

Full Length Research Paper

Programmed cell death-like behavior in photoperiod-sensitive genic male sterile (PGMS) rice

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In photoperiod-sensitive genic male sterile rice (*Oryza sativa* L.) pollen abort in long day and high temperature, and revert to fertility in short day length and low temperature growth conditions. This type of control of rice plant fertility can facilitate production of hybrid rice using two lines (photoperiod-sensitive genic male sterile (PGMS) and restorer) system. Our objective in this study was to determine anatomical changes in PGMS rice pollen cells induced by long day length and high temperature growth conditions that are responsible for the cell abortion. All materials in this study were sown at Zhejiang University research field at Hangzhou, China 30°15' N. Seeds of ZAU11S106, a PGMS rice line, were sown so as to obtain two bulks, 1) Extreme sterile pollen bulk and 2) fertile pollen bulk. Rice plants for extreme sterile pollen bulk were sown on March 18th and those for fertile pollen bulk on July 15th, so that they flowered under 13 h or more and under less than 13 h respectively of day light. Transmission electronic microscopes were used to study histological sections of anthers while light microscopes were used to examine whole pollen cells. The tapetal layers and the cytoplasm of the sectioned ZAU11S106 rice anthers from extreme sterile pollen bulk were deformed but those from fertile pollen bulk were normal when compared to the control rice lines ZAU11F121 and Xusui163. Pollen cells from anthers of extreme sterile pollen bulk had eroded exine and intine, missing or damaged nucleus, and disintegrating cytoplasm that lead to cell abortion. Our conclusion is that, cell abortion observed in extreme sterile pollen bulk of photoperiod genic male sterile rice, display “programmed cell death-like behavior”.

Key words: Pollen cell abortion, photoperiod genic male sterile rice, programmed cell death-like, tapetum.

INTRODUCTION

Cell death in a programmed manner has been reported in both plants and animals. Cells systematically die due to environmental conditions such as pathogen attack, aging, and for development and homeostasis (Bouillet and Strasser, 2002; Pozo and Lam, 1998; Vaux and Strasser, 1996). These intra- and extra-cellular stimuli induce

production of cytotoxins which instigate apoptosis.

For example in *Caenorhabditis elegans*, the *ced* genes code for cysteine proteases Ced-3 and Ced-4, that are essential for programmed cell death (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992). Apoptosis, the major type of programmed cell death (PCD), has been reported in both plants and animals (Greenberg, 1996; Mittler et al., 1997; Yamada et al., 2001; Bouillet and Strasser, 2002). In animals, cysteine proteases called caspases regulate animal programmed cell death (Whyte, 1996) and in plants, protease(s) that participate in hypersensitive plant responses to pathogens have been reported (Pozo and Lam, 1998).

Pollen cell abortion in plant male sterility, just like in PCD or apoptosis is a genetically controlled cellular death (Li et al., 2006; Hernould et al., 1998; Kaul, 1988; Xu et

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Abbreviations: PCD, Programmed cell death; PGMS, photoperiod-sensitive genic male sterile; LD, long day; HT, high temperature; SD, short day; LT, low temperature; PBS, phosphate buffered solution; TEM, transmission electron microscope; CMS, cytoplasmic male sterile; FPR, first photo reaction; SPR, second photo reaction.

al., 1995). Sterile pollen in plants like *Arabidopsis thaliana* L., *Nicotiana tabacum* L. (tobacco), *Oryza sativa* L. (rice), and *Brassica napus* L. (oil seed rape) have a deformed pollen cell tapetum layer, which is believed to be the genesis of pollen cell death (Xu et al., 1995; Mariani et al., 1990). In artificially-induced (through chemical gametocides) cytoplasmic male sterile (CMS) rice, pollen deformity is very predictable and deformities of various kinds are initiated from the pollen mother cell stage to mature pollen (Tamaru and Kinoshita, 1985). By the time pollen grains are mature they are extremely deformed, which usually results in abortion. Gene and gene products have been reported that cause sterility in plants. In Tobacco and rapeseed, two genes *TA29-Rnase* and *TA29-barnase* are responsible for tapetum deformity and male sterility (Martine et al., 1993; Mariani et al., 1990). In Petunia plant, flavonoid proteins destroy the tapetum and this leads to pollen cell death and male sterility (Yamazaki et al., 2002; van der Meer et al., 1992).

In photoperiod-sensitive genic male sterile (PGMS) rice too, pollen cell sterility is under genetic control (Zhang et al., 1994; Mei et al., 1999). Plants are sterile but only in long day (LD) length and high temperature (HT) growth conditions (Shi and Deng, 1986; Shi, 1985, 1981). Sterility is controlled in such a way that under LD and HT, growth conditions the pollen cells are 100% abortive and under short day (SD) length and low temperature (LT) growth conditions the pollen cells begin to recover their vitality and are fertile (Zhang and Yuan, 1987). Use of PGMS rice to produce hybrid seeds has not been exploited to full capacity because of inadequate knowledge on the mechanism of how LD length and LT growth conditions, induce pollen abortion and the timing of the abortion program. Our knowledge in PGMS rice genetics, led us to predict that pollen cell abortion in this type of rice when grown in LD length and HT conditions may be under a program similar to PCD. One of our major objectives in this study was to determine anatomical changes induced by LD length and HT growth conditions in PGMS rice pollen cell, that are responsible for cell abortion. Also, we want to quantify in days the specific stage in the growth phase when the program controlling pollen abortion is executed. Evidence obtained in this study shows that, pollen cell in PGMS rice under LD length and HT growth conditions abort in a manner similar to PCD.

MATERIALS AND METHODS

Plant material

PGMS rice ZAU11S106 developed from japonica line N5047S protoplasts Xue et al. (1999) was used as a pollen source for this study, while rice lines ZAU11F121, and Xusui163 were used as controls. Rice line ZAU11F121 is a reverse mutant of the ZAU11S106 rice line and the two are the same in all traits apart from PGMS character (Xue et al., 1999). Xusui163 is an ordinarily

fertile rice line. In this study, unless otherwise mentioned, monthly averages of natural day-light-lengths and temperatures were used. A natural long day was 13 h or more and a natural short day was less than 13 h of sunlight. High temperatures used in this study were minimums of 33 and 26°C during day and night times respectively, and low temperatures were less than 30 and less than 22°C, during day and night times, respectively. ZAU11S106 rice plants for artificial SD length treatment were sown on May 14th, so that the flowering was in the month of August which coincided with LD and HT growth condition. All materials were sown at Zhejiang University research field at Hangzhou, China 30° 15'N.

TEM Observation of tapetum

ZAU11S106 rice was sown in two bulks, extreme sterile pollen bulk and fertile pollen bulk. Extreme sterile pollen bulk was obtained from ZAU11S106 sown on March 18th, so that the last 26 days before flowering were under LD length and HT growth conditions. Fertile pollen bulk was obtained from ZAU11S106 sown on July 15th, so that the last 20 days before flowering were under SD length and LT growth conditions. In both bulks, ZAU11F121 and Xusui163 were included as the control lines. Anthers were harvested from the two bulks just before the panicle emerged out of the flag leave and fixed. This included primary fixation in 2 to 4% glutaraldehyde solution at 4°C for more than 6 h followed by washing in phosphate buffered solution (PBS) (0.1 M, pH 7.0) 3 times for 15 min each. Post fixation was done in 1% osmium oxide (OsO₄) solution at room temperature for 1 to 2 h and washing was done in PBS as previously described. Specimens were then dehydrated once in ascending alcohol concentrations of 50, 70, 80, 90 and 95% for 15 min in each step and then twice for 20 min in pure alcohol. Specimens were infiltrated in Spurr (Spi Supplies, West Chester, PA, USA) as follows Spurr : alcohol=1:1 for 1 h; Spurr : alcohol=3:1 for 3 h and in pure Spurr overnight. Embedding was done at 70°C overnight. Ultra sectioning was done using a microtome (Reichert Jung Ultracut E) and staining was done using 1% staining solutions of uranyl acetate and alkaline lead citrate for 15 min each. Sections were observed under Transmission Electron Microscope (TEM) Jem-1200 Electron microscope (Jeol Ltd., Japan) at magnification of X6000. Photographs were scanned into the computer and formatted using Adobe Photo Element, Read Me computer software version 1.

Photoperiod regulation of pollen abortion

ZAU11S106 rice seedlings sown on May 14th were divided into nine blocks designated Block 1, Block 2, up to Block 9 each with twenty plants. SD treatment was done by covering plants with opaque black cloth starting from 3.30 pm each day and uncovered when darkness fell, so that they received only 10.5 h of sunlight. On the 58th day after sowing (before boot stage) Block 1 was exposed to SD treatment, followed by Blocks 1 and 2 four days later and Blocks 1, 2 and 3 after the next four days. After every four days, a new block was included into the ones receiving SD treatment until August 4th when plants in Block 1 flowered. At this time, Blocks 1 to 7 were under SD treatment. Blocks 8 and 9 did not receive SD treatment and were used as controls. After August 4th, plants in all blocks were allowed to grow under natural growth conditions (which at this time were LD and HT) until maturity. Plants in all the blocks were scored for pollen sterility by staining with 1% I/KI staining solution. Testing of pollen sterility was done in five replicates, before flowers emerged from flag leave, at emergence from flag leave and at 5, 50 and 100% flowering rates.

Anthers were picked in the field and immediately put in Canvoy's fixing solution II. In the laboratory, a drop of 1% I/KI staining solution was put on a microscope glass slide, macerated to release

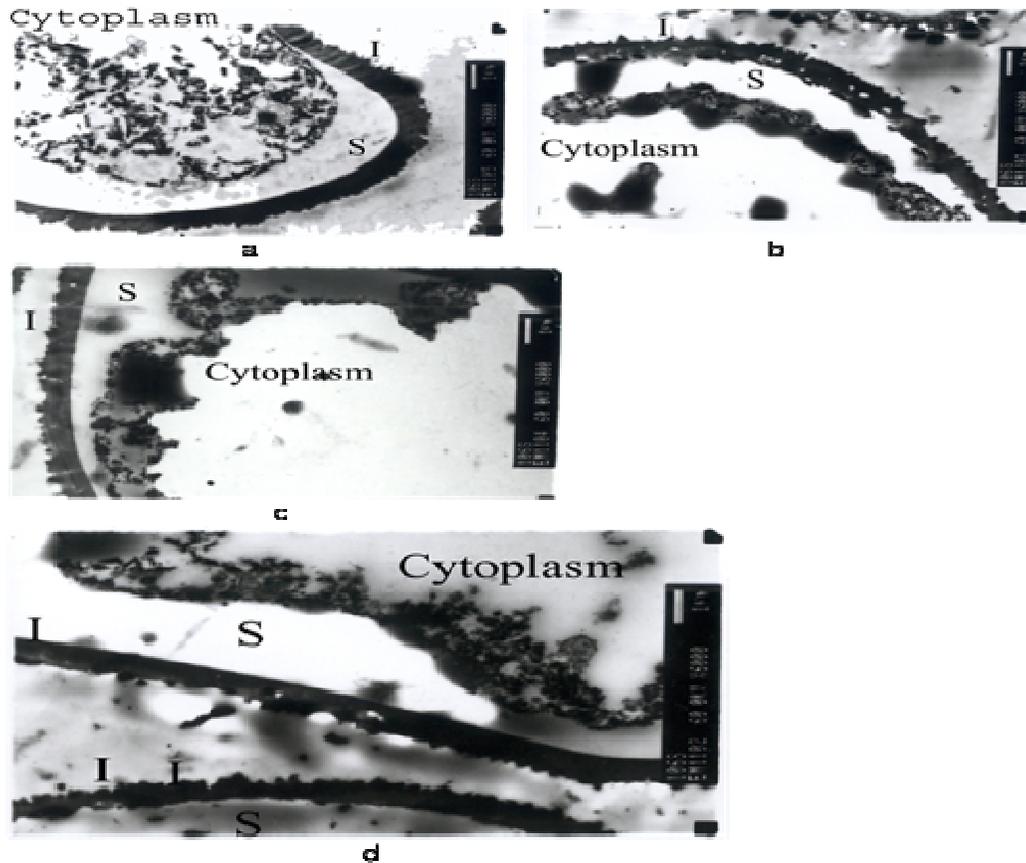


Figure 1. Transmission electron microscope photographs of sections of anthers from ZAU11S106 rice plant grown under LD length and HT conditions. Letters S and I indicates space and intine. Figure 1a and d shows space between intine and the contracting cytoplasm, 1b and c shows cytoplasm that is forming clumps and a thinning intine layer.

pollen cells and the cells were observed under X10 objective of light microscope. To observe the morphology of whole pollen cells and that of the nucleus, anthers were placed on a glass slide in the presence of acetocarmine staining solution. After maceration to release pollen, anther husks were removed from the slide leaving the microspores only and a cover slip was placed on the slide. Observation and photometry were done using Olympus 35AD2 light microscope (Japan). Photographs were scanned and formatted as previously described. Seed set rate was determined by getting the ratios of whole seeds in the spikes to total glumes per spike for each of the plants under the study.

Flowers to study whole anther locule were picked from both ZAU11S106 and ZAU11F121 rice lines, one day after the panicles emerged out of the flag leaves. They were fixed in Canvo's fixing solution II at 4°C for 3 h after which they were washed in 95% ethanol to improve clarity. Anthers were then carefully removed from glumes using forceps and placed on a drop of 1% I/KI staining solution on a glass slide. A cover slip was applied on the anther and a squash was made by pressing gently on top of the cover slip using a finger. Photographs were scanned and formatted as earlier described.

Statistical analysis

This was for plants given artificial SD treatment. Data was analyzed

Using analysis of variance (ANOVA) procedure (Tukey's studentized range test, "proc anova" program) using the Statistical Analysis System (SAS) computer software version 8 (The SAS systems – USA). The SAS model was as follows:

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Title 'PCD in PGMS'; data pgms; input Block Rep Line PAR SSR;
datalines; proc anova; class Block Rep Line PAR SSR; model PAR
SSR = Block Rep Line PAR SSR; means PAR SSR Block Rep Line
/tukey; run;
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In the program, Block = "ZAU11S106 given SD treatment, and ZAU11S106 not given SD treatment"; Rep = "short day length treatment, 1, 2,... to 9"; Line = "5 replicates of pollen sampled and tested for sterility which were as follows; before the flowers emerged from the flag leaves, at emergence of the flowers from the flag leaves, at 5, 50 and 100% flowering"; PAR = "pollen abortion rate"; SSR = "seed set rate".

RESULTS

TEM Observation of tapetum

Figures 1a to d shows histological sections of anthers picked from extreme sterile pollen bulk of ZAU11S106

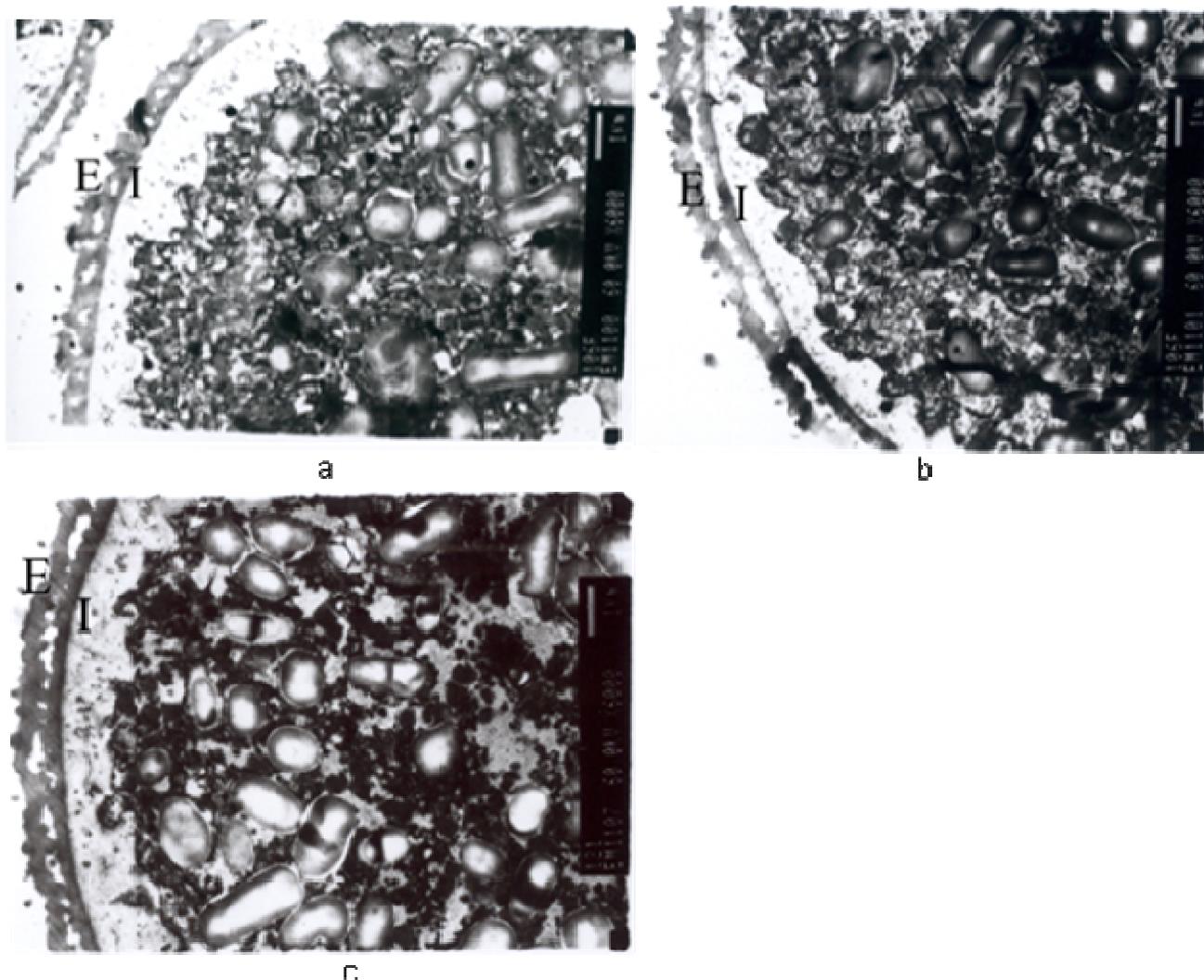


Figure 2. Transmission electron microscope photographs of sections of pollen cells from ZAU11S106 grown under SD length and LT conditions, and from control rice lines. Letters E and I designate exine and intine. Figures a, b and c shows tissue sections displaying exine and intine layers from extreme fertile bulk of ZAU11S106, ZAU11F121, and Xusui163 respectively.

observed at magnification of X6000 under TEM. The tapetum and the immediate exine layers of the individual pollen cells were completely disintegrated leaving only the intine layer which is also losing some structures within its outer and inner lining. Figures 1a and d clearly shows space between intine and the contracting cytoplasm, while Figures 1b and c shows cytoplasm that is forming clumps and a thinning intine layer.

Histological sections of ZAU11S106 anthers from fertile pollen bulk are shown in Figure 2a, and those from fertile controls are represented in Figures 2b (ZAU11F121) and 2c (Xusui163). The exine and the intine of ZAU11S106 anthers from fertile pollen bulk are thicker than the ones from extreme sterile pollen bulk. Also, the space between the intine and cytoplasm, and the clump-like structures observed in pollen cells from ZAU11S106 extreme sterile pollen bulk, are evidently absent in the cells from

ZAU11S106 fertile pollen bulk. There was no remarkable difference observed among the three sections in Figure 2.

Photoperiod regulation of pollen abortion

Tables 1 and 2 shows the result of ZAU11S106 PGMS rice given artificial SD length treatment and their controls. Seed set rate in Blocks 1 to 5 was significantly higher than that in Blocks 6 to 9 (Table 2), while pollen sterility of plants in Block 5, 6 and 9 was significantly higher than in the other blocks. Although, plants in Blocks 7 and 8 had significantly higher pollen fertility than Block 9, the three blocks recorded seed set rate of 0% (no significant difference). A squash of the whole anthers from ZAU11S106 grown under LD length and HT conditions

Table 1. ANOVA procedure using Tukey's studentized range test for percentage pollen abortion rates in ZAU11S106.

Tukey's grouping	Pollen sterility rate (%)	SDL Treatment date	Duration after sowing to SDL treatment (days)	Duration from SDL treatment to heading (days)
A	100.0	Block9-CK (paddy field)	No SDL	No SDL
A	100.0	Block6-July 31	78	22
A	100.0	Block5-July 27	74	26
B	99.6	Block7-August 4	82	21
C	84.8	Block8-CK (wire mesh paddy)	No SDL	No SDL
D	77.8	Block3-July 19	66	22
E	69.0	Block2-July 15	62	22
F	67.0	Block1-July 11	58	23
G	32.4	Block4-July 23	70	24

Critical value of studentized range was 5.51915; SDL, short day length.

Table 2. ANOVA procedure using Tukey's studentized range test for percentage seed set rates in rice line ZAU11S106 all under SD length treatment and the control lines (no SD treatment).

Tukey's grouping	Seed set rate (%)	SDL Treatment date	Duration after sowing to SDL treatment (days)	Duration from SDL treatment to heading (days)
A	14.8	Block2-July 15	62	22
B	13.2	Block1-July11	58	23
C	11.6	Block4-July 23	70	24
D	6.6	Block3-July 19	66	22
E	5.2	Block5-July 27	74	26
F	0.0	Block6-July 31	78	22
F	0.0	Block7-August 4	82	21
F	0.0	Block8-CK (wire mesh paddy)	No SDL	No SDL
F	0.0	Block9-CK (Paddy field)	No SDL	No SDL

Critical value of studentized range was 5.51915; SDL, short day length.

and that from ZAU11F121 are shown in Figure 3. In ZAU11S106 rice under LD and HT growth conditions, the pollen locules were empty or occupied by shriveled pollen cells (Figure 3a), but in the control round pollen cells were observed (Figure 3b).

Pollen cells of ZAU11S106 rice from extreme sterile bulk were seen to leak and bleb (Figures 4a and b), disintegrate (Figure 4c), shrivel (Figure 4d), or did not stain blue/black with 1% I/KI staining solution (Figure 4e). Pollen cells from the fertile pollen bulk of ZAU11S106 rice plants stained blue/black with 1% I/KI staining solution (Figure 4f) just like in the control (Xusui163) rice line (Figure 4g). When stained with acetocamine staining solution pollen cell nuclei from ZAU11F121 (Figure 5a), and that from the fertile pollen bulk of ZAU11S106 (Figure 5b) rice plants appeared oval or round under light microscope. However, nuclei of pollen cells from extreme sterile pollen bulk of ZAU11S106 rice were deformed in appearance (Figure 5c).

DISCUSSION

In LD length and HT growth conditions, ZAU11S106 rice had deformed abortive pollen. Cytological observation of pollen cells from ZAU11S106 extreme sterile pollen bulk shows exine part of tapetal layer that has disintegrated leaving a thin intine layer (Figures 1a to d). Plants with these characteristics were all sterile. Tapetum provides nutritive support tissue for pollen, and it is the source of enzymes and the proteins that regulate pollen cell (Ku et al., 2003). Therefore, deformities on the tapetal layer lead to the dysfunction of the whole pollen cell. Studies on cytoplasmic male sterile rice indicate that, destruction of tapetum layer leads to cell death (Mariani et al., 1990; Kaul, 1988; Tamaru and Kinoshita, 1985).

In CMS, plants are sterile unless fertility is restored by a restorer line (Pradhan and Jachuck, 1999). However, in the PGMS rice, plants are sterile as long as they are grown under LD length and HT conditions but regain

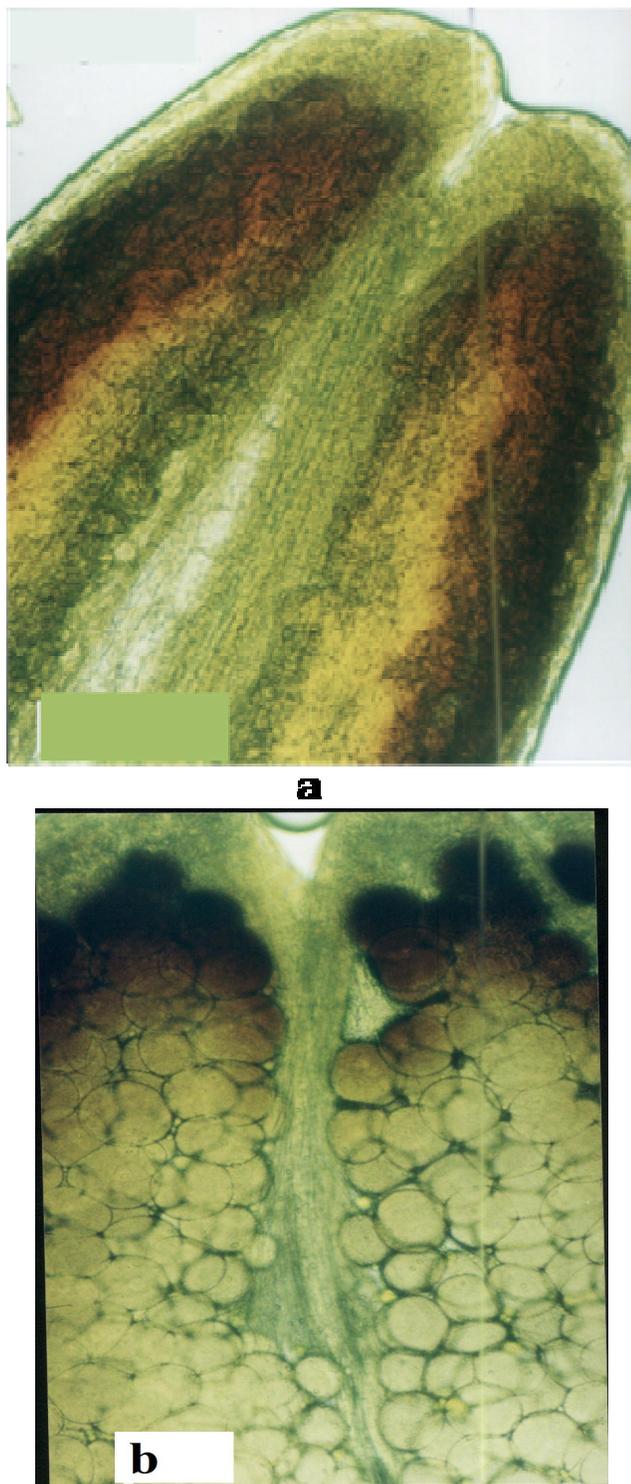


Figure 3. Light microscope squash photograph from sterile and fertile anthers. (a) Shows an anther from extreme sterile pollen bulk of ZAU11S106 rice line and (b) shows an anther of ZAU11F121 line. In (a), the anther locules were empty or shriveled pollen were observed. Locules in (b) were occupied by round or oval fertile pollen. Pollen were stained using I/KI staining solution however, staining was not typical blue-black (because before emergence of flag leaves enough starch had not accumulated).

fertility under SD length and LT growth conditions (Zhang and Yuan, 1987). For PGMS rice, restoration to fertility under SD length and LT growth conditions eliminates the need for a maintainer line in rice hybrid breeding programs. Therefore, production of hybrid rice will only require PGMS line, and the fertile restorer line. This is a two “two-line system” which is unlike the three-line system that requires CMS, restorer and maintainer lines (Virmani and Sharma, 1992). The genes controlling the PGMS trait have been identified as *pms1*, *pms2*, and *pms3* on chromosomes 7, 3 and 12 respectively of the PGMS rice genome (Zhang et al., 1994; Mei et al., 1999). Also, leaf proteins that are present in LD and HT (sterility inducing conditions) and disappear in SD and LT (fertility inducing conditions) have been reported in PGMS rice (Bi et al., 1997). These are proteins that are likely to induce sterility and in their absence under SD length and LT growth conditions fertility results. In tobacco tapetum, deformity is executed by *TA29-RNase* and *TA29-barnase* genes (Mariani et al., 1990), while in petunia, flavonoids whose biosynthesis is regulated by *chalcone synthase* (*chs*) gene, are responsible (van der Meer et al., 1992). Evidence in these crops illustrates the relationship between sterility in CMS and in PGMS plants and the dysfunction of tapetal layer.

In our experiments, when ZAU11S106 rice was covered on July 27th to August 4th, so that it received 10.5 h of sunlight for a total of only 8 days, 100% of its pollen was stained yellow but 5.2% seed set was recorded (Tables 1 and 2). The ZAU11S106 rice given SD length treatment on July 31st had 100% pollen sterility and 0% seed set rate. After this stage, ZAU11S106 rice plants given SD length treatment had over 99% of their pollen cells stained yellow with 1% I/KI staining solution and seed set was 0%. This indicates that, the critical phase determining pollen fertility lie within the four days between 27th and 31st July (Table 2). After July 27th, once exposed to LD length growth conditions, the pollen aborted, and SD length growth conditions were found not to reverse it. ZAU11S106 rice plants that received SD length treatment on July 27th had its first flowers emerge out of the flag leaves on August 22nd. This shows that under these growth conditions, the photo-induced program that led to pollen abortion took place around the 26th to 24th day before the flowering date. According to Yuan et al. (1993), PMGS rice has two photoreactions, first photo reaction (FPR) and the second photo reaction (SPR). FPR is responsible for vegetative growth and SPR is responsible for male sterility. SPR takes place in the time surrounding microsporogenesis after which sterility is irreversible, even if the plant is put in fertility inducing conditions. This observation is a confirmation of results from our study. We went further to quantify time after sowing in days when SPR is executed for the purpose of developing hybrid rice breeding programs.

Our observations show that, seed set rate in Block 5 (5.2%) was significantly higher than that in Block 6 (0%).

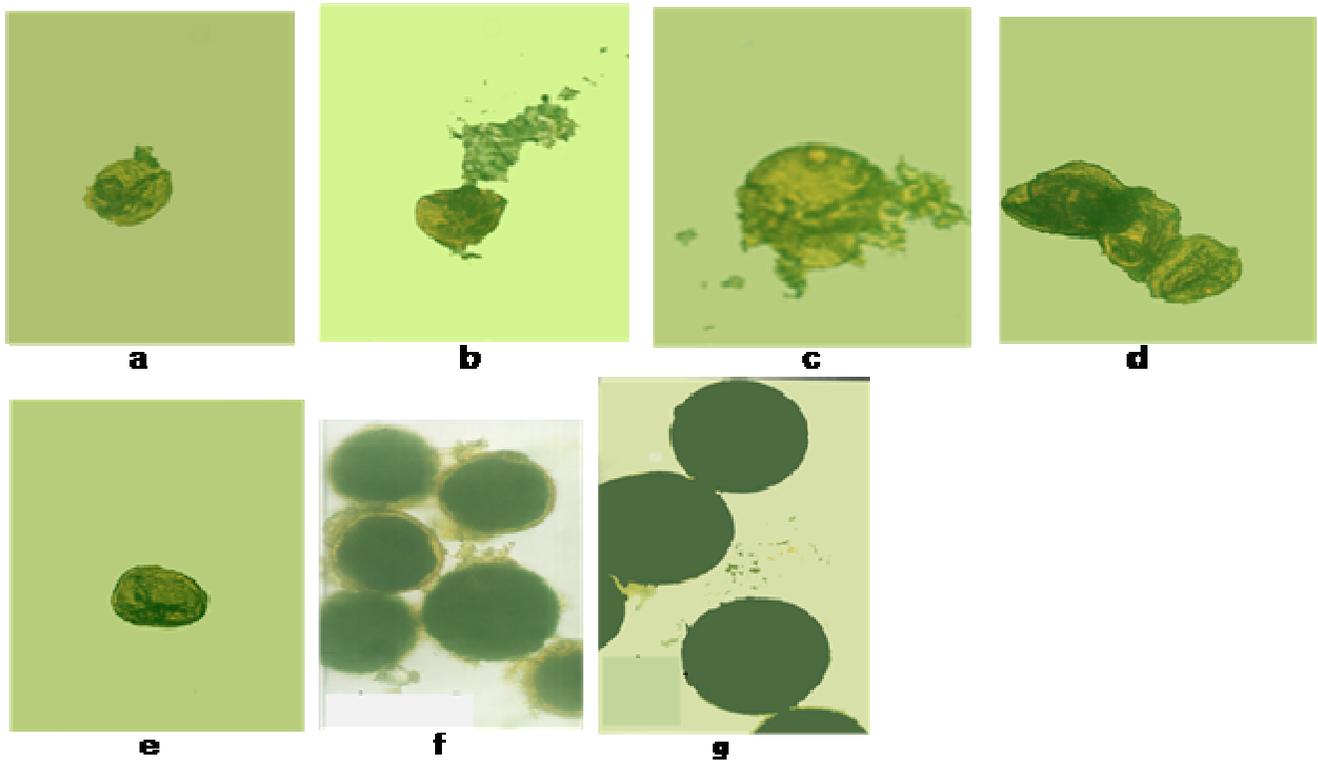


Figure 4. Pollen cells stained with 1% I/KI as viewed under light microscope. (a) to (e) represent pollen cells of ZAU11S106 line under LD length and HT growth conditions. Figures above represent, blebbing and leaking pollen cells (a and b), disintegrating pollen cells (c), shriveling cells (d and e), pollen from ZAU11S106 rice fertile pollen bulk (f) and Xushui163 (g).

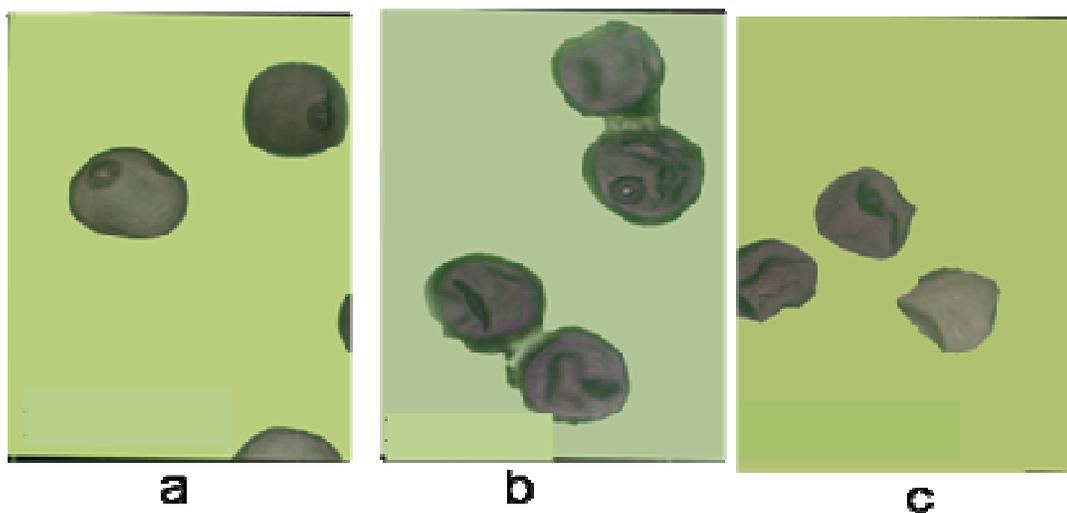


Figure 5. Pollen cell nuclei stained with acetocamine staining solution as viewed under light microscope. Figures show pollen cells from ZAU11F121 (a), ZAU11S106 rice fertile pollen bulk (b), and ZAU11S106 rice extreme sterile pollen bulk (c).

Therefore, the critical point of execution of the program that determines pollen cell abortion was calculated to be within four days that separated Blocks 5 and 6. This was derived from the observation that Blocks 5 and 6 were

given SD treatment on the 74th and 78th day, respectively after sowing or 24 to 26 days before flowering (Tables 1 and 2). The difference between these two is four days and the activities that determine sterility were found to be

within this growth period. Growth conditions in Block 6 could not reverse the effects of LD length and the plants were 100% sterile. In our conditions, 24 to 26 days before flowering of the PMGS rice plants is the best time to produce hybrid seeds with minimum contamination from self bred seeds.

PGMS trait is under genetic control (Zhang et al., 1994), and the results from our study of ZAU11S106 rice under sterility inducing conditions, show the pollen cells had blebs, shrivels or leaking cell wall (Figures 4a to c), and the pollen cell nuclei were shriveled or disintegrating (Figure 5c). All these are traits associated with PCG (Krishnamurthy et al., 2000). Therefore, we conclude that pollen cell abortion in PGMS rice grown in LD and HT display “programmed cell death-like behavior”.

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