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 cystein 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, 1996). 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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Sterility is controlled in such a way that under LD and HT, growth conditions induce pollen abortion and the timing of the LD 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 leaf 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 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 leaf 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-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.
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 Canvoy’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:

**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 respectfully. 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 ZAU11106 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
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

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**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-July 11     | 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.
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 sterili
ty 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 acetocarmine 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 PCD (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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