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RA68 is required for postmeiotic pollen development in *Oryza sativa*

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Abstract Postmeiotic development is a unique characteristic of flowering plants. During the development, microspores undergo two cycles of mitosis (PMI and PMII) and a subsequent maturation process to finally produce the mature pollen, but the mechanism underlying the development is still largely unknown. Here, we report on the roles of a novel gene, *RA68*, in postmeiotic pollen development in *Oryza sativa*. *RA68* was expressed preferentially in shoots and flowers. In flowers, the transcript persisted from the floral organ differentiation to the mature pollen stages and showed preferential accumulation in male meiocytes, developing pollen and tapetal cells. RA68-deficient RNAi lines showed reduced seed setting and pollen viability but not an aberrant phenotype in vegetative organs. Knockdown of *RA68* led to arrested PMI, smaller pollen grains with little or no starch, and aborted pollen but not severely distruped male meiosis. Additionally, no abnormality of anther wall development was observed in RA68-RNAi lines. RA68 may be required for postmeiotic pollen development by affecting PMI and starch accumulation.

Keywords *RA68* · Tapetum · Pollen · Postmeiosis · *Oryza sativa*

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Introduction

Pollen development is an important process in the plant life cycle and directly determines sexual reproduction. As well, pollen development relates to a series of events in cell division, cell fate determination, cell polarity, and cell signaling; therefore, the male spore is an excellent biological microcosm to study cellular development (Twell 2002). In contrast to animals, in which products of meiosis differentiate directly into sperm cells, flowering plants undergo unique postmeiotic pollen development after meiosis of pollen mother cells (PMCs) to give rise to sperm cells. During postmeiotic development, microspores released from tetrads generated by meiosis become vacuolated, enlarge in size, and then undergo pollen mitosis I (PMI) and mitosis II (PMII).

PMI represents asymmetric division that generates a large vegetative cell with the dispersed nucleus and most of the cytoplasm; the small generative cell has highly condensed chromatin and little cytoplasm and is totally encased within the vegetative cell, for a unique "cell-within-a-cell structure" of bicellular spores. PMI also represents determinative division, with the resulting two cells having distinct cell fates. The vegetative cell exits the cell cycle, whereas the generative cell undergoes further symmetric PMII to generate two sperm cells. The place and time of PMII depends on the species. In the plant families of Cruciferae and Gramineae, PMII occurs during pollen maturity in the anther and mature pollen grains are tricellular, whereas in other plant families such as Solanaceae and Liliaceae, PMII occurs at the growing pollen tube after

pollen pollination, so the mature pollen is bicellular at anthesis (Ma 2005; McCormick 1993, 2004; Singh and Bhalla 2007; Twell et al. 1998).

The sporophytic tissues of anthers surrounding spores play important roles in normal pollen development. The tapetum, the innermost layer of the anther wall, is the most important layer of the anther wall for pollen development. It directly provides essential nutrition, synthesizes required secondary metabolites, and deposits contents of pollen exine (Ma 2005; McCormick 1993; Twell 2002). Mutation in several genes expressed specifically/preferentially in tapetal cells results in aberrant structure and/or degeneration of tapetum and eventually aborted pollen. *Arabidopsis MS1*, *AMS*, and *MS2* expressed in tapetal cells are essential for normal pollen development (Aarts et al. 1997; Ito et al. 2007; Ito and Shinozaki 2002; Sorensen et al. 2003; Wilson et al. 2001). In rice, *UDT1* and *TDR* are required for tapetum development (Jung et al. 2005; Li et al. 2006), whereas *RTS* seems to directly regulate pollen development (Luo et al. 2006).

In addition, several gametophytic genes involved in pollen development in *Arabidopsis* have been identified by mutant analysis. In the *sidecar pollen* mutant, uninucleate microspores undergo additional cell division before PMI to generate two vegetative cells (Chen and McCormick 1996). The gametophytic mutant *solo pollen* is defective in nuclear division and cytokinesis at PMI and thus generates uninucleate pollen at the mature pollen stage (Twell et al. 1998). Both the *gemini pollen 1* and *gemini pollen 2* mutants show disrupted formation of the PMI cell plate and thereafter cytokinesis, which results in uninucleate pollen, partially divided pollen, and equally divided pollen (Park et al. 1998, 2004; Park and Twell 2001). *GEM1*, encoding a microtubule-associated protein MOR1, participates in the formation of phragmoplast by binding microtubules in cytokinesis (Twell et al. 2002). Likewise, *TIO*, which encodes an ortholog of the FUSED protein kinase family, plays a role in cytokinesis during PMI (Oh et al. 2005). By contrast, both the *duo1* and *duo2* mutants show arrested PMII but have no effect on PMI (Durbarry et al. 2005; Rotman et al. 2005).

Although great progress has been made in understanding the molecular regulation of postmeiotic pollen development in the dicot model plant *Arabidopsis* related to the complicated cellular events leading to functional pollen, the mechanism underlying the development is still largely unknown (Ma 2005). Only a few genes were identified to function in pollen development in rice, which is a kind of important staple food as well as an important monocot model plant. Forward and reverse genetic approaches have revealed the function of several genes in postmeiotic pollen development in rice. For example, *OsRAD21-3*, an rice orthologue of yeast *RAD21*, is required for both PMI and PMII (Tao et al. 2007).

In previous study, we separated the full-length cDNA of *RA68* (AY568677 in the DNA Databank), a flower-preferential gene, by PCR-mediated RNA subtraction hybridization in rice (Wu and Wang 2004). The single-copy gene is located in chromosome 2 and encodes a protein of 219 amino acid residues of 22.8 kDa with a putative signal peptide of 22 amino acid residues at the N terminal and several posttranslational modification sites. Domain/motif searching revealed the protein to have no significant homology with any known proteins, except 38% overall sequence similarity with *Arabidopsis* PROTODERMAL FACTOR1 (PDF1), which indicates that RA68 is a novel protein in rice (Wu and Wang 2004). Here, we reveal that the gene is required for postmeiotic pollen development in rice.

Materials and methods

Plant materials and growth conditions

Seedlings of the rice cultivar Zhonghua 10 (*Oryza sativa* L. ssp. *japonica*) were planted as described previously (Tao et al. 2007). For expression analysis, roots and shoots were collected from 6-day-old plantlets grown in a growth chamber. Leaves, sheathes, lamina joints, stems, shoot apical meristems (SAMs), panicles, and spikelets were collected from adult plants.

Semi-quantitative RT–PCR

Total RNA was isolated with use of Trizol Reagent (Invitrogen, USA). For the first-strand cDNA synthesis, 5 μg of total RNA was reversely transcribed in a 20-μl system with use of ReverTra Ace (TOYOBO, Japan), with Oligo(dT) used as a primer. PCR was performed in a 25-μl system with 2.5 μl cDNA used as a template for 30 cycles. Primer pairs for *RA68* were P1 (5′-TCT ACT ACT CAG TAT GGT GGC TCC C-3′) and P2 (5′-ATG AGC TCC AGT GCC ATC TGT GAT G-3′). The *Tubulin A* cDNA (Accession no. X91806) was amplified as an internal standard (Ding et al. 2002).

In situ hybridization

For in situ hybridization, DIG-labeled antisense and sense RNA probes for RA68 were synthesized in vitro with SP6 polymerase (Roche, Germany) with use of a cDNA fragment spanning nucleotides 364-560 of *RA68* as a template. Flowers of different stages were fixed and further processed as described (Ding et al. 2002).

RNA interference and plant transformation

For RNA interference (RNAi) construction, a 463-bp fragment was first amplified from *RA68* cDNA by use of the primer pairs P3 (5′-GGA CTA GTA CTA CTC AGT ATG GTG GCT-3′, *Spe*I) and P4 (5′-GAG AGC TCC CTG TTG GGT GGT AAA A-3′, *Sac*I) and then the primer pairs P5 (5′-GGG GTA CCA CTA CTC AGT ATG GTG GCT-3′, *Kpn*I) and P6 (5′-GAA GAT CTC CTG TTG GGT GGT AAA AG-3′, *Bgl*II). After enzyme digestion, the two cDNA fragments were inserted into the RNAi vector pTCK303 (Wang et al. 2004) to generate the RA68-RNAi construct pRA68i. pRA68i was introduced into *Agrobacterium tumefaciens* EHA105 and then transformed into rice calli to generate RA68-RNAi plants as described previously (Hiei et al. 1994). The seeds of the T0 or T1 generation were selected on 1/2 MS medium with 25 mg/l hygromycin B (Roche, Germany), and the surviving seedlings were transferred to soil as the T1 or T2 generation, respectively.

PCR identification and Southern blotting

For PCR identification, genomic DNA was extracted from transgenic lines and the wild-type by use of Edwards buffer (Edwards et al. 1991). The primer pairs P3 and P7 (5′-GCG GGA CTC TAA TCA TAA AAA CC-3′, located in the NOS terminator of T-DNA), P8 (5′-ATG CTC TAA CCT TGA GTA CCT ATC-3′, located in the ubiquitin promoter of T-DNA) and P6 were used to amplify the inserted sense or anti-sense *RA68* cDNA fragments.

For Southern blot analysis, genomic DNA was extracted from the PCR-positive lines by use of the CTAB method (Murray and Thompson 1980) and digested by *EcoR*I, which has no cut site in the inserted hygromycin sequence. Southern blot hybridization was as described (Zhang et al. 2006).

Pollen viability and in vitro germination

To examine pollen viability, mature pollen grains were spread from anthers, stained in Alexander staining solution (Alexander 1969) or 0.1% I_2-KI solution, and observed on light microscopy. Viable pollen grains were counted, and the viable pollen percentage for each line represented the mean of three independent experiments. The number of counted pollen grains was >2,000. Pollen in vitro germination was as described previously (Tao et al. 2007).

Cytology

Male meiotic spreading, DAPI staining and nuclear observation of microspores or bi- or tricellular pollen grains were as described previously (Tao et al. 2007). For nuclear observation of pollen grains, 90 spikelets from three independent plants of each line were randomly selected, and pollen grains were observed. The total number of counted pollen grains from each line was between 2,000 and 4,000.

For microscope observation of anther wall, flowers at different developmental stages were fixed at room temperature overnight in a solution of 50% ethanol, 5% acetic acid and 3.7% formaldehyde, dehydrated in a graded ethanol series to 100% ethanol, and embedded in paraffin (Sigma, USA). Ten micrometer transverse sections were cut, mounted onto poly-L-lysine-treated glass slides (Sigma, USA), and stained with 1% toluidine blue. The resultant transverse sections were observed and photographed after dehydration and mounting.

Results

Identification of sequences similar to RA68 protein

Previous study showed that RA68 had no significant homologue except for the *Arabidopsis* PDF1 (Wu and Wang 2004). Following addition of newly sequenced genomes, we reanalyzed potential sequences similar to RA68 using the full-length amino acid (AA) sequence as a query in the current National Center for Biotechnology Information (NCBI) databases. The analysis revealed 11 potential homologues of RA68 (*e* value ≦ 6E-21, identity >40%) from *Zea mays* (4 entries), *Sorghum bicolor* (2), *Oryza sativa* (2), *Arabidopsis thaliana* (1), *Ricinus communis* (1), and *Vitis vinifera* (1), respectively. Consistent to the previous result, the homologue from *Arabidopsis* was PDF1. All these sequences are predicted and functionally unknown. Further multiple sequence alignments revealed that RA68 and its homologues had conversed C-termini with highly conserved amino acid sites, while their N-termini are changeable (Fig. 1). Therefore, these proteins may represent a type of novel proteins and the conserved C-terminus may be potential function region.

Pairwise alignment analysis by use of needle program in EBI website (http://www.ebi.ac.uk/Tools/emboss/align/index.html) indicated that RA68 was more similar to sorghum XP_002451823 and maize NP_001148678 (80.6% overall sequence identity with XP_002451823 and 70.0% with NP_001148678) than to other sequences (<45% identity). Correspondingly, RA68 was organized into a branch with its homologues from maize and sorghum, and formed a clade with XP_002451823 and NP_001148678 in a phylogenetic tree (Supplemental Figure S1).

RA68 is expressed preferentially in young seedlings and throughout flower development

To investigate the function of *RA68*, we used semi-quantitative RT–PCR to analyze its expression during rice

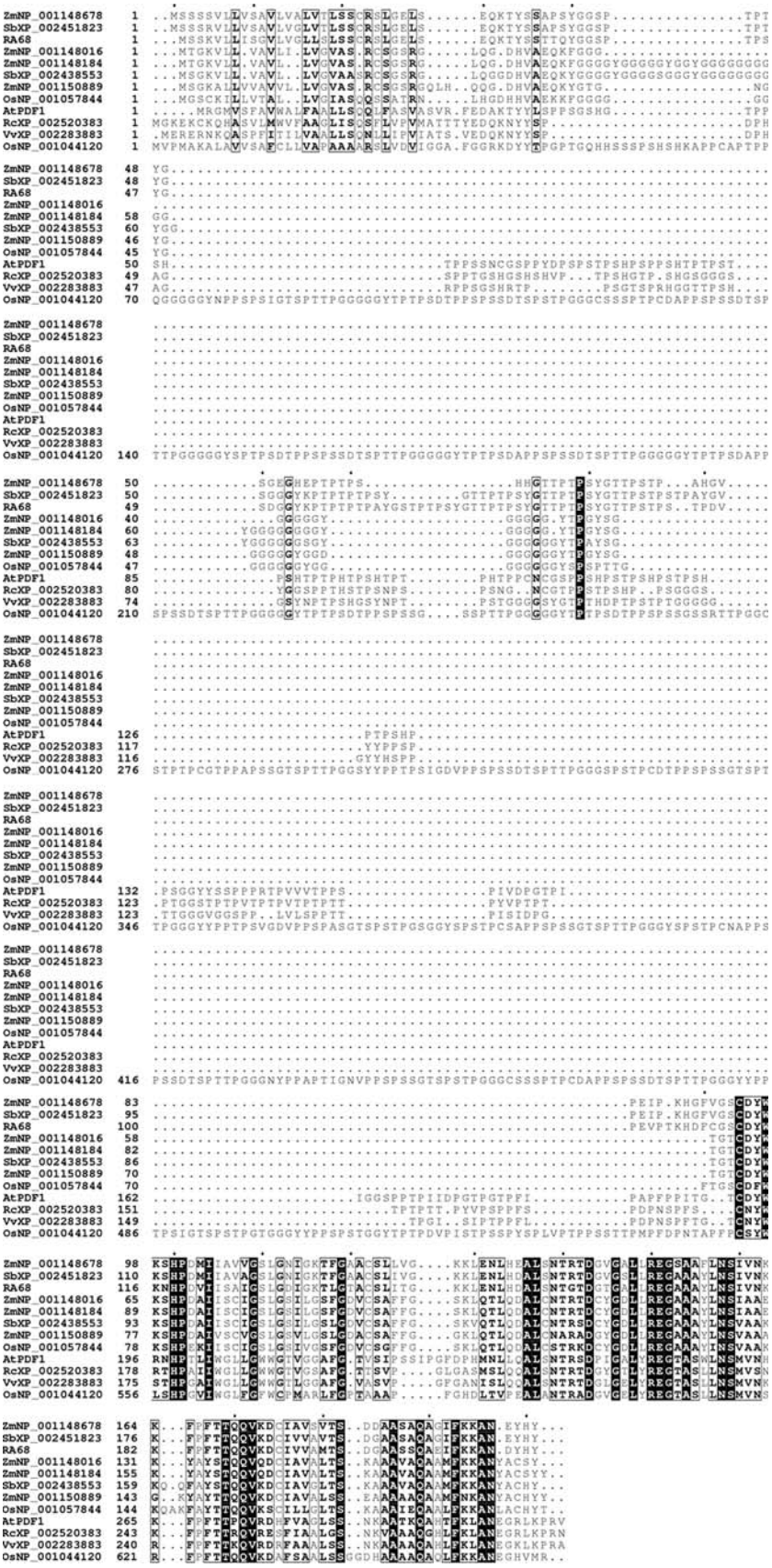

Fig. 1 Multiple alignment of full-length amino acid sequences of RA68 and its homologues using ClustalX software. *Black boxes* indicate identical residues, and *white boxes* indicate similar residues. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Rc, *Ricinus communis*; Sb, *Sorghum bicolor*; Vv, *Vitis vinifera*; Zm, *Zea mays*

development. In the vegetative phase, *RA68* mRNA was accumulated preferentially in shoots, weakly in lamina joints, stem nodes, and stem internodes, and was not detected in roots, young leaves, young sheaths, mature leaves, mature sheaths, and SAMs (Fig. 2a). Therefore, *RA68* is preferentially expressed in aerial tissues of young seedlings.

When rice entered the reproductive phase, we collected panicles and spikelets with different lengths. From the characteristics of panicle development described previously (Itoh et al. 2005), panicles to 80 mm could be divided into 4 flower developing stages: the formation of branches (panicles of 0–1 mm), differentiation of floral organs (panicles of 1–2, 2–3, 3–4, 4–10 mm), spore mother cells (panicles of 10–20, 20–30, 30–40 mm), and meiosis (panicles of 50, 80 mm). Because panicles of the early stage are tiny and vegetative tissues around the panicles may be not removed completely, to avoid the contamination of vegetative organs around the panicles, we used young leaves, young stems around the panicles and SAMs of the vegetative phase as negative controls. We collected spikelets of the uninucleate, bicellular and tricellular pollen stage after meiosis by following the division standards of Tao et al. (2007). *RA68* mRNA was detectable from the differentiation of floral organs to the tricellular pollen stage, except for the earliest stage (formation of branches), and was undetectable in negative controls (Fig. 2b). The *RA68* mRNA level was relatively high at floral organ

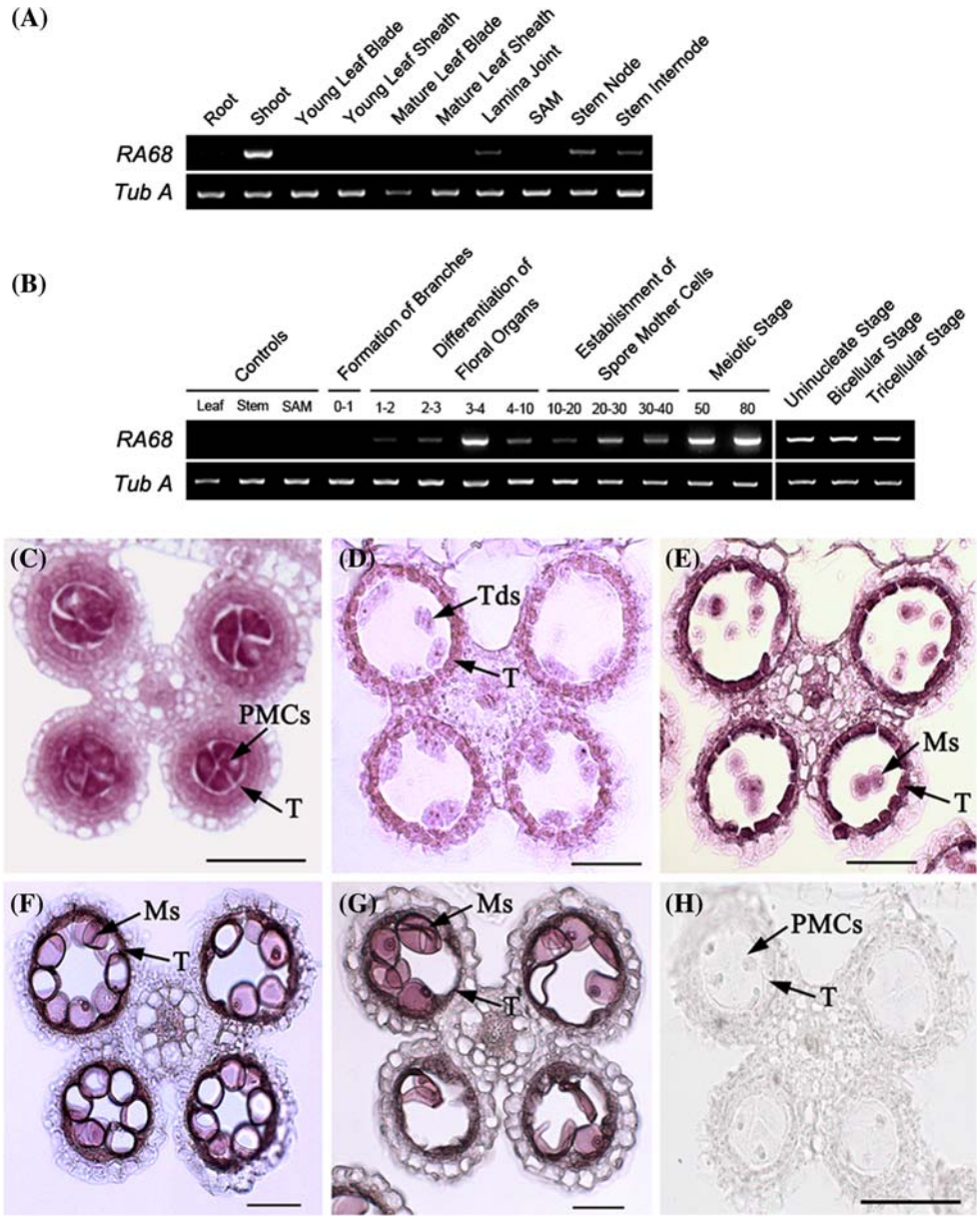

Fig. 2 *RA68* expression pattern. **a** and **b**, RT–PCR analysis of *RA68*. **a**. *RA68* mRNA accumulation in vegetative organs. SAM, shoot apical meristem. **b**. *RA68* mRNA accumulation in flowers at different stages. The numbers represent panicle length (millimeter). Leaf and stem around young panicle and SAM of the vegetative phase were used as negative controls. *Tub A* was amplified as an internal standard. **c** to **h**, In situ hybridization analysis of *RA68* mRNA. Transverse sections of flowers in meiotic PMC stage (**c**, **h**), tetrad stage (**d**). early microspore stage (**e**), late microspore stage (**f**), and bicellular pollen stage (**g**) were hybridized with DIG-labeled antisense (**c**–**g**) or sense RNA probe (**h**). PMCs, pollen mother cells; Ms, microspores; Tds, tetrads; T, tapetum. Scale bars in **c**–**h** = 50 μm

differentiation (3- to 4-mm panicles), meiotic (50- and 80-mm panicles) and postmeiotic stages (Fig. 2b).

Furthermore, we analyzed the expression pattern of *RA68* in anthers using in situ hybridization. *RA68* was expressed in meiotic PMCs (Fig. 2c), tetrads (Fig. 2d), early microspores (Fig. 2e), late microspores (Fig. 2f), and bicellular pollen (Fig. 2g). Additionally, the gene was expressed in tapetal cells until tapetum was degraded (Fig. 2c–g). Only background signals were detected by the sense probe (control) (Fig. 2h). Therefore, *RA68* expression is associated with the tapetum and developing spores.

Generation and molecular characterization of RA68-RNAi lines

We used RNAi to address the functions of *RA68*. A 463-bp fragment spanning 170-632 nt of *RA68* cDNA with no similarity to any other sequences in the rice genome was used to generate the RA68-RNAi construct. The *RA68* cDNA fragment was amplified and inserted into RNAi vector pTCK303 (Wang et al. 2004) in a sense and antisense orientation separated by a rice intron fragment and under the control of a ubiquitin promoter. We obtained 55 T0 independent transformants by introducing the resulting pRA68i construct into rice calli.

PCR analysis by use of primer pairs located in the ubiquitin promoter and the antisense sequence or in the NOS terminator and sense sequence revealed that 35 of the 55 transformants contained the insertion of both antisense and sense sequences. Furthermore, we examined the copy number of the transgene in PCR-positive lines by use of Southern blotting and found that 13 transgenic lines (L1, L3, L6, L8, L12, L15, L36, L40, L43, L46, L50, L53, and L54) had only one copy of the transgene and the other lines had two or three copies. The insertion sites differed in the 13 single-copy lines (Supplemental Figure S2).

The sterility of RA68-RNAi lines is associated with downregulation of endogenous *RA68*

The T0 generation of PCR-positive RA68-RNAi lines showed no abnormalities in vegetative growth or flower development. However, in contrast to the wild-type, which showed >90% seed-setting rate, the seed-setting rate in 26 of the 35 transgenic lines was decreased, ranging from 3% to 74%. Among the 26 lines, 21 had seed-setting rates <50% (<30% for 15 of 21 lines). T1 (Fig. 3a, b) and T2 generations (Fig. 3c) showed stable and heritable phenotypes. The seed-setting rates of the T0, T1, and T2 generations of 6 single-copy-insertion RNAi lines (L1, L3, L6, L8, L15, and L40) was largely from 30% to 50%, except for L1, which had a seed-setting rate similar to that of wild-type (about 90%) (Fig. 3d). Therefore, the 6 lines were used for further analysis.

The transcribed antisense-intron-sense sequence from the RNAi construct can form a double-stranded RNA hairpin structure and ultimately knock down the expression of a target gene in RNAi plants (Chuang and Meyerowitz 2000; Wesley et al. 2001). To investigate whether the endogenous *RA68* transcript was knocked down in RA68-RNAi lines, we used semi-quantitative RT–PCR to examine the mRNA level of endogenous *RA68* in uninucleate microspore-staged spikelets of wild-type plants and the 6 RA68-RNAi lines. The endogenous *RA68* mRNA level was downregulated in 5 (L3, L6, L8, L15, and L40) of the 6 lines, but almost unaffected in L1, as compared to that of the wild-type control (Fig. 4). Therefore, L1 was used as transgenic negative control (TNC) in further analysis, along with wild-type control. These results indicated that the downregulation of endogenous *RA68* was associated with the sterile phenotypes in the RA68-RNAi lines.

Pollen viability is reduced in RA68-RNAi lines

Pollen is a key regulator of plant fertility. Since RA68-RNAi lines showed no abnormalities in flower development and morphology, we examined pollen viability at the mature pollen stage by Alexander staining (Alexander 1969) and I_2-KI staining. On Alexander staining, more than 90% of control pollen grains were viable (97% for wild-type, $n = 2{,}560$; and 95% for TNC, $n = 2{,}195$) (Supplemental Figure S3a and S3e), with a uniform round shape (Supplemental Figure S3c). However, RA68-RNAi lines showed a high proportion of "nonviable" pollen grains (Supplemental Figure S3b and S3e), and the "nonviable" pollen grains were irregular and shrunken (Supplemental Figure S3d). On I_2-KI staining, about 90% of control pollen grains were stained dark blue-black and had regular shapes (91% for wild-type, $n = 5{,}297$; and 86% for TNC, $n = 3{,}621$) (Fig. 5a, c), whereas most pollen grains of RA68-RNAi lines were variable in shape and did not be stained or be stained little (Fig. 5b), with reduction of pollen viability percentages (Fig. 5c); these results agreed with those from Alexander staining. For example, only 30% ($n = 5{,}743$) of L8 pollen grains were viable (Fig. 5c). Furthermore, results of in vitro pollen germination assay to examine pollen viability were similar to those from pollen staining. Most mature pollen grains of control plants could germinate normally, whereas only a small proportion of mature pollen grains from RA68-RNAi lines could germinate (data not shown). Thus, pollen viability was severely reduced in RA68-RNAi lines and the sterile phenotype was related to reduced pollen viability.

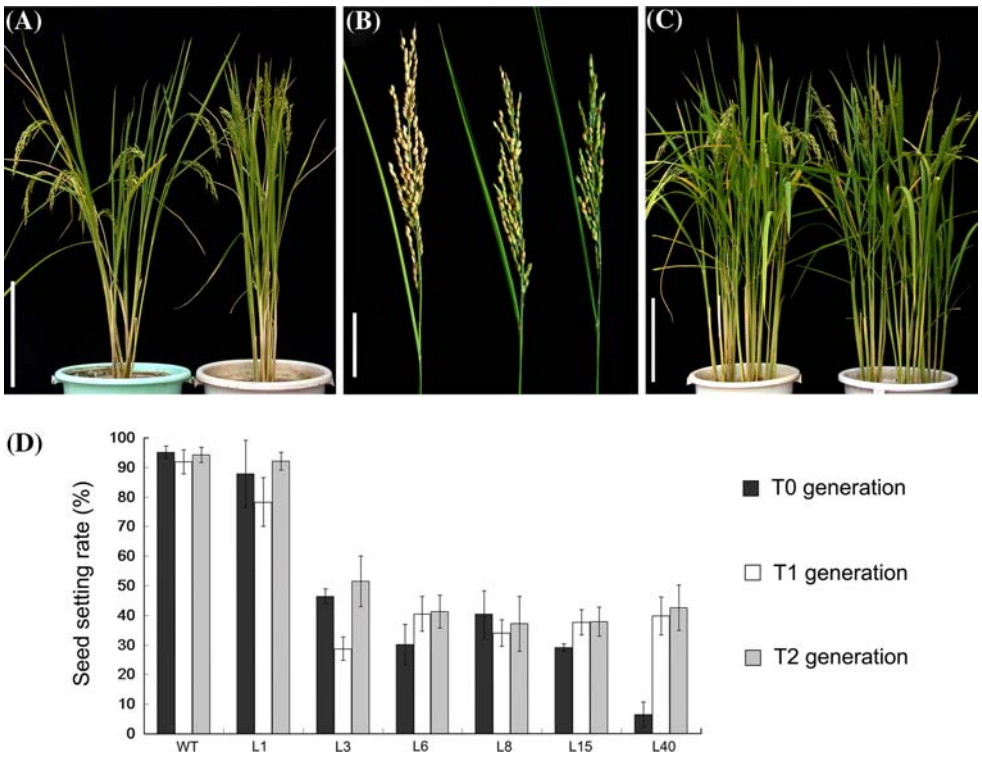

Fig. 3 RA68-RNAi lines show decreased fertility. **a** The plant morphology of wild-type (*left*) and RA68-RNAi line T1 generation (*right*). Wild-type panicles (*left*) bent as grains matured, whereas RA68-RNAi panicles (*right*) remained upright because of decreased fertility. **b** The panicle morphology of wild-type (*left*), medium sterile RA68-RNAi line (*middle*), and severely sterile RA68-RNAi line (*right*). At grain maturity, most caryopses of wild-type panicle (*left*) became yellow with grain stuffing, whereas most caryopses of RA68-RNAi panicles (*middle and right*) remained green because they were empty. **c** The plant morphology of wild-type (*left*) and RA68-RNAi line T2 generation (*right*). **d** The seed-setting rates of the T0 generation, T1 generation and T2 generation of 6 single-copy insertion RA68-RNAi lines (L1, L3, L6, L8, L15, and L40). The seed-setting rates of wild-type plants growing at the same time were also evaluated. *Scale bars* in $a = 25$ cm, $b = 5$ cm, and $c = 20$ cm

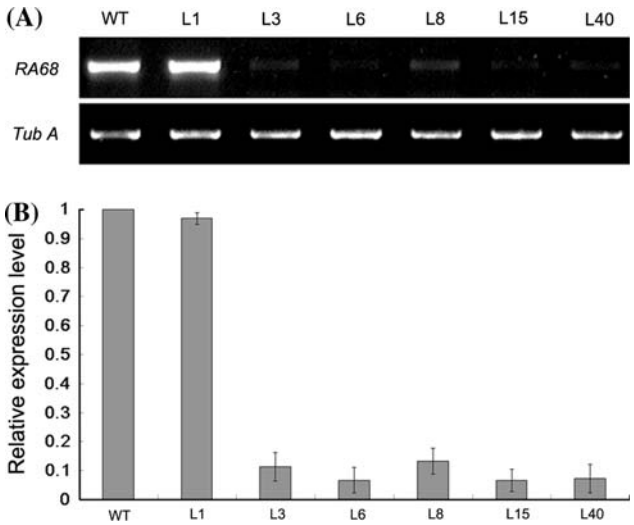

Fig. 4 The expression of endogenous *RA68* is decreased in RA68-RNAi lines. **a** Semi-quantitative RT–PCR analysis of endogenous *RA68* mRNA levels in wild-type and RA68-RNAi lines. *Tub A* was amplified as an internal standard. All PCR products were separated on 1% agarose gels. **b** The relative *RA68* mRNA levels of RA68-RNAi lines versus the wild-type quantified after normalization to *Tub A* level. Data show mean ± SD of triplicates

Male meiosis is not severely disrupted in RA68-RNAi lines

Because *RA68* is expressed at a relatively high level in flowers of the meiotic stage and downregulation of *RA68* results in reduced pollen viability, we first examined male meiosis in male meiocytes from RA68-RNAi line L6 (the line with relatively low pollen viability) and wild-type plants. In wild-type male meiocytes, homologous chromosomes accomplished pairing and synapsis from leptonema to diplonema (Fig. 6a–d) and formed 12 bivalents after condensation at diakinesis (Fig. 6e). Afterwards, highly condensed 12 bivalents aligned at metaphase I (Fig. 6k), underwent reduction division at anaphase I (Fig. 6l), and reached poles at telophase I (Fig. 6m), thus generating a dyad (Fig. 6n). After meiosis II, the two daughter cells divided simultaneously and equally to generate a tetrad (Fig. 6o).

In male meiocytes of L6, the chromosome behaviors from leptonema to diplonema were similar to that of the wild type (Fig. 6f–i). Pairing and synapsis of chromosomes did not differ from that of the wild-type. At diakinesis,

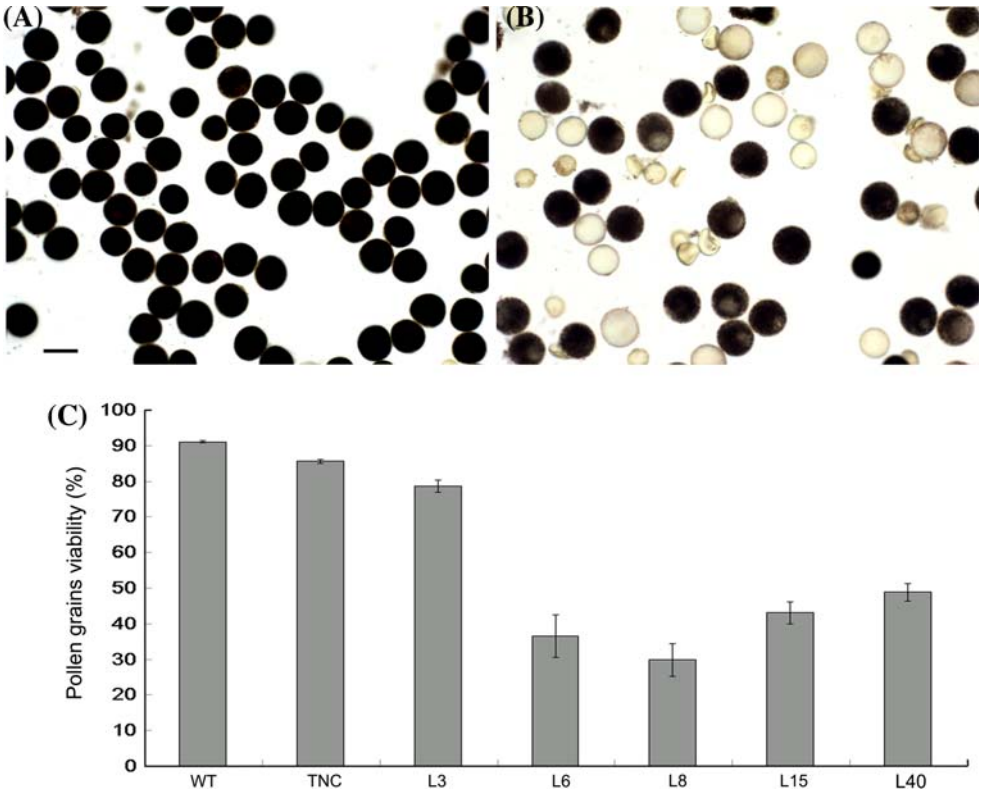

Fig. 5 I_2-KI staining showing reduced pollen viability of RA68-RNAi lines. **a** Control-plant mature pollen grains. **b** RA68-RNAi mature pollen grains. **c** The viability percentage of pollen grains at the mature pollen stage in control plants and RA68-RNAi lines. The data show mean ± SD of three independent assays. *Scale bar* in $a = 50$ μm for *b*

most male meiocytes had 12 recognizable bivalents, whereas only 13% of cells ($n = 231$) had univalents (Fig. 6j). At metaphase I, some desynapsed univalents could not align at metaphase plate (approximately 12% of cells, n = 43) (Fig. 6p). Lagging chromosomes appeared at anaphase I (Fig. 6q) and telophase I (Fig. 6r) in some male meiocytes (approximately 9% and 8%, $n = 46$ and 58, respectively), which resulted in micronuclei in some dyads (Fig. 6s). After meiosis II, a few male meiocytes produced abnormal tetrads with unequal cells (Fig. 6t). Therefore, male meiosis may not have been severely disrupted in RA68-RNAi lines, and male meiotic disruption is not the main reason for pollen abortion.

RA68-RNAi lines show aberrant pollen development after male meiosis

We further examined postmeiotic pollen development in RA68-RNAi plants and control plants on DAPI staining. In wild-type plants, uninucleate microspores released from the tetrad entered the early microspore stage (Fig. 7a). Accompanying vacuolation and enlargement in microspores, the nucleus gradually migrated to the periphery of the cell at the mid-microspore stage (Fig. 7b) and finally located at the side opposite of the aperture at the late microspore stage (Fig. 7c), where PMI took place. The late uninucleate microspore underwent PMI to generate bicellular pollen comprising a large vegetative cell with a dispersed nucleus and a small generative cell with a condensed nucleus (Fig. 7d). The generative cell then underwent PMII to generate 2 sperm cells. Pollen development ended with the production of tricellular pollen (Fig. 7e).

Most microspores of RA68-RNAi lines were indistinguishable from those of control plants at early (Fig. 7f), mid (Fig. 7g), and late microspore stage (Fig. 7h); only a few microspores were shrunken, with unstained nuclei. The percentages of normal microspores of RA68-RNAi lines at the mid and late microspore stage were 91% (L3, $n = 205$), 85% (L6, $n = 166$), 96% (L8, $n = 350$), 86% (L15, $n = 342$), and 94% (L40, $n = 315$). However, as the spore advanced to the bicellular stage, RA68-RNAi lines showed arrested uninucleate microspores (Fig. 7k, l), as well as normal bicellular pollens (Fig. 7i). At the mature pollen stage, 93% ($n = 3{,}062$) of pollen grains in wild-type plants were tricellular pollen, with only 1% uninucleate microspores, 0.03% bicellular pollen, 6% small pollen, and 0.2% aborted pollen; a similar pattern was observed in TNC plants (Fig. 7p). In contrast, a high proportion of spores showed aberrant phenotypes in RA68-RNAi lines at the same stage (Fig. 7p), including arrested mid microspore (Fig. 7k), arrested late microspore (Fig. 7l), arrested bicellular pollen (Fig. 7m), small pollen (tricellular pollen with diameter <38 μm) (Fig. 7n), and aborted pollens (nuclei-undetectable and shrunken) (Fig. 7o). For example, only 23% ($n = 3{,}601$) of L8 pollen grains were tricellular (Fig. 7j, p), whereas the remaining spores were uninucleate

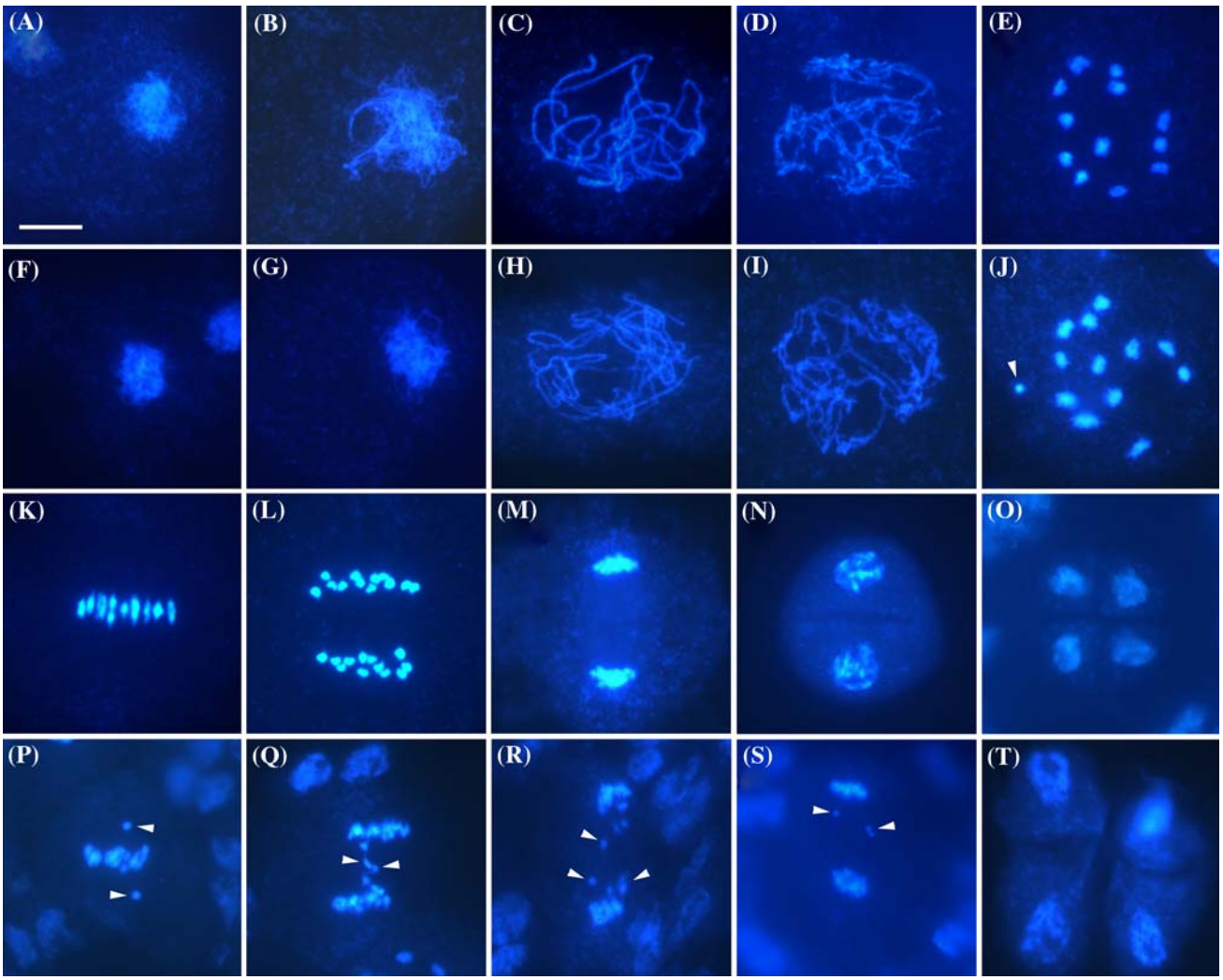

Fig. 6 Male meiosis in wild-type and RA68-RNAi plants. Male nuclear spreads were prepared from wild-type (**a**–**e**, **k**–**o**) and RA68-RNAi (**f**–**j**, **p**–**t**) plants and stained with DAPI. **a** and **f**, leptonema. **b** and **g**, zygonema. **c** and **h**, pachynema. **d** and **i**, diplonema. **e** and **j**, diakinesis. **k** and **p**, metaphase I. **l** and **q**, anaphase I. **m** and **r**, telophase I. **n** and **s**, dyad. From diakinesis to dyad, univalent (**j**, *arrowhead*), lagging chromosomes (**p**–**r**, *arrowheads*), and micronuclei (**s**, *arrowheads*) observed in some male meiocytes of RA68-RNAi lines. **o** and **t**, tetrad. Some abnormal tetrads with unequal cells (*t*) observed in RA68-RNAi line. *Scale bar* in **a** = 10 μm for **b**–**t**

(41%, Fig. 7k, l, p), bicellular (1%, Fig. 7m, p), small (28%, Fig. 7n, p), or aborted (7%, Fig. 7o, p). Most of the aberrant spores were arrested at PMI (12% in L3, $n = 2{,}831$; 38% in L6, $n = 3{,}380$; 41% in L8, $n = 3{,}601$; 40% in L15, $n = 2{,}613$; and 37% in L40, $n = 3{,}155$) or were small (28% in L3, $n = 2{,}831$; 11% in L6, $n = 3{,}380$; 28% in L8, $n = 3{,}601$; 23% in L15, $n = 2{,}613$; and 13% in L40, $n = 3{,}155$). The mean diameter of spores at the mature pollen stage was 41.7 ± 1.5 μm (range 37.6–47.5 μm, $n = 620$) in wild-type plants but decreased to 37.9 ± 3.4 μm (range 29.3–49.4 μm, $n = 364$) in RA68-RNAi lines. Therefore, the deficiency of *RA68* in the RA68-RNAi lines led to PMI arrest and defective cell expansion during pollen development.

RA68-RNAi lines show normal anther wall development

Anther wall, mainly the innermost tapetum, is considered to have a nutritive function for the developing spore (Twell 2002). During normal pollen development, shortly after microspores release from the tetrad, tapetal cells begin to degenerate. The cell components from the degenerating tapetum are also important nutrients for the growth and maturation of the spores (Wilson and Zhang 2009). In wild-type anthers, each anther locule was enclosed by innermost tapetum, middle layer, endothecium and outmost epidermis at meiotic stage (Fig. 8a). Tapetum began to degenerate at tetrad stage (Fig. 8b), and appeared thin at microspore

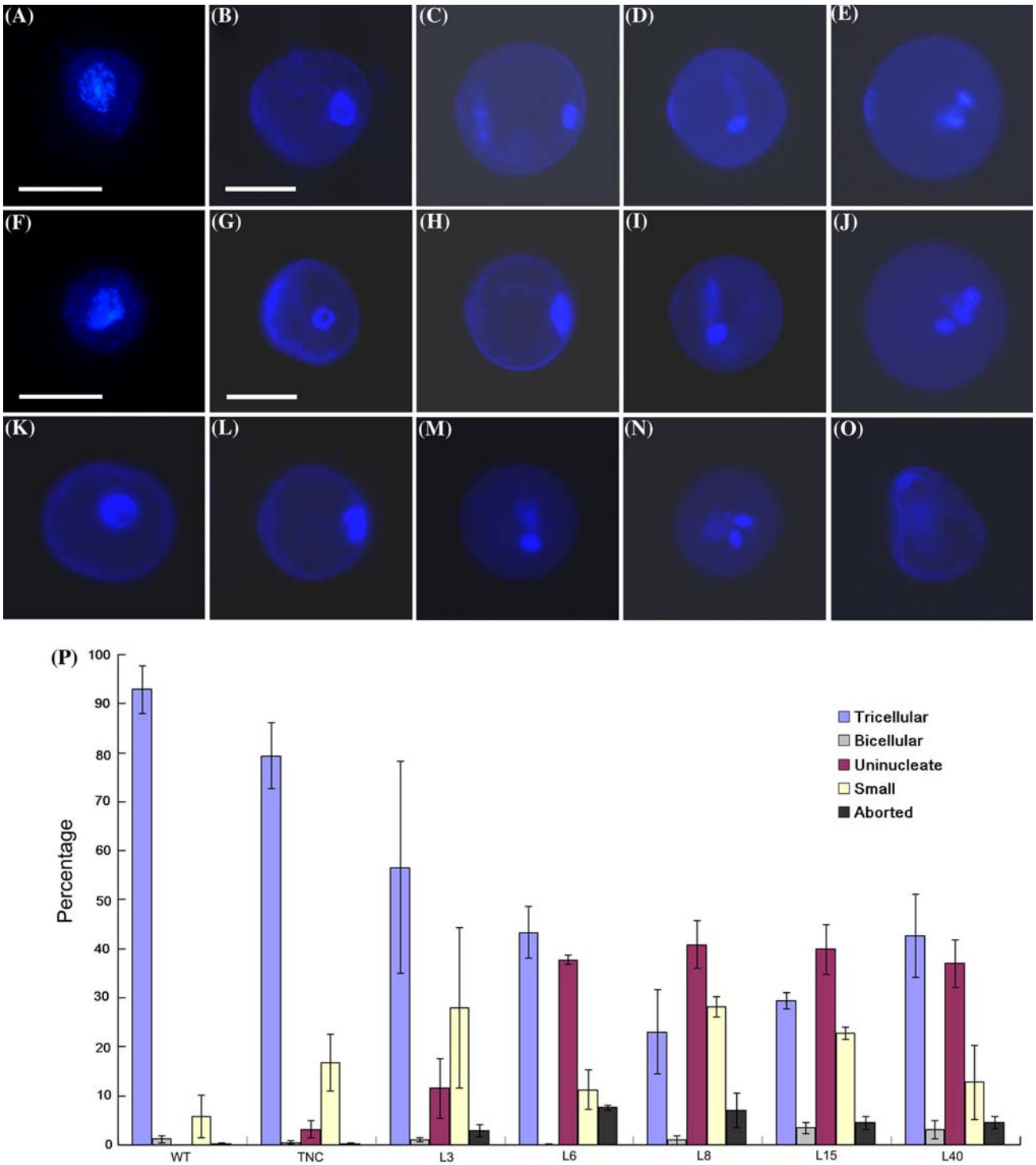

Fig. 7 RA68-RNAi lines showing abnormal pollen development. Pollen development in wild-type (**a**–**e**) and RA68-RNAi (**f**–**o**) plants by DAPI staining. **a**–**e**, wild-type pollen development. **a**, early microspore. **b**, mid microspore. **c**, late microspore. **d**, bicellular pollen. **e**, tricellular pollen. **f**–**o**, RA68-RNAi pollen development. **f**, early microspore. **g**, mid microspore. **h**, late microspore. **i**, normal bicellular pollen. **j**, normal tricellular pollen. **k**–**o**, abnormal pollen at mature pollen stage in RA68-RNAi lines. **k**, arrested mid microspore. **l**, arrested late microspore. **m**, arrested bicellular pollen. **n**, small pollen. **o**, aborted pollen. **p**, frequencies of different pollen types at mature pollen stage in control plants and RA68-RNAi lines. "Uninucleate" includes arrested mid and late microspores. The data show mean ± SD of three biological repeats. *Scale bars* = 20 μm and **b** for **c**–**e**, and **g** for **h**–**o**

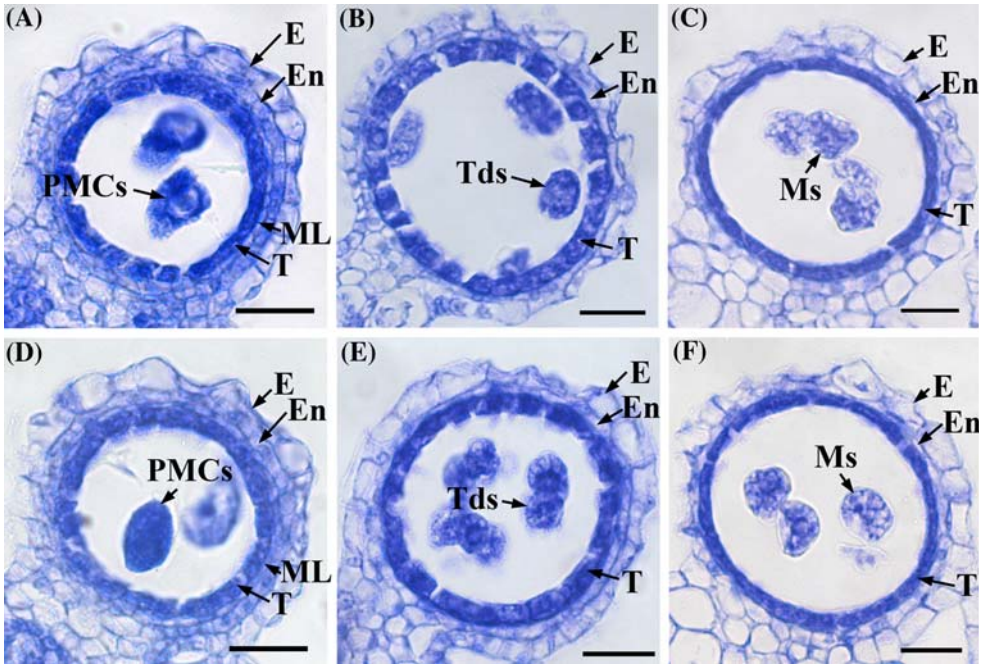

Fig. 8 RA68-RNAi lines showing no abnormality in anther wall development. Anther transsections of wild-type and RA68-RNAi lines were compared from the meiotic stage to the microspore stage. **a** to **c**, wild-type anther wall development. **d** to **f**, RA68-RNAi anther wall development. **a** and **d**. The meiotic stage. **b** and **e**. The tetrad stage. **c** and **f**. The microspore stage. E, epidermis; En, endothecium; ML, middle layer; Ms, microspores; PMCs, pollen mother cells; T, tapetum; Tds, tetrads. *Scale bar* = 20 μm

stage (Fig. 8c). Anther wall layers from RA68-RNAi lines showed cytological features similar to those from wild-type (Fig. 8d–f).

Discussion

The predicted RA68 protein has no conserved functional domains known in the present database. However, we found that the protein hit 11 functionally unknown polypeptides from different sequenced-genomes in the present database. RA68 and its homologues have conversed C-termini with highly conserved amino acid sites, whereas their N-termini are changeable. Therefore, these proteins may represent a type of novel proteins and the conserved C-terminus may be potential function region. Obviously, RA68 and its homologues from maize and sorghum can be organized into a branch, suggesting the biological function of RA68 is conserved at least in monocots.

In the vegetative development phase, *RA68* is expressed in shoots, lamina joints, and stems of rice. However, we found no obvious phenotype of vegetative development in RA68-RNAi lines as compared with the wild-type, which indicates that *RA68* is not a key gene or is redundant to the function of other genes in rice vegetative development. In the productive development phase, *RA68* is expressed throughout flower development, with a relatively high level at floral organ differentiation, meiotic and postmeiotic stages. In situ hybridization of anthers showed that *RA68* is expressed in meiotic PMCs, developing spores and tapetal cells. In general, severe disruption in male meiosis causes a marked reduction in number of pollen grains (Zhang et al. 2006); however, we did not observe a notable difference in number of pollen grains between the wild-type and RA68-RNAi lines. As well, only a few male meiocytes in RA68-RNAi lines showed an aberrant meiotic appearance. *RA68* may not play an important role in male meiosis.

Postmeiotic development of the male gametophyte involves PMI and PMII, and the fate of daughter cells is determined after PMI. PMI is a vital event for correct germ cell differentiation (Borg et al. 2009). Genetic studies of *Arabidopsis* have identified several genes required for PMI, such as *GEM1* (Park et al. 1998; Park and Twell 2001; Twell et al. 2002), *TIO* (Oh et al. 2005), and *Kinesin 12A/12B* (Lee et al. 2007), which function in phragmoplast organization to regulate asymmetric division and male germline formation (Borg et al. 2009). RA68-deficient RNAi rice showed a large accumulation of uninucleate microspores at the stage corresponding to the mature pollen stage of the wild-type control, which suggests the arrest of PMI.

Besides showing arrest in PMI, RA68-deficient RNAi lines showed substantially decreased pollen diameter. Correspondingly, I_2-KI staining showed more than half of the spores with little or no accumulation of starch. Carbohydrate metabolism plays an important role in pollen development, and starch accumulation is a significant characteristic of pollen grains in Gramineae. Starch, mainly accumulating in the vegetative cell, is the key source for generation of the carbon skeleton and energy used for spore development, germination, and pollen tube growth (Baker and Baker 1979; Clément et al. 1994; Franchi et al. 1996;

Pacini et al. 1992). The starch deficiency by disturbing carbohydrate metabolism in tapetal cells and/or developing spores leads to abnormal pollen development. For example, extracellular invertase isoenzymes cleave sucrose to monosaccharides and are important in male gametophyte development (Roitsch et al. 2003; Roitsch and González 2004). Downregulation of *Nin88*, an extracellular invertase gene in tobacco, results in changed pollen size, reduced starch accumulation in pollen, and male sterility; further analysis revealed pollen development was arrested before PMI (Goetz et al. 2001). A similar phenotype appears when extracellular invertase gene *OSINV4* is downregulated in rice (Oliver et al. 2005). Another example is *SnRK1*, which encodes a sucrose non-fermenting-1-related protein kinase. *SnRK1* antisense barley lines show blocked pollen development at the bicellular stage, with many small pollen grains containing little or no starch (Zhang et al. 2001). The tapetum is responsible for nutrition of pollen development by its secreting carbohydrates to the anther locule, and aberrant tapetum led to defect in pollen development (Goldberg et al. 1993; Mascarenhas 1990; Twell 2002). However, microscope observations of the RNAi anther sections did not detect obvious abnormality of the tapetum and other anther wall layers, suggesting that *RA68* should not be a component of the molecular network regulating tapetum development and the arrest of pollen development is not due to abnormality of anther development. Together, the lines of evidence suggest that *RA68* is required for PMI and pollen development via carbohydrate metabolism and/or sugar signals, although the biochemical mechanism of the gene function needs further study.

In summary, this work demonstrates that *RA68* is required for postmeiotic pollen development in rice. Downregulation of *RA68* results in arrest of PMI and reduced pollen size. Our results provide a novel insight into and expand our knowledge of the regulation mechanism of postmeiotic pollen development.

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