 58S (Table 1).

3.1.2 Meiosis and tetrad stage

When the PMCs reached meiosis stage, their shape gradually changed from polygon to ellipse. In fertile rice 58N,

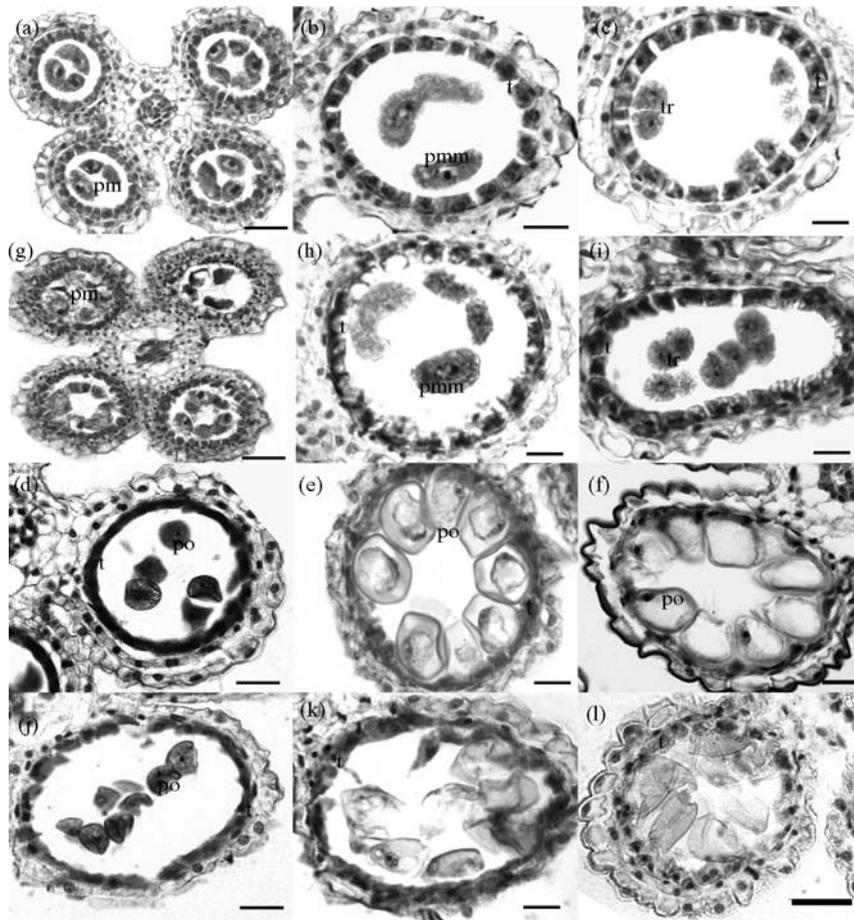


Plate 1 Light-microscopic observation of the anther development of the PSGMS rice (Nongken58S)

Note: a–f: anther locules of the fertile rice Nongken 58 N; g–i: anther locules of the fertile rice Nongken 58 S; a, g: pollen-mother cell stage; b, h: pollen-mother cell meiosis; c, i: tetrad stage; d, j: early microspore; e, k, f, l: late microspore stage; po: pollen; t: tapetum; tr: tetrad; pmm: pollen mother meiosis; Bars = 30 μm for c, e, f, i, k, l; Bars = 20 μm for a and 10 μm for b, d, g, h, j.

the PMCs underwent two consecutive divisions giving rise to tetrads, the tapetal cells showed a big volume with dense cytoplasm, and the middle layer began to disintegrate at this stage (Plate 1 b–c). While the majority of PMCs could complete meiosis in PSGMS rice 58S, abnormality was observed in about 19% of the anther observed (Table 1). Such abnormality included further condensation and vacuolation in the protoplasm of PMCs, and some of the PMCs even became aggregated. Tapetum disintegration became more apparent with distinct signs of cytoplasmic degradation at meiosis and the tetrad stage (Plate 1 h–i).

3.1.3 Uninucleate microspore stage

After release from the tetrad, the separated cells developed into microspores. It was observed that at the early microspore stage, a large portion of the microspores in 58S had an abnormal shape compared with those of 58N, although most of them still had dense cytoplasm with a centrally located nucleus as in 58N (Plate 1 d). While the tapetal cells of 58N had a prominent poly-nucleus and rich cytoplasm at this stage, the tapetal cells in 58S had vacuolated and crimped cytoplasm indicating a disintegrative state (Plate 1 j).

Table 1 Numbers of anthers examined from the PSGMS rice in different stages of pollen development

observation	developmental stage				
	pollen mother cell	meiosis	early uni-microspore	middle uni-microspore	late uni-microspore
total	370	173	132	115	185
normal	130	140	120	70	8
abnormal	240	33	12	45	177
abnormality/%	64.86	19	9.09	39.13	95.68

With the development of the microspore, the degeneration of tapetal cells initiated in 58N, manifested by aggregated cytoplasm and blebs. The degenerating tapetal cells connected to form a circle (Plate 1 c), while the middle layer was almost completely disintegrated. In 58S, the degeneration of tapetal cells and the middle layer proceeded at a much slower pace.

At the late microspore stage, a microspore normally contains a large centralized vacuole with a nucleus on the side. The microspore exine of 58N incrassated substantially, accompanied by a manifold increase of the microspores by volume, as well as rapid disintegration of tapetal cells and complete degeneration of middle layer cells (Plate 1 e).

Although the majority of microspores in 58S could form a centralized vacuole, their exines did not develop to the same extent as in 58N. Subsequently the microspores became crimped and empty, gradually losing shape. Rather than complete disintegration as in 58N, the tapetal cells in 58S disintegrated slowly, with abundant unexhausted cytoplasm. Some middle layer cells also persisted (Plate 1 k).

3.1.4 Mature pollen stage

In 58N, the first mitotic division of a microspore resulted in a large vegetative cell and a small generative cell, forming a bicellular pollen grain. Further division of the generative cell led to a tricellular pollen grain, also referred to as mature pollen (Plate 1 f). The tapetum was completely disintegrated at the bicellular pollen stage.

In 58S, however, pollen development was arrested at the late microspore stage. The majority of the microspores at this stage were empty with an irregular shape and thus abortive. These pollen grains were subsequently degraded into debris (Plate 1 l). In contrast, most of the tapetal cells still persisted though partially disintegrated.

Taken together, pollen abortion in PSGMS rice 58S first occurred at/or before the PMC stage and continued in the entire process of pollen development till the eventual degradation of the pollen. This abortive process was closely associated with the abnormal behavior of the tapetum. Although the tapetum degeneration in 58S initiated at as early as the PMC stage, it proceeded slowly and did not complete until the breakdown of the pollen (corresponding to mature pollen stage in normal rice). This is in sharp contrast to the rapid disintegration of the tapetal layer in 58N during the late microspore to bicellular pollen stage.

3.2 DNA fragmentation in the PSGMS rice

A distinct feature of PCD is the cleavage of nuclear DNA into oligonucleosome-sized fragments (about 180 base pair), which can be viewed by fluorescent labeling of the TUNEL assay (Balk and Leaver, 2001; Li et al., 2004). In

order to determine whether premature DNA fragmentation occurs in the PSGMS rice, we conducted a TUNEL assay of anthers from both 58N and PSGMS 58S during PMC meiosis to the late microspore stage.

At the PMC meiosis stage, the nuclei of tapetal cells in anthers from 58S showed an intense yellow-green stain (Plate 2 c), whereas no positive signal was detected in anthers from 58N (Plate 2 a), suggesting that molecular events of PCD in 58S had already initiated at this stage. At the tetrad stage, the staining in tapetal cells was more intense in 58S than its fertile counterpart (Plate 2 b, d).

4 Discussion

PSGMS is a very interesting phenomenon for biological study and PSGMS rice has provided a very useful germplasm resource for hybrid rice development. However, the exact stage when abnormality leading to pollen abortion first occurs has not been determined in previous studies. For example, results from a number of studies on PSGMS rice have suggested that microspore abortion occurs at the late uninucleate microspore stage, and the abnormal behavior of tapetal cell results in pollen abortion (Wang and Tong, 1992; Li et al., 1993; Tian et al., 1993; Sun and Zhu, 1995). However, Zhang et al. (1994a) and Mei et al. (1997) proposed that the pollen abortion in PSGMS rice initiated at the sporogenous cell to PMC stage. It is likely that the different conclusions were the results of different experimental methods used in the studies, and also partly due to the angles from which the results were viewed.

It is known that in the process of PCD, the nuclear DNA ruptures into oligonucleosome-sized fragments, chromatin separates into masses, and cytoplasm condenses, leading to the final death of the cells (Papini et al., 1999). Thus, time-wise, there is a lag period from the onset of the molecular event to cleavage of nuclear DNA and to observable cytological abnormality of the anthers that are usually taken as the evidence of abortion. Such inference is in agreement with the results from previous studies. For example, Li et al. (2004) reported that fragmentation of the nuclear DNA of tapetum in the Honglian type of CMS rice was observed at the meiosis stage, whereas cytological abnormality was observed at the late microspore stage. Therefore, results of cytological observation and TUNEL assay can be combined to reinforce each other when inferring the stage of abortive development.

Based on the combined information of cytological observation and TUNEL assay, we infer that the abortive development of PSGMS anthers initiated no later than the PMC stage, which corresponds well to the most sensitive stage of male fertility induction by the photoperiod that lies in between secondary branch differentiation and PMC formation, as determined for PSGMS rice by Yuan et al. (1988).

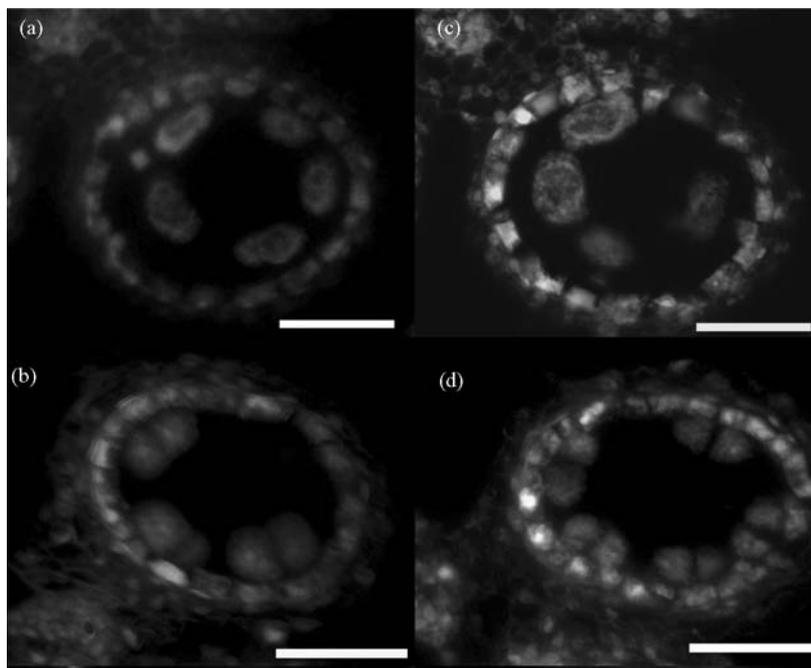


Plate 2 DNA Fragmentation observation of PSGMS rice anthers

Note: a, b: Male-fertile anther locules; c, d: Male-sterile anther locules; Observations were made at two different developmental stages of anthers; a, c: Pollen-mother cell stage; b, d: Tetrad stage; pm: Pollen mother cell; t: Tapetum; Tr: Tetrad; Bars = 30 μ m.

Furthermore, the results also demonstrate that the premature PCD of tapetum was the main cause for microspore abortion. In fertile rice 58N, degeneration of the tapetal layer occurred quickly during the vacuolated microspore to bicellular pollen stage which is in agreement with the timing of PCD revealed by the TUNEL assay. This is also consistent with the general conclusion that tapetum degeneration begins shortly before the first pollen mitosis and usually completes at the bicellular pollen stage (Yang et al., 2005). It was previously suggested that pollen abortion of PSGMS rice was caused by the delayed disintegration of the tapetum (Zhang et al., 1987; Wu and Wang, 1990; Tian et al., 1993). However, our study unequivocally revealed that the tapetum degeneration process in the PSGMS rice 58S initiated at as early as the PMC stage, but proceeded slowly and did not complete until breakdown of the pollen (corresponding to mature pollen stage in normal rice). This timing is also in good agreement with the occurrence of PCD detected by the TUNEL assay. Such coincidence clearly indicates that PCD accounts for the abnormal behavior of the tapetum, which in turn may cause abortive development of the anther and pollen.

The tapetum plays a crucial role in pollen development. This secretory tissue produces numerous nutritive substances, such as sporopollenin, protein and lipid, which are essential for pollen development and maturation (Jin et al., 1997; Taylor et al., 1998). Thus the functional loss of the tapetal cells would certainly cause pollen abortion. Indeed, many studies indicate that male sterility is associated with tapetum dysfunction. Taylor et al. (1998)

ascribed the cause of pollen abortion in the *ms7* male sterility mutant of Arabidopsis to the earlier disintegration of the tapetum. In the PET1-CMS system of the sunflower, the sterility of PET1-CMS sunflower was also proved to be associated with premature PCD of the tapetal cells (Balk and Leaver, 2001). A similar observation was reported in thermo-sensitive genic male-sterile rice by Ku et al. (2003) who, using the TUNEL assay, found that male sterility was associated with premature PCD of the tapetum.

Kapoor et al. (2002) reported that silencing of TAZ1, a zinc finger protein, caused the functional loss of the tapetum, such that the tapetum showing premature PCD could result in cell collapse when TAZ1 was silenced. However, it can be inferred that interrupting the function of any gene that has a nonredundant role in tapetum development would impair pollen development. Thus, molecular understanding of the cause for the premature PCD of the tapetum in the PGSMS rice would await the isolation of the genes, two of which have been mapped genetically (Zhang et al., 1994; Mei et al., 1999a, b; Liu et al., 2001; Lu et al., 2005).

The development and maturation of pollen are dependent on the mutual effect of sporophytic and gametophytic cells. It has long been thought that the tapetum supports pollen development by supplying signals and nutritional substances (Ma, 2005). Recent investigation suggested that tapetum development and differentiation may be regulated by signals from generative cells as well (Ma, 2005). Direct evidence has come from the molecular study of *TPD1* and *EMS1*, which are critical genes for signal

transduction essential for early stage anther development (Ma, 2005). Microspores have direct contact with the tapetal cells. At the uninucleate microspore stage, microspores induce the initiation of tapetum degradation, and tapetal cells disaggregate rapidly driven by the absorbability of the germination pore of the late microspores (Takeoka et al., 1993). Thus tapetum degeneration is also dependent on microspores. This clearly forms a biochemical cross-talk between these two types of cells.

This relationship may also have implications in the understanding of pollen abortion in PSGMS rice. Although the meiotic process of the PMCs controlled by gametophytic genes is normal, incomplete exine formation on early microspores of PSGMS rice indicates an insufficient deposition of pollenkit which is supplied by the tapetum (Tian et al., 1993; Sun and Zhu, 1995; Tian et al., 1998), presumably due to the premature PCD of tapetal cells. Consequently, the abortive microspores cannot consume the degrading substances from the tapetal cells and are thus unable to facilitate tapetum cell degradation, which eventually delays tapetum disintegration. This cytological dynamics may provide an explanation for pollen abortion, although a full understanding of the underlying biological processes would not be possible until the characterization of the genes regulating PSGMS.

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