

Full Length Research Paper

Tracking the expression of photosensitive genic male sterility genes in rice

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Photoperiod sensitive genic male sterile rice lines contain genes that induce complete sterility in high temperature and long day light length period, and revert to fertility in optimum low temperature and short day light length period. These lines are good candidates for hybrid rice seed production. The main challenge limiting their use in production of hybrid rice seeds is that of determining the exact time of their growth period when sterility gene(s) is expressed. The objective of this study was to determine the time in the rice growth period when the sterility gene(s) are expressed. Rice line ZAU11S106, a photoperiod sensitive genic male sterile line was used to test the hypothesis: it is possible to estimate time within ± 2 days when photosensitive genic male sterility (PGMS) gene is expressed. Sowing was done in 9 rows in Hangzhou, China in the month of May and matured in August when day light length was over 14 h and day temperatures were over 30°C. At 57 days, old plants in row 1 were given short day length treatment and after every four days, the next row was included in the treatment. This was done until plants in row 1 flowered when the treatment was stopped. Plants given short day length treatment at 73 and 77 days old realized 6 and 0% seed set, respectively. At 73 and 77 days old, plants were at dyad and tetrad stages of pollen development, respectively. The conclusion was that, the sterility gene was expressed between the dyad and tetrad stage of pollen development.

Key words: Photoperiod, pollen abortion, PGMS rice.

INTRODUCTION

Heterosis or hybrid vigour realized in F_1 has been used to increase yield in many out-crossing crops like maize (*Zea mays*). In particular, hybrid seed technology has been practiced in maize since 1939s and it accounts for over 30% increase in yield (Duvick, 1992, 1997). Two inbred genetically fixed varieties of a particular crop are crossed to obtain hybrids seeds. Plants from such seeds are special in that they express what is called "heterosis" or hybrid vigor. The basic principle is that, if two parents are crossed, which are genetically distant from each other,

the offspring will be "superior", particularly in terms of yield. Hybrid seed technology is practiced in many other crops including wheat, sunflower, cotton and rice (Igor and Hari, 1969; Burke and Arnold, 2001; Xie and Hardy, 2009). Search for high yielding rice (*Oryza sativa* L.) line has gone through a number of breeding metamorphic stages. The first major breakthrough was the incorporation of a semi-dwarfing gene (*sd-1*) in Chinese variety Dee-geo- woo-gen (Khush, 1994, 1995) into the ordinary rice plant around 1955 providing a plant

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Abbreviations: PGMS, Photosensitive genic male sterile line; SDLLT, Short day light length treatment; LDLL, Long day light length; HYVs, High yielding varieties; IRRI, International Rice Research Institute; CMS, Cytoplasmic male sterile; I/KI, Potassium iodide.

architecture that can accommodate use of more nitrogenous fertilizer hence, higher yield (Hu, 1993). Later came the high yielding varieties (HYVs) such that the International Rice Research Institute (IRRI) produced IR8 (Hossain et al., 1999). This was nicknamed “*miracle rice*” because of its improved yield. IR8 varieties churn out 10 metric tones/hectare in research stations, and for a long time this remained the yield ceiling. To break this barrier, two routes were adopted “*Super Rice*” or IRRI 15-tonner, which was achieved by a radical restructuring of the rice plant architecture, and the other route is by hybridizing rice. Super hybrid rice (Asia-Pulse, 1999; Yuan, 1997) has so far demonstrated the capacity to break the yield ceiling established by IR8 and other high yielding varieties (Heiling, 1999; Yuan, 1997). Production of hybrid rice started in 1970s with the discovery of cytoplasmic male sterile, “*wild rice with abortive pollen*”, or WA rice line (*Oryza sativa f. spontanea*) (Virmani, 1996). This is what is called the “three line system” because it involves a sterile, a maintainer and a restorer line. Yield increase due to crossing breeding in rice exploits hybrid vigour or *heterosis* just like in maize. For rice, *heterosis* has been reported to increase rice yield by between 20 to 30% above the current dwarf lines (Kush et al., 1994; Virmani et al., 1996; Virmani et al., 2003). The yield gains led China to start commercial production of hybrid rice in 1976 and lines that can yield up to 17 tons per hectare have been developed (Yuan, 1997; Kuyek, 2000). Most available estimates suggest that China’s hybrid rice yields average 15 to 20% more than the high-yielding inbred varieties (Yuan, 1994; Xie and Hardy, 2009).

To produce hybrid seeds, a sterile female parent and fertile male parent (pollen donor) are needed. This is achieved by male emasculation of the pollen recipient or the female parent which makes it labour and skill-intensive and increases cost of production especially if it is done manually (Virmani and Kumar, 1997). Despite this, hybridization remains the major method of increasing the rice yield. According to Yuan Longping, China has reached the yield plateau for hybrid rice (IRRI, 1998) using the three line system, however another major boost is expected from adopted “super hybrid” rice program (Xie and Hardy, 2009). The Green Revolution, led by IRRI’s high-yielding varieties (HYVs), led to dominance of a few lines such that by the mid-1980s just two HYVs occupied 98% of the entire rice growing area of the Philippines (Kuyek, 2000) leading to genetic erosion and reduced biodiversity. The problem has been increased by use of cytoplasmic male sterile (cms) lines in hybrid seed production that has led to increase of cytoplasmic uniformity leaving the hybrids vulnerable to disease and other environmental catastrophe (Levings, 1990).

Photosensitive genic male sterile (PGMS) rice is expected to reduce the problem of genetic degradation because it can be used with many restorer lines (both indica and japonica rice lines) to produce hybrid seeds, unlike in

CMS where they are limited due to maternal (female parent) and paternal (male parent) incompatibility, which lead to F₁ sterility (Oka, 1974; Lin et al., 1992). Wide compatibility is realized because PGMS (female) lines with diversified germplasm background can be produced unlike in cms whereby wild abortive (WA) is the major maternal line used (Virmani, 1996). Since the cost of production of hybrid rice seed using PGMS is expected to be lower than, PGMS lines are suitable candidates in Hybrid rice seed production technology.

Discovery of PGMS rice line in 1970s (Shi, 1981, 1985) ushered in the use of two-line system as a major method of producing hybrid seeds (Mao and Deng, 1993). PGMS rice lines are completely sterile in long day light length (LDLL) and revert to fertility in short day (Shi, 1985; Shi and Deng, 1986). They do not require a maintainer line like in the case of cytoplasmic male sterile plants since they maintain themselves. In LDLL (above 14 h) and in temperature of above 26°C it is completely sterile and reverts to fertility in optimal day length and temperature (Yuan et al., 1993; Ku et al., 2001).

To effectively use PGMS rice lines in hybrid rice seed production, a good breeding program needs to be developed. This will include evaluation and monitoring of PGMS genes to determine the time when it is switched on and off. Under sterility inducing conditions, the fertility gene is off and the pollen is completely sterile and seed set rate is zero (Xu et al., 1995, Ku et al., 2001, Njiruh and Xue, 2011). When PGMS are grown in short day, low temperature plants revert to fertility; a time when they are used to propagate themselves for the next generation. The PGMS character is genetically controlled and can be inherited from one generation to another (Shi, 1985; Virmani, 1994). The trait is controlled by genes *pms1* and *pms2* in chromosomes 7 and 3, respectively (Zhang et al., 1994) and *pms3* on chromosomes 12 (Mei et al., 1999).

This has enabled breeding for new lines with the PGMS trait and among them include W6154s, W7415s, NS5047S, 31111s, WD1S, 8801S, 6334s, N5047S (Virmani, 1994; Xue et al., 1999). The objective of this research was to track PGMS gene and determine when it is expressed in rice growth cycle as this will enable development of a rice breeding program that ensures optimum sterility inducing condition, prevail at the time of gene expression. In this report, the PGMS gene has been tracked to identify the critical four days within rice growth cycle in which switching off and on of the gene is determined.

MATERIALS AND METHODS

Plant materials used were Sterile (PGMS) line ZAU11S106 and a fertile line ZAU11F121 (control- ck). ZAU11S106 is a PGMS developed from japonica line N5047S protoplasts (Xue et al., 1999). Sowing was done on May 14th, 2003 at the Zhejiang University–Huajiachi Campus experimental fields at Hangzhou in China, 30°15N. Plants were grown under natural conditions and sowing

was programmed so that the plants headed in summer during the LDLL and high temperature. In this research, LDLL refer to day length of over 14 h day time including morning and evening twilight while short day refer to 11 h daylight including the morning and the evening twilight. High temperature refer to $>33^{\circ}\text{C}$ and $>26^{\circ}\text{C}$ during day and night respectively and low temperature refer to 26 and 20°C during day and night, respectively.

Sowing and Short day length treatment of PGMS

Rice lines ZAU11S106 and control ZAU11F121 were sown on May 14th in nine rows each with six plants and allowed to grow up to 57th or the stage just before the primordial stage. After 57 days, the first row was covered with an opaque black cloth at 4.00 pm and uncovered at 9.00 pm Hangzhou-China time, so that it experienced only 11 h of normal daytime light. This is what is referred to as short day light length treatment (SDLLT) throughout this research. Time when first row was given SDLLT was referred to as day zero (0) and after four days, the first and the second row were put under SDLLT. After every four days, a new row was included in the SDLLT. This was done as described in Table 1 until plants in the first row flowered when SDLLT was stopped.

Relation between PGMS gene expression and Pollen development

Before plants in each row were given SDLLT, a panicle was collected and fixed in Canoy's solution II for pollen analysis. Panicles were collected on days 57, 61, 65, 69 and 73 after sowing for rows 1, 2, 3, 4, and 5, respectively. At 73 days old and after, pollen had completely matured and therefore, pollen were collected from 77 days old plants or row 6 represents rows 7 and 8. A whole panicle from each sample was directly scanned using Uniscan 2100k scanner (Tsinghua, China). The fixed spikelets were stored at 4°C till use. This was followed by washing the glumes in 95% ethanol to wash away any residues of Canoy's solution that could create artifacts. Anthers were extracted from the glume using forceps or a dissecting needle for very young glumes and placed on a microscope glass slide with a drop of 1% potassium iodide (I/KI) solution after which, they were macerated using the forceps to release the pollen cells. Anther-husks were removed from the glass slide leaving the microspores only after which a cover slide was placed on a glass slide, observed, and photographs taken under x40 of light microscope (Olympus 35AD2, Japan). Photos were scanned using Uniscan 2100k scanner (Tsinghua, China) and processed using Adobe Photo Element version 2.

Determination of plant fertility

At post ripening stage, three tillers with full grown panicles were picked from each hill in each row for seed set evaluation. Glumes with filled up grains were counted and fertility was calculated as a percentage of total number of grains per spike to total number of glumes per spike $\times 100$.

RESULTS

Effects of short day length treatment on ZAU11S106 rice panicle fertility

ZAU11S106 plants in rows 1, 2, 3, 4 and 5 were given SDLLT for 28, 24, 20, 16 and 12 days respectively

(Table 1). These plants recorded seed set of 16, 18, 8, 20 and 6%, respectively. The plants given SDLLT on 57, 61, 65, 69 and 73 days old flowered at 81, 81, 90, 96 and 101 days old after sowing respectively. In ZAU11S106 rows 6, 7 and 8 (Ck) were given SDLLT for 8, 4 and 0 days, or at 77, 80 and 80(Ck) days old all recorded 0% seed set and panicle appeared seedless (Figure 2b). These plants flowered after 101, 102 and 105 days of age in this respect. The boundary on which seeds set was recorded and complete sterility was 77 days old or at 8 days of SDLLT or in row 6. ZAU11F121 plants in all row recorded over 39% seed set except row 2, which recorded 15% (Table 1).

A sample of panicles from ZAU11S106 plants under SDLLT in lines 1 to 5 and that from 6 and 7 lines are shown in Figure 2a and b, respectively. Glumes of panicle shown in Figure 2b were completely seedless while those from panicle shown in 2a recorded an average of 45% seed set.

Linkage of PGMS gene expression to Pollen developmental stages

Samples of panicles picked from rows 1 and 2 (corresponds 57 and 61 days old plants) did not have observable glumes. When these samples were stained with 1% I/K, no pollen grains were noticed (Figure 3a and b). However, samples obtained from rows 3 to 5 had distinctively grown glumes and after staining with 1% I/K, pollen were observed. These are the plants that received SDLLT on 65, 69 and 73 days old (Figure 3c to e).

Between days 57 and 61 after sowing panicles had no differentiated glumes and no pollen was observed for both ZAU11S106 (Figure 3f and g) and ZAU11F121 (Figure 3k and l). On 65th day after sowing pollen mother cell had formed for both ZAU11S106 and ZAU11F121 (Figure 3h and m) respectively. Dyads were observed in both ZAU11S106 (Figure 3i) and ZAU11F121 (Figure 3n) on day 69th after sowing; while on day 73 after sowing tetrad were observed for both ZAU11S106 and ZAU11F121 (Figure 3j and o, respectively).

Mature panicle from rows 5, 6, 8 (sterile control) and ZAU11F121 are shown in Figure 4a to d. Some pollen grains of plants in row 5 stained blue-black like the pollen from ZAU11F121 (Figure 4e and h). Plants in row six which was given SDLLT at 77 days old had their pollen all staining yellow same to those in rows 8 (ZAU11S106 control line). Pollen and panicle for row 7 were similar to those from row 6 and were not included.

DISCUSSION

Effects of Short length treatment on ZAU11S106 fertility

PGMS rice line ZAU11S106 given SDLLT in row 1 to 5

Table 1. Parameters used to track Critical Point of PGMS gene expression. Table a shows data collected from ZAU11S106 while b show data collected from ZAU11F121

| Day length (h) | Plant Row | Date of initiation of SDLLT (Days after sowing) | Heading date | Total days of SDLT | Days from sowing to Heading | Seed set % |
|----------------|-----------|---|--------------|--------------------|-----------------------------|------------|
| 11 | 1 | 11-July (57) | 4-Aug | 28 | 81 | 16 |
| 11 | 2 | 15-July(61) | 4-Aug | 24 | 81 | 18 |
| 11 | 3 | 19-July(65) | 11-Aug | 20 | 90 | 8 |
| 11 | 4 | 23-July(69) | 17-Aug | 16 | 96 | 20 |
| 11 | 5 | 27-July(73) | 22-Aug | 12 | 101 | 6 |
| 11 | 6 | 30-August(77) | 23-Aug | 8 | 102 | 0 |
| 11 | 7 | 3- August(81) | 26-Aug | 4 | 105 | 0 |
| 14 | 8 | CK (81) | 26-Aug | 0 | 105 | 0 |
| 11 | 1 | 11-July (57) | 4-Aug | 28 | 81 | 39 |
| 11 | 2 | 15-July(61) | 4-Aug | 24 | 81 | 15 |
| 11 | 3 | 19-July(65) | 11-Aug | 20 | 88 | 41 |
| 11 | 4 | 23-July(69) | 11-Aug | 16 | 88 | 53 |
| 11 | 5 | 27-July(73) | 17-Aug | 12 | 94 | 64 |
| 11 | 6 | 30-August(77) | 17-Aug | 8 | 94 | 67 |
| 11 | 7 | 3- August(81) | 17-Aug | 4 | 94 | 51 |
| 14 | 8 | CK (81) | 11-Aug | 0 | 91 | 54 |

In both Tables a and b, column 1 shows number of hours of daylight the plants were exposed to SDLLT while "plants row" in column 2 refer to order of sowing in the concrete trough. Column three indicate the dates on which SDLLT was initiated and figures in brackets indicate the age of plant at the time of SDLLT. Heading dates in column 4 refer to date when all plants in each row flowered. Column 5 and 6 shows total number of short day length treatment and days from sowing to maturity respectively. Plant fertility (%) or seed set (%) shown in column 7 indicate percentage seed of all plants in each row treatment.

recorded a seed set rate of between 6 and 20% with an average of 14%. These were plants given SDLLT on days 57, 61, 65, 69 and 73 after sowing. For rows 6 and 7 seed set rate of 0% was recorded same as ZAU11S106 control line. There was a drastic reduction of 20% seed set recorded in row 4 to 0% seed set recorded in rows 6 and 7. Plants in row 5 received 12 days of SDLLT and recorded 6% seed set while plants in row 6 received 8 days of SDLLT and recorded seed set of 0%. At initiation of SDLLT, plants in row 5 were 73 days old; this was followed with 12 days of the SDLLT treatment after which the plants were left to grow under LDLL growth condition for 16 days when they flowered. The total time in days taken to completion of flowering was 73 days at initiation of SDLLT +12 days of SDLLT +16 days under LDLL growth conditions or 103 days.

Despite the 16 days under LDLL growth conditions before flowering, plants in this row still recorded 6% seed set (Table 1) and some pollen stained blue-black with 1% I/K same as non PGMS control plants of ZAU11F121 (Figure 4e and h). This implies that fertility gene must have been expressed within the 12 days of SDLLT and before the plants were left to grow under LDLL growth conditions. Plants in row 6 received SDLLT for 8 days starting at 77 days old and stopped when they were 85days old. After 8 days of SDLLT these plants were left to grow under LDLL growth conditions and flowered 17 days later, recorded a seed set of 0% (Table 1a) and

mature pollen stained yellow with 1% same ZAU11S106 control plants in row 8 (Figure 4f and g) . Difference in SDLLT was 4 days (12 to 8 days) and within this period sterility inducing gene had already been expressed thus a seed set of 0% in row 6. Therefore the critical period when sterility genes were expressed lie between days 73 and 77 after sowing. At 77 days of age, exposure of plants to SDLLT for only 8 days resulted to complete sterility just like plants in the control row 8. At 73 days old, pollen corresponds to tetrad stage of meiosis (Figure 3); at this stage when the PGMS were exposed to SDLLT for only four days above 73 they were irreversibly fertile and LDLL growth conditions could not reverse it. Similarly, plants exposed to LDLL growth conditions for four days above 73 days old became completely sterile and a follow up with SDLLT could not induce any seed set. Growth of ZAU11S106 under LDLL growth conditions or under SDLLT before 73 days of age did not determine if the plant was to be completely sterile or fertile. This is why plants given SDLLT at 73 days old displayed spikelet fertility just like the ones given SDLLT at 57days old.

Panicle development in Figures 3a to e corresponds to growth in row 1 to 5 or to 28 to 12 days of SDLLT or 57 to 73 days of plants' age at commencement of SDLLT. Pollen from plants in these growth stages had normal meiotic pollen development for ZAU11S106 (Figure 3f to j) same as ZAU11S121 (Figure 3k to o). Pollen from ZAU11S106 plants given SDLLT at 73 days old stained



Figure 1. Sowing pattern of ZAU11S106 and ZAU11F121 for SDLLT. PGMS rice ZAU11S106 was sown in concrete troughs in rows each with 6 plant and allowed to grow for 57 days after which SDLLT was started. Plants were covered from 4.00 to 9.00pm when complete darkness set in so as to be exposed to only 11 h of daylight. Figure a shows plants at initiation of SDLLT and Figure b shows plants at cessation of SDLLT. In Figure 1 a only four rows appear (others were not captured) but Figure b show the total size of trough and number of rows. Plants given SDLLT flowered earlier than those which did not (Figure b).

yellow or colourless with 1% I/KI indicating that they were of abortive type (Figure 4b). Therefore, SDLLT after 73 days old could not induce fertility and pollen cells were of abortive type. When plants were given SDLLT between 69 and 73 days old for 12 days they were fertile and allowing them to grow under LDLL growth conditions for 16 days could not prevent some pollen to stain blue-black with 1% I/KI solution indicating that they are of fertile type. Transformation of pollen from sterile to fertile and vice versa was found to occur between days 73 and 77 old after sowing. This is the critical period of pollen transformation. Once sterility or fertility reaction take place during the critical period, transformation is irrever-



A

B

Figure 2. Effects of SDLT and LDLT on PGMS rice ZAU11S106 panicle fertility. Panicle in Figure 2a was a sample of plants in row 1 to 5 shown in Table 1. These plants which recorded some seed set received SDLLT for 12 to 28 days or when plants were between 57 and 73 days old. Panicle shown in Figure 2b with seedless glumes was obtained in ZAU11S106 sown in row 6 as recorded in Table 1. Plants in this row received SDLLT for only 8 days when they were 77 days old.

sible despite the conditions prevailing later. According to Yuan et al. (1993), at critical period, SDLLT make PGMS rice irreversibly fertile while LDLL treatment makes them irreversibly sterile.

Microscopic observation of pollen development from pollen mother cell to tetrad stage from both ZAU11S106 and ZAU11F121 (control) plants with or without SDLLT displayed same meiotic features up to tetrad. ZAU11S106 plants given SDLLT at tetrads stage (row 5) of pollen cell development recoded 6% seed set but those given SDLLT after tetrad (row6) had 0% seed set (Table 1). Apparently, the decision whether glumes will be fertile or sterile is taken between days 73 and 77 (4 days difference). Therefore, ZAU11S106 needed for production of self-breed seed for its own maintenance need to be given SDLLT before 77days old and those needed to be completely sterile for hybrid seedproduction need be given LDLL growth conditions between 73 and 77 days after sowing.

In photosensitivity male sterility (PGMS) rice, LDLL and high temperature induce up to 100% pollen sterility while in short day length growth conditions pollen recover their vitality and become fertile (Xue et al., 1999). Sterility is controlled by three major genes; *pms1*, *pms2* and *pms3* that have been mapped on chromosomes 7, 3 and 12, respectively (Zhang et al., 1994; Mei et al., 1999).

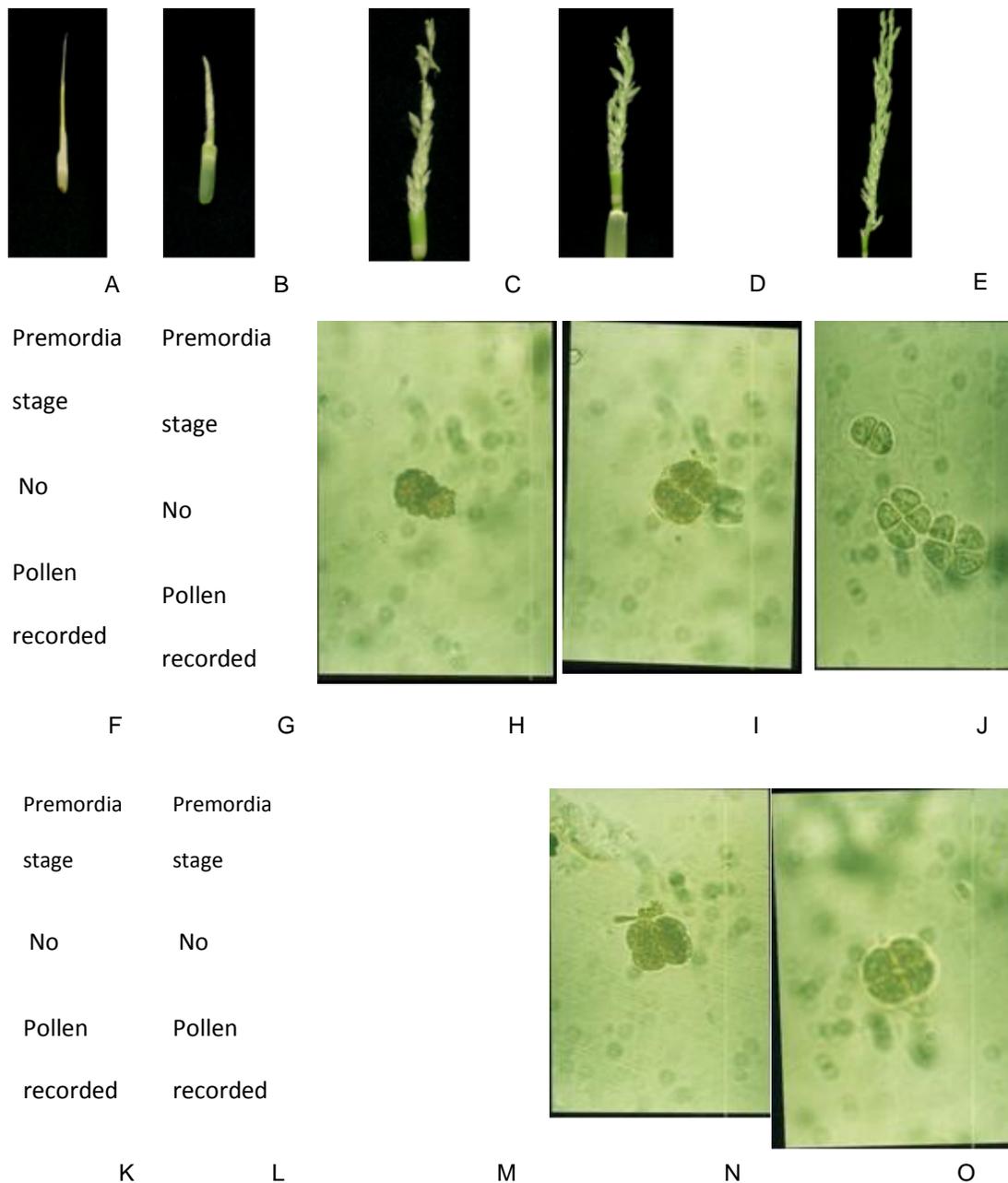


Figure 3. ZAU11S106/ZAU11F121 rice panicles and pollen cells at various stages of development. a to e shows samples of panicles obtained from ZAU11S106 in rows 1 to 5 taken before SDLT initiation. At this stage panicles from ZAU11S106 and ZAU11S121 did not display any observable difference among them (therefore the samples represent both lines). Meiotic pollen from ZAU11F106 are presented in f to j while those from ZAU11F121 are presented in k to o. Samples were taken up to completion (tetrad stage) of meiosis.

According to Yuan et al. (1993) the critical time determining sterility or fertility in PGMS rice is the time from primary premordia through secondary premordia differentiation to differentiation of stamen and pistillate. All pollen (100%) from ZAU11S106 rice given SDLT at 77 days stained yellow with 1% I/KI and 0% seed set was realized (Figure 4f and g). At 73 days old, a time when pollen development was at tetrad stage of meiosis,

SDLT for four days gave 6% seed set. This is an indication that PGMS gene(s) were expressed between days surrounding tetrad and cytokinesis stages of pollen development or between 73 to 77 days old after sowing.

When breeding for hybrid rice seeds female parent need to be 100% sterile to prevent contamination of hybrid seeds with self-breed seeds. Precise determination of the most critical stage of sterility expressing genes

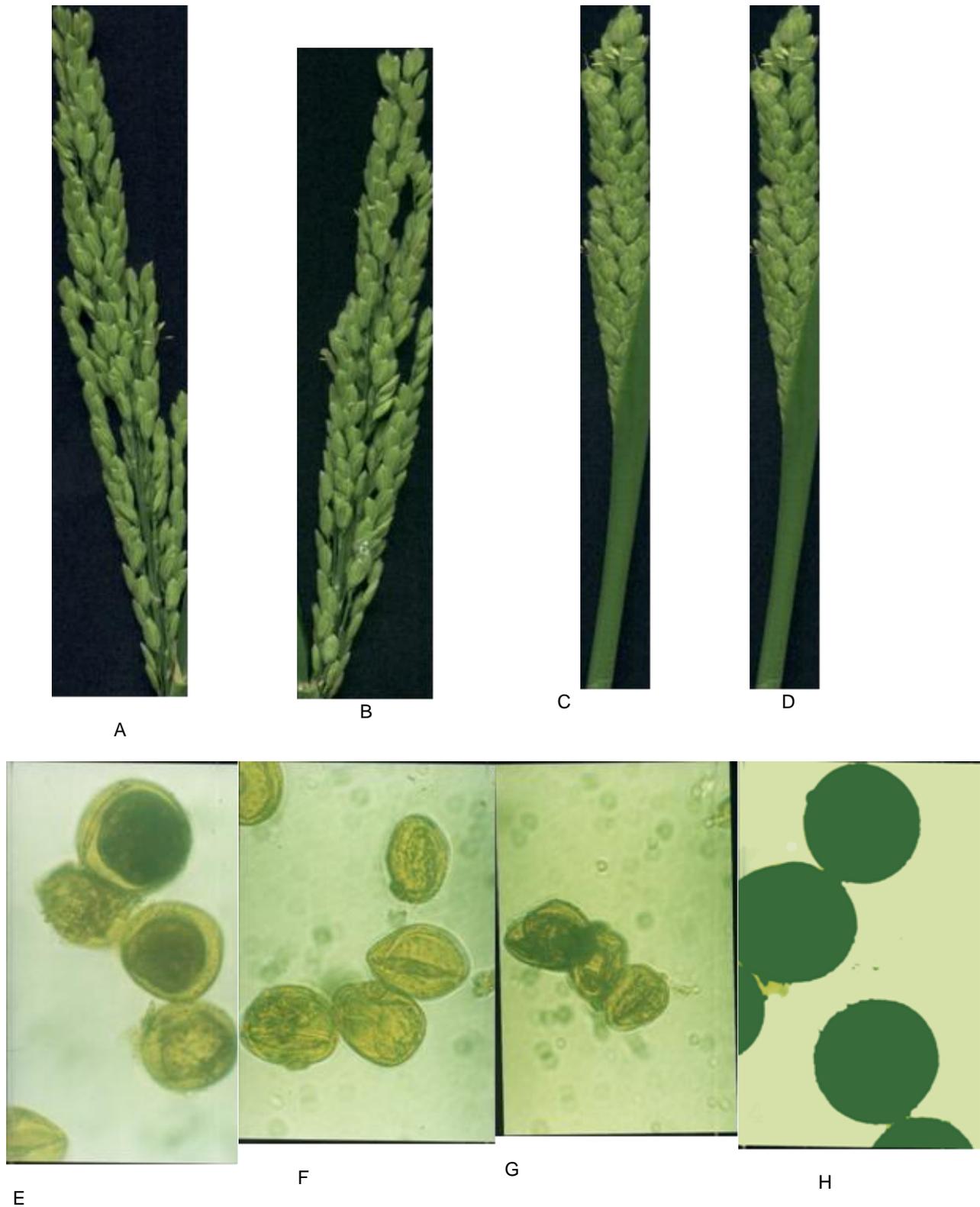


Figure 4. Manifestation of ZAU11S106 and ZAU11F121 panicles and pollen cells after critical point of fertility/sterility determination. Figures a, b, c and d shows mature PGMS panicle collected from rows 5,6, 8(sterile control) and Fertile control (ZAU11F121). Pollen extracted from glumes were stained in 1% K/I. Figure e show pollen collected from mature panicle from row5 which shows effect of SDLLT at critical point of fertility determination and some pollen have stains of blue-black, resembling pollen from ZAU11F121 rice panicles in Figure h which stain blue black with 1% K/I. Figures f and g indicate pollen from row and row 8 (control), respectively.

will enable synchronization of sowing, so plants enter primordial stage of flowering in LDLL growth conditions. PGMS require sterility inducing conditions only at the critical sterility determining stage, once this is realized pollen become irreversibly sterile (Njiruh and Xue, 2011). Once expressed, the PGMS gene leads to deformed tapetum and exine (Kaul, 1988; Xu et al., 1995; Njiruh and Xue, 2011). Tapetum is the innermost wall of microsporogium that provide enzyme, hormones and food to the growing pollen mother cells (PMC). Biochemical substances interfere with the normal functioning of the tapetum by cutting off the pollen nourishment system and starve them to death since under sterility inducing conditions anther locules of PGMS plants are occupied by deformed pollen (Ku et al., 2001); such biochemical reaction may have taken place under SDLL growth conditions.

Conclusion

ZAU11S106 was completely sterile when given LDLL growth conditions at dyad and tetrad stage of pollen development. This corresponds to period 73 to 77 days old. Therefore, fertility or sterility was determined within these 4 days.

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