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RESEARCH ARTICLE

Programmed cell death may act as a surveillance mechanism to safeguard male gametophyte development in *Arabidopsis*

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ABSTRACT

Programmed cell death (PCD) plays an important role in plant growth and development as well as in stress responses. During male gametophyte development, it has been proposed that PCD may act as a cellular surveillance mechanism to ensure successful progression of male gametogenesis, and this suicide protective machinery is repressed under favorable growth conditions. However, the regulatory mechanism of male gametophyte-specific PCD remains unknown. Here, we report the use of a TdT-mediated dUTP nick-end labeling-based strategy for genetic screening of *Arabidopsis* mutants that present PCD phenotype during male gametophyte development. By using this approach, we identified 12 mutants, designated as *pcd in male gametogenesis* (*pig*). *pig* mutants are defective at various stages of male gametophyte development, among which nine *pig* mutants show a microspore-specific PCD phenotype occurring mainly around pollen mitosis I or the bicellular stage. The *PIG1* gene was identified by map-based cloning, and was found to be identical to *ATAXIA TELANGIECTASIA MUTATED* (*ATM*), a highly conserved gene in eukaryotes and a key regulator of the DNA damage response. Our results suggest that PCD may act as a general mechanism to safeguard the entire process of male gametophyte development.

KEYWORDS programmed cell death, male gametophyte, *PIG*, TdT-mediated dUTP nick-end labeling, *ATM*

INTRODUCTION

In *Arabidopsis thaliana*, male gametogenesis consists of several distinct stages. In a specialized male reproductive organ called anther, diploid pollen mother cells undergo meiosis and then give rise to tetrads of four haploid microspores. The microspores in the tetrad are released by the action of an enzyme (callase) secreted by the tapedum, the inner layer of the anther. The unicellular microspores then undergo an asymmetric mitotic division known as pollen mitosis I (PM I), resulting in the formation of the bicellular pollen grains consisting of a smaller generative cell and a larger vegetative cell. The smaller generative cell has a condensed nucleus and then undergoes a second pollen mitosis (PM II) to form the two sperm cells, while the larger vegetative cell exits the cell cycle. The *Arabidopsis* pollen grains contain three cells at the tricellular stage: two sperm cells and one vegetative cell which eventually will form the pollen tube. Male gametogenesis is completed when the sperm cells are released from the anther (McCormick, 1993; Sanders et al., 1999; McCormick, 2004; Ma, 2005).

Programmed cell death (PCD) is a genetically regulated process by which unwanted cells deliberately suicide themselves (Lockshin, 1969; Lockshin and Zakeri, 2004). The common phenotype of PCD contains membrane blebbing, cytoplasmic shrinkage, nuclear condensation and DNA fragmentation (Bursch et al., 2000). TUNEL (TdT-mediated dUTP nick-end labeling) method, using TdT (terminal deoxynucleotidyl transferase) to label 3'-OH groups during DNA fragmentation, is considered as a good specific method to detect cell death by PCD (Gavrieli et al., 1992). In plants, PCD plays an important role in not only the response to the biotic or antibiotic stress, but also the developmental processes including leaf senescence, xylem development, the removing of root cap cells and disappearance of aleurone cells (Pennell and Lamb, 1997; Kuriyama and Fukuda, 2002).

In addition, PCD has been proposed to act as a cellular surveillance mechanism to safeguard the meiosis and the second mitosis progression of male gametophyte development (Yang et al., 2003; Teng et al., 2008). Under normal growth condition, *MMD1* (*Male Meiocyte Death1*), encoding a PHD-finger protein, may ensure the male gametophyte development by repressing a meiocytic cell death pathway. Mutation of *MMD1* causing the meiosis defection directly or indirectly triggers a cell death pathway (Yang et al., 2003). Similarly, mutation in *FBR11* (*Fumonisin B_1 Resistant11*), encoding a rate-limiting enzyme in *de novo* sphingolipid synthesis, causes cellular disruption and PCD features during the second mitosis of male gametophyte development. However, it is still unclear whether a similar mechanism occurs at the first mitosis stage of male gametophyte development, and whether other genes are involved in the regulation of male gametophyte-specific PCD. In this study, we identified 12 *pig* (*pcd in male gametogenesis*) mutants all exhibiting PCD phenotype detected by TUNEL analysis, but they are defective at various stages of male gametophyte development including the first mitosis stage. These results suggest that PCD may act as a common cellular surveillance mechanism to safeguard the entire process of male gametophyte development.

RESULTS

Identification of *pcd in male gametogenesis* (*pig*) mutants

Previous studies revealed that PCD might play an important role in the regulation of male gametophyte development (Yang et al., 2003; Teng et al., 2008). To explore whether PCD is a general regulatory mechanism in male gametophyte development, we performed a genetic screen for mutants that show PCD phenotype during male gametogenesis (Fig. 1). To this end, we first identified mutants with defective gametogenesis by screening a T-DNA mutagenized population showing a distorted segregation ratio for the kanamycin resistance phenotype conferred by the T-DNA insertion. Because kanamycin-resistance is a dominant trait, the phenotype should be segregated in a typical Mendelian pattern of 1:3 (sensitive to resistant). However, a gametophytic mutation caused by a single T-DNA insertion, either female- or male-specific, will alter the segregation ratio to 1:1. This distorted segregation ratio has allowed the identification of many gametophytic mutations from foreign DNA insertional mutants (Howden et al., 1998; Shi et al., 2005). Based on this strategy, we identified approximately 350 putative mutants with a segregation ratio of 1:1 (kanamycin sensitive: resistant) by screening over 20,000 transgenic T2 families (Fig. 1).

Because putative gametophytic mutants identified by this method may be at least both female- or male-gametophyte-specific, we therefore analyzed pollen development of these

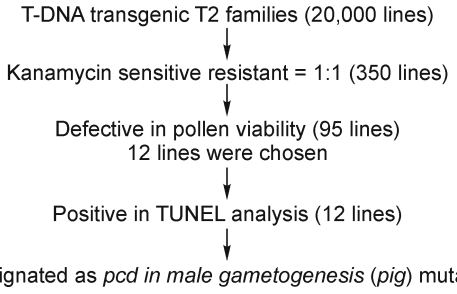

Figure 1. The flow chart of the genetic screen of *pcd in male gametogenesis* (*pig*) mutants.

putative mutants by Alexander staining. After the staining, normal pollens exhibited dark-brown color, whereas abnormal or dead pollens showed green color (Fig. 2A). Among the 350 putative mutants, we identified 95 lines, of which more than half of the examined pollens stained with green color (see representative examples in Fig. 2B and 2C). Of these mutants, several lines showed extremely severe phenotype with only a few viable pollen grains (Fig. 2B).

For this study, we selected 12 mutant lines for further characterization. To rule out the possibility that PCD phenotype is correlated with the severity of the pollen defection, we chose 12 mutants containing both severe like *pig1* and non-severe like *pig4* phenotype based on Alexander staining. Data presented below demonstrated that these mutants showed PCD phenotype during male gametophyte development, and we therefore designated these mutants as *pig1*, *pig2*…to *pig12*.

Genetic analysis of the *pig* mutants

The putative *pig* mutants were initially identified by the distorted segregation ratio of kanamycin-resistance, which may not co-segregate with the pollen abortive phenotype. Therefore, we examined the genetic nature of these 12 putative mutants by analyzing pollen development using the Alexander staining method. Pollens were collected from progenies derived from self-pollinated putative *pig*/+ heterozygous plants, and then subjected to Alexander staining. Among the 12 putative *pig* mutant plants, progenies derived from self-pollinated *pig1*/+ and *pig2*/+ plants showed a 3:1 ratio with the pollen development phenotype, i.e., 75% progenies showed normal pollen development, whereas 25% progenies showed defective pollen development. This result suggests that *pig1* and *pig2* are defective in sporophytic development, which consequently causes defective pollen development (see also below).

The other ten mutants (*pig3*–*pig12*) showed a 1:1 segregation ratio, similar to that observed in the analysis of kanamycin-resistance. In these ten mutants, approximately 50% progenies produced normal pollens, whereas the remaining half of the progenies had both normal and aborted

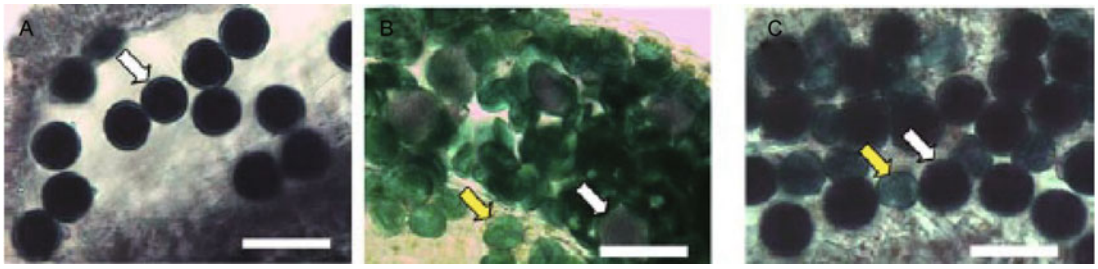

Figure 2. Pollen viability determined by Alexander staining. (A) Mature pollen grains from wild type plants; (B) Mature pollen grains from *pig1* mutants; (C) Mature pollen grains from *pig4* mutants. The viable pollens are stained dark-brown (white arrow), whereas the unviable pollens are stained green (yellow arrow). Bars, 50 μm.

pollens with approximately 50% each. These results indicate that *pig3*–*pig12* are male gametophytic mutants.

Analysis of DNA fragmentation in male gametophytes of the *pig* mutants

DNA fragmentation is a key cellular feature of PCD, which produces a free 3′-OH group (Williams et al., 1974; Wyllie, 1980). The free 3′-OH group can be detected by the TdT-mediated dUTP nick-end labeling (TUNEL) method to assay possible DNA fragmentation in a cell (Gavrieli et al., 1992). To ask whether pollen abortion in the putative *pig* mutants is related to PCD, we performed a whole-mount TUNEL analysis in preparations containing male gametophytes (Teng et al., 2008). Flowers at developmental stages 1–6 (the developmental stages were defined according to (Lalanne and Twell, 2002)) were collected, which contained male gametophytes at the unicellular, bicellular and tricellular stages (Lalanne and Twell, 2002). In the preparations made from wild-type flowers, although non-specific signals were often observed at the cell wall of pollen grains, no fluorescent signal was detected in the nuclei of the pollen grain at various stages (Fig. 3A–C). In contrast to that observed in wild type pollen grains, positive TUNEL signals were observed in the nuclei of the pollen produced by all 12 mutants (Fig. 3D, 3E and Fig. S1). These results indicated that all these 12 *pig* mutants showed PCD phenotype.

The *pig* mutants are defective at different stages of male gametogenesis

Data presented above suggest that mutations in the *PIG* genes cause PCD in male gametophytes. In particular, the *pig1* and *pig2* mutations resulted in sporophytic defects. On the other hand, *pig3*–*pig12* mutants showed male gametophyte-specific defects. Since TUNEL analysis was performed in a group of flowers with stages 1 to 6, we could not distinguish the defective stages in mutant pollens. Therefore, we examined the defective stages in 10 mutants of

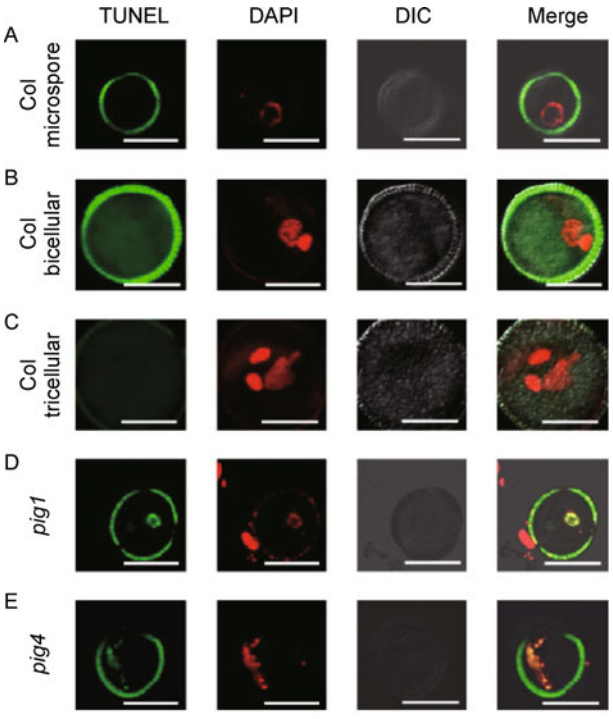

Figure 3. DNA fragmentation detected by TUNEL analysis in *pig1* and *pig4* mutants. (A) Unicellular stage of wild type pollen grains; (B) Bicellular stage of wild type pollen grains; (C) Tricellular stage of wild type pollen grains; (D) Pollen grains from *pig1* mutants; (E) Pollen grains from *pig4* mutants. Fluorescence signal after TUNEL analysis is shown in the left panel. DAPI and phase images (DIC) of the corresponding fields are shown in the middle panels as indicated. The right panel shows the merged images of the above channels. Bars, 10 μm.

pig3–*pig12* by DAPI (4',6-diamidino-2-phenylindole) staining, a fluorescent dye specifically binding to DNA and labeling the nucleus. In the ten *pig* mutants, no abnormality was observed at the unicellular stage (representative example from *pig4* is

shown in Fig. 4A). However, nine mutants from *pig3* to *pig11* showed apparent defects at the bicellular stage, as indicated by diffusely stained nuclei (representative example from *pig4* is shown in Fig. 4B). These observations indicate that the *pig3*–*pig11* mutants are defective at PM I stage. The *pig12* mutants did not have apparent defects at the bicellular stage (Fig. 4C), but were abnormal at the tricellular stage with small-sized or empty pollen grains without visible nuclei (Fig. 4D). This result suggests that *pig12* is defective at PM II stage. Our results suggest that microspore-specific PCD phenotype occurs mainly around PM I or the bicellular stages.

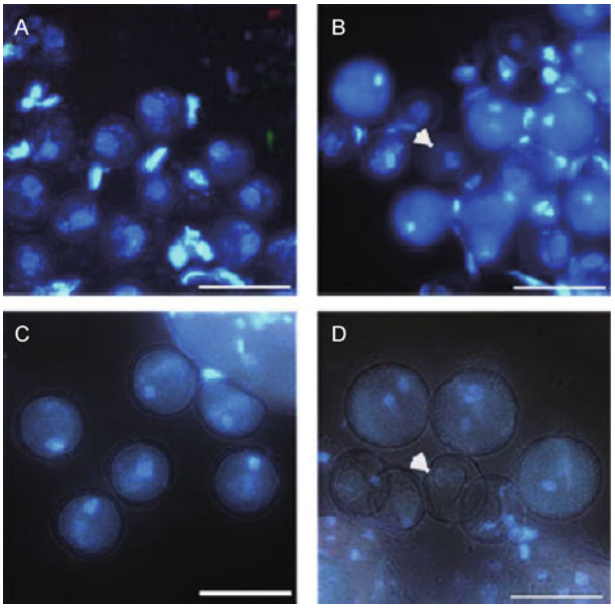

Figure 4. Stages of pollen grain abortion examined by DAPI staining. (A) The microspore stage of pollen grains from *pig4* mutants. All pollens show normal DAPI staining. (B) The bicellular stage of pollen grains from *pig4* mutants. Normal pollens are stained as single bright generative nucleus, whereas abnormal pollens show diffusely stained nucleus as arrow indicated. (C) The bicellular stage of pollen grains from *pig12* mutants. All pollens are stained normally with single bright generative nucleus. (D) The tricellular stage of pollen grains from *pig12* mutants. Normal pollens are stained as two bright generative nuclei, whereas abnormal pollens are empty without visible nuclei as arrow indicated. Bars, 50 μm.

To better understand the microspore-specific PCD at PM I stage, we examined other PCD features in *pig3* mutants. Semi-thin sections were performed from anthers collected at various developmental stages of wild-type and *pig3* mutants, and then analyzed by light microscopy. No abnormality was observed at unicellular stage, whereas small and misshaped pollens were observed at bicellular and tricellular stages (Fig. 5A–C). To obtain the nuclear morphology, the section samples were analyzed by fluorescence microscopy after DAPI staining. At unicellular stage, all microspores from *pig3* mutants showed one nucleus and normal speckled cytoplasm because of the staining of the mitochondrial and plastid DNA (Fig. 5D and 5G) (Regan and Moffatt, 1990). However, at bicellular and tricellular stages, only half of pollens showed wild type brightly stained generative nuclei, whereas half of pollens showed abnormal nuclei with diffusely DAPI staining (Fig. 5E and 5F). In comparison with the wild type pollen (Fig. 5H and 5I), PCD features including cytoplasmic shrinkage and nuclear degeneration were observed at bicellular and tricellular stages in the mutant pollen (Fig. 5J and 5K). Therefore, our further analysis confirmed that PCD is present at first mitosis stage and might act as a cellular surveillance mechanism to ensure the successful complementation of this progression.

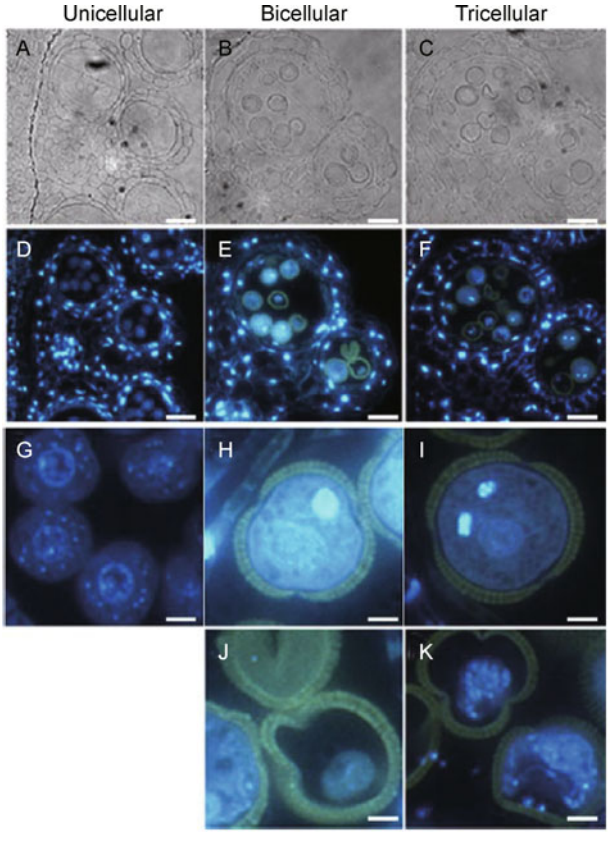

Figure 5. Developmental defects of *pig3* mutants during male gametogenesis. (A–C) Semi-thin section of *pig3* anthers under a light microscope; (D–F) Fluorescence observation of (A–C) after DAPI staining; (G–K) Magnified pictures of (D–F); (G–I) showing the wild type pollen grains, while (J) and (K) showing the defective pollen grains. Developmental stages of male gametophyte are showed on the top of the figures. Bars for (A–F), 50 μm; Bars for (G–K), 10 μm.

Taken together, these results indicate that defective male gametophyte development occurs at all major developmental

stages of the *pig* mutants, including meiosis, PM I and PM II, with a relatively higher frequency at PM I.

PIG1 is allelic to *ATAXIA TELANGIECTASIA MUTATED* (*ATM*)

To get more insight into the molecular mechanism of *PIG*-regulated male gametophyte development, we attempted to identify and characterize representative *PIG* genes. Results obtained from partial characterization of *PIG1* are reported below.

The pollen defective phenotype of *pig1* mutants is constant after the backcross. In an F1 population obtained from the backcross, the progenies showed a wild-type phenotype. In F2 plants, approximately 25% progenies showed a *pig1* phenotype, characteristics of a Mendelian segregation pattern (*pig1*:wild-type = 14:48, χ^2 = 0.38). This result indicates that *PIG1* mutation is recessive in a single nuclear gene. Subsequent studies indicated that the *pig1* mutation was not tagged by the T-DNA insert. Therefore, we performed a positional cloning experiment to identify the *PIG1* gene. A genetic mapping population was generated by crossing *pig1* (Col-0) with wild type L*er* plants. Using an F2 population of 133 *pig1* mutant plants, *PIG1* was mapped onto chromosome III between two SSLP markers T17F15 and T24C20.2, in an interval of 169 Kb (Fig. 6A). This region contained 37 annotated genes. DNA sequencing analysis of the *pig1* genome revealed a large fragment of 13,071 bp in the *ATAXIA TELANGIECTASIA MUTATED* (*ATM*) gene was deleted and replaced by a small fragment of filler DNA (Fig. 6B). Consistent with the mutation nature of *pig1* in the *ATM* gene, reverse transcription (RT)-PCR analysis revealed that no transcript was detected in the deleted region of *ATM*. However, an *ATM* transcript was detected using primer pairs upstream of the deletion region (Fig. 6C and 6D), suggesting that *PIG1* mutation most likely causes the generation of a truncated transcript of *ATM*.

The *pig1* mutant shows a similar phenotype as several *atm* mutants previously characterized (Garcia et al., 2003). To verify whether the *pig1* mutant phenotype is caused by the deletional mutation in *ATM*, we performed an allelism analysis of *pig1* with *atm-2* (Garcia et al., 2003). From a cross between *pig1/+* (female) and *atm-2/+* (male), two *pig1*/*atm-2* plants were identified out of 13 F1 progenies assessed by phenotyping and genotyping. Pollen grains produced from *pig1*/*atm-2* plants were stained as green by Alexander's staining solution (Fig. 7A and 7B). The siliques of *pig1*/*atm-2* were shorter than that of wild type (Fig. 7C and 7D). These results demonstrate that the allelism of *pig1* and *atm-2,* and that the *pig1* mutant phenotype are caused by the mutation in *ATM* gene.

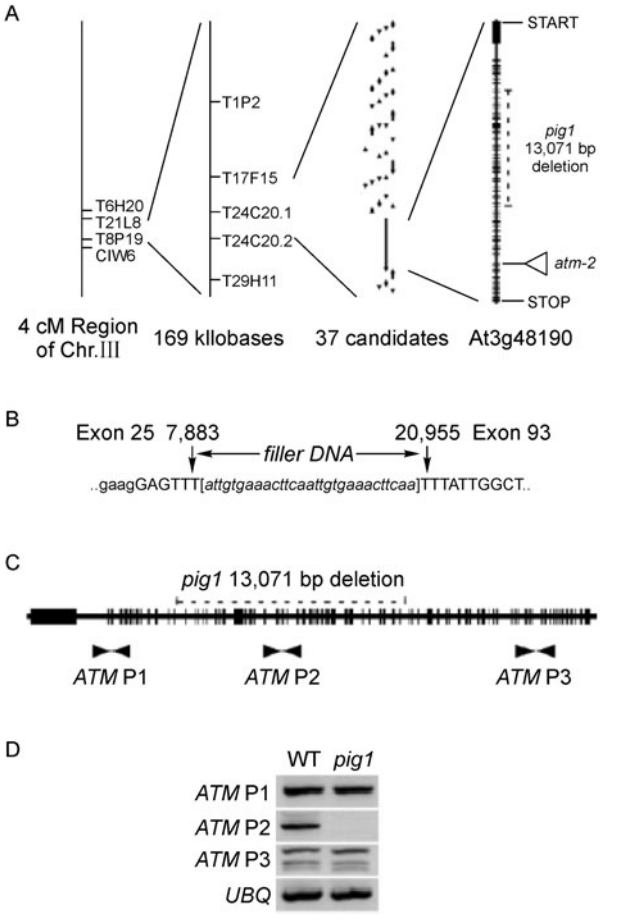

Figure 6. Molecular characterization of the *PIG1* gene. (A) The genetic and physical map of *PIG1* gene. The *PIG1* locus was mapped to a 4 cM region with flanking markers T6H20 and CIW6 on chromosome III. Subsequent mapping with 133 *pig1* homozygotes showed that *PIG1* was mapped to a 169 kb region with flanking markers T17F15 and T24C20.2. Thirty seven candidate genes were identified in that region according to the Col-0 sequence in GenBank. Directly sequencing indicated that a 13,071 bp DNA deletion in the open reading frame (ORF) of *ATAXIA TELANGIECTASIA MUTATED* (*ATM*, At3g48190) may cause the mutation of *PIG1* gene. Filled boxes represent the predicted exons of the *PIG1*/*ATM* gene. The dot line indicates the missing region in *pig1* mutants. The triangle represents the T-DNA insertion site in *atm-2* mutants. (B) The filler DNA sequence in *pig1* mutants. Number above indicates the position in wild type *PIG1*. The filler DNA sequence found in the *pig1* mutants are indicated in lowercase italic within the square brackets. (C) Schematic diagram of *ATM* gene. Filled boxes represent the predicted exons of the *PIG1*/*ATM* gene. The dot line indicates the deleted sequences in *pig1* mutants. (D) *ATM/PIG1* expression was analyzed by semi-quantitative PCR in *pig1* and wild type. *Ubiquitin* (*UBQ*) was used as an internal control, and the position of primer pairs are indicated in (C).

DISCUSSION

Male gametogenesis is an essential process for the formation of pollen grains and regulated by precisely regulatory

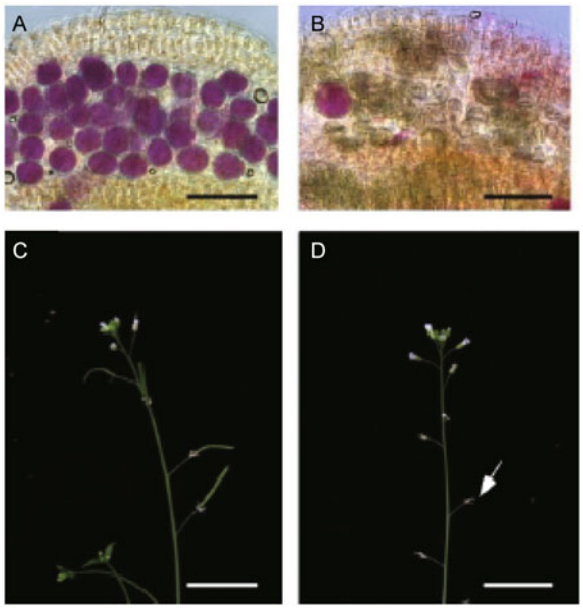

Figure 7. Phenotype analysis of *pig1atm-2* double mutants. (A) Alexander's test of mature pollen grains from wild type; (B) Alexander's test of mature pollen grains from *pig1atm-2* double mutants; (C) Flowering stems of wild type (6-week-old); (D) Flowering stems of *pig1atm-2* double mutants (6-week-old). Siliques of *pig1atm-2* double mutants are shorter than that of the corresponding wild type as arrow indicated. Bars for A and B, 50 μm. Bars for C and D, 2 cm.

mechanisms including PCD (Bakeeva et al., 2005). During male gametophyte development, the tupedum, the endothecium and the epidermis all undergo PCD, eventually leading to the release of pollen grains (Papini et al., 1999; Varnier et al., 2005; Li et al., 2006; Vizcay-Barrena and Wilson, 2006). In those cases, PCD is a developmental program to eliminate the unwanted cells, but not triggered by the extensive damage of male gametophyte. The recent studies of *mmd1* and *fbr11* mutants in *Arabidopsis* indicated that PCD could act as a surveillance mechanism to remove the damaged cells at meiosis and PM II stages during male gametophyte development (Yang et al., 2003; Teng et al., 2008). In this study, we isolated 12 *pig* mutants which all showed PCD phenotype detected by TUNEL analysis, but were defective at various stages of male gametophyte development including the PM I stage. Our results suggest that PCD is a general surveillance mechanism for monitoring the entire development of male gametophyte. However, except *MMD1* and *FBR11*, the molecular study of this mechanism is still in its infancy. *MMD1* gene encodes a PHD-finger protein probably associated with chromatin-remodeling, and its mutation results in meiotic defect and apoptosis-like phenotype, suggesting that *MMD1* may regulate the male gametophyte-specific PCD at meiosis stage (Yang et al., 2003). *FBR11* encodes a key enzyme for the synthesis of sphingolipid which is an important signal for cell death. The cellular defection and PCD features observed at the PM II stage of the male gametophyte development in *fbr11* mutants suggest that sphingolipid may be involved in the male gametophyte-specific PCD during the second mitosis (Teng et al., 2008). Therefore, in addition to *MMD1* and *FBR11*, further studies of *pig* mutants might provide new insight into the molecular mechanism of the male gametophyte-specific PCD in pollen development.

ATM (*Ataxia-telangiectasia mutated*) gene is a key factor in damage response to DNA double-strand breaks, first reported in ataxia-telangiectasia disease (Boder and Sedgwick, 1958; Centerwall and Miller, 1958). Studies of *ATM* genes in yeast, fly and mice indicated that *ATM* gene is not only important for the response to DNA damage, but also essential for meiosis (Carpenter, 1979; Kato and Ogawa, 1994; Lydall et al., 1996). Similarly, Arabidopsis *atm* mutants are hyposensitive to some DNA-damaging treatments and partially sterile due to the extensive chromosome fragmentation in meiocytes (Garcia et al., 2003). It has been reported that DNA damage-induced apoptosis is a common surveillance mechanism to maintain genomic stability in many organisms (Gartner et al., 2000; Norbury and Zhivotovsky, 2004). In mice, *atm*-deficiency causes meiosis arrest during male gametogenesis followed by apoptotic degeneration (Barlow et al., 1997). Here, we isolated *pig1* mutants, an allele of Arabidopsis *atm*, showing PCD phenotype during male gametophyte development. Combined with the meiosis defects in *atm* mutants with PCD feature in *pig1* mutants, *ATM*/*PIG1* might regulate the DNA damage-induced apoptosis during male gametophyte development.

In summary, we isolated 12 *pig* mutants which are defective at various stages of male gametophyte development, exhibiting the male gametophyte-specific PCD features. This study demonstrate that PCD may play a surveillance role during the entire process of male gametophyte development.

MATERIALS AND METHODS

Plant materials, growth conditions and genetic screening

Unless otherwise indicated, the Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in this study. *atm-2* mutants (SALK_006953) were obtained from the Arabidopsis Biological Resources Center (ABRC).

The genetic screen was performed in a T-DNA mutagenized population which is generated by a pER16 vector containing kanamycin resistance gene (Zuo et al., 2000). Approximately 20,000 independent T2 families were screened for male gametophytic mutants on the basis of their distorted segregation ratios of kanamycin resistance. Seeds were surface-sterilized in 10% sodium hypochlorite for 10 min, washed thoroughly with sterile water and then evenly distributed on plates containing 1/2 Murashige-Skoog (MS) agar (1/2 × MS salts, 1% sucrose, 0.8% agar) supplemented with 50 mg/mL kanamycin (Murashige and Skoog, 1962). Plates were sealed with surgical type, placed at 4°C in the dark for two days, and then

transferred to continuous fluorescent white light. After two weeks, the kanamycin phenotype (resistance or sensitive) was scored and kanamycin resistance seedlings were transferred to pots containing vermiculite saturated with Gamborg's B5 liquid medium. Plants were grown under a 16 h light/8 h dark cycle at 22°C and seeds were collected from T2 individuals.

Reciprocal crosses and genetic analysis of male gametophytic mutants were performed essentially as previously described (Howden et al., 1998).

Alexander test and DAPI (4',6-diamidino-2-phenylindole) staining

Alexander staining was performed as previously described (Alexander, 1969). Released pollen grains were directly immersed in Alexander solution, and then were observed under a light microscope (Olympus BX51). DAPI staining was carried out following the procedures described in (Regan and Moffatt, 1990). For whole-mount DAPI staining, pollen nuclei were stained with 1 mg/mL DAPI for 5 min at room temperature, and then mounted temporarily on a slide with 50% glycerol in PBS (phosphate-buffered saline) buffer. Samples for semi-thin section were fixed in formaldehyde acetic acid (FAA) overnight. After dehydration in gradual ethanol series, the samples were embedded in historesin (Leica). Semi-thin sections (3 μm) were stained with 1 mg/mL DAPI for 5 min. Pollen viability after DAPI staining was analyzed under a fluorescent microscope (Olympus BX51).

Whole-mount TUNEL (TdT-Mediated dUTP Nick End Labeling) assay for pollen grains

For TUNEL assay, pollen grains were fixed in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) at 4°C overnight. After PBS washing, pollen grains were dehydrated through a graded methanol series (30%, 50%, 70%, 100%, and 100%) and 100% xylene with 30 min incubation for each step, and then rehydrated through a series of methanol (85%, 70%, 50%, 30%, 10% and water) with 10 min incubation for each step. Pollen grains were digested with 20 μg/mL proteinase K at 37°C for 35 min and subsequently washed twice with 2 mg/mL glycine in PBS. The TUNEL assay was performed using the *In Situ* Cell Death Detection Kit-Fluorescein (Roche Diagnostics Hong Kong). After TUNEL staining, pollen grains were stained with 1 mg/mL DAPI for 5 min at room temperature. Images were taken using a confocal microscope (Olympus FV1000).

Map-based cloning of *pig1*

An F2 mapping population was generated by crossing *pig1* (Col-0) with wild type L*er* plants. The *PIG1* gene were mapped by using simple sequence length polymorphisms (SSLP) markers and fine mapped using specific markers (Supplementary Table 1).

RNA isolation and RT-PCR

Total RNA was extracted from flowers of wild type and *pig1* mutants by RNase easy kit (Invitrogen) according to the manufacturer's instructions. RT-PCR was carried out as previously described with modification (Zuo et al., 2000). The reaction was cycled at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, 28–30 times.

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