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# Cell Biological Characterization of Male Meiosis and Pollen Development in Rice

Chang-Bin CHEN[1*], Yun-Yuan XU[1], Hong MA[2] and Kang CHONG[1**]

(1. *Research Center for Molecular and Developmental Biology*, *Key Laboratory of Photosynthesis and Environmental Molecular Physiology*, *Institute of Botany*, *the Chinese Academy of Sciences*, Beijing 100093, China;
2. *Department of Biology*, *Pennsylvania State University*, *University Park*, PA 16802, USA)

**Abstract**: Little systematic analysis has been undertaken in rice (*Oryza sativa* L.) on the stages of male meiosis from leptotene to telophase II or of pollen development from microspores to mature pollen grains. The present study describes multiple stages in detail from analysis of rice chromosome spreading with staining of 4',6-diamidino-2-phenylindole. The description of normal wild-type male meiosis provides an important morphological reference for analyses of meiotic mutants. Meiosis in rice is largely similar to those of the well characterizing model plants *Arabidopsis thaliana* L. and *Zea mays* L. However, rice meiosis differs from that in *Arabidopsis* in that rice meiosis I is followed by the formation of a cell plate, instead of an organelle band that forms between the two nuclei and persist through meiosis II. This suggests a difference in the control of organelle biogenesis and distribution and cytokinesis. Our results should facilitate studies of rice meiosis and pollen development using molecular genetic and cell biological approaches.

**Key words**: chromosome; meiosis; *Oryza sativa*; pollen grains.

Since the availability of large volume of genomic sequences, rice (*Oryza sativa* L.) is becoming an increasingly popular model for comparative plant genomics (Shimamoto and Kyozuka 2002). Rice is not only an excellent model species for a major group of flowering plants, the monocotyledons, but also the most important food staple for the world's population. Rapid progress in rice genomics (Goff *et al.* 2002; Yu *et al.* 2002; Han and Xue 2003) has made it possible to undertake detailed structural and functional comparisons of genes in rice that are involved in various biological processes, as it has in the eudicotyledonous model plant *Arabidopsis thaliana* L.

Meiosis is a critical process in the life cycle of sexual plants. In rice breeding, the fertility of pollen grains and/or ovules is paramount. In the 1930s, Morinaga and his colleagues began their research on the behavior of meiotic chromosomes for rice and its related species, with a focus on the metaphase and anaphase stages. Those studies involved the use of light microscopes and resulted in hand-drawn illustrations (Morinaga and Fukushima 1937; Morinaga 1941, 1942). Relatively little progress has been made in this area of research over the subsequent three decades. Since the 1970s, more reports have appeared on meiosis of rice. Still focusing on chromosome behaviors during metaphase I, II and anaphase I, II, researchers used light microscopy and recorded their results with photographs (Hu 1970; Katayama 1977).

Genetic control of various aspects of the meiotic process has been uncovered by studies of meiotic genes, particularly in *Arabidopsis,* including *TES*

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* Present address: Department of Biology and the Huck Institutes of Life Sciences, the Pennsylvania State University, University Park, PA 16802, USA.
** Author for correspondence. Tel: +86 (0)10 6283 6225; Fax: +86 (0)10 8259 4821; E-mail: <chongk@ibcas.ac.cn>.

(Spielman *et al.* 1997), *AtDMC1* (Klimyuk and Jones 1997; Couteau *et al.* 1999), *ASK1* (Yang *et al.* 1999), *DYAD* (Siddiqi *et al.* 2000), *TAM* (Magnard *et al.* 2001), *SDS* (Azumi *et al.* 2002), *ATK1* (Chen *et al.* 2002), *MMD1* (Yang *et al.* 2003), *AtMSH4* (Higgins *et al.* 2004), *AtRAD51* (Li *et al.* 2004), *AtXRCC3* (Bleuyard and White 2004), and *AtRAD51C* (Bleuyard *et al.* 2005). In maize, the meiosis process was described as early as the 1950's (Rhoades 1950). Many meiotic mutants have been isolated and characterized and detailed cell biological studies have been conducted that revealed important insights into the control of meiosis (Golubovskaya *et al.* 2002; Pawlowski *et al.* 2003). In addition, meiotic genes are beginning to be cloned in maize (Franklin *et al.* 1999; Pawlowski *et al.* 2004; Zhang *et al.* 2005), although cloning is still rather difficult and time consuming in maize. Molecular genetic and cell biological studies of meiosis in a number of organisms, including maize and *Arabidopsis* have been extensively reviewed (Dawe 1998; Moore 2000; Kurata *et al.* 2002; Armstrong and Jones 2003; Caryl *et al.* 2003; Jones *et al.* 2003; Schwarzacher 2003; McCormick 2004; Ma 2005).

Rapid improvements have been made over the past few years in the understanding of rice chromosomes in the pachytene phase of meiosis I as a result of the introduction of fluorescence *in situ* hybridization methods and image enhancement (Peterson *et al.* 1999; Li *et al.* 2001; Cheng *et al.* 2002; Ziolkowski and Sadowski 2002; Kato *et al.* 2003; Lysak *et al.* 2003). A small number of rice meiosis genes have been studied, including *DMC1* (Ding *et al.* 2001; Kathiresan *et al.* 2002), *PAIR1* (Nonomura *et al.* 2004a), and *PAIR2* (Nonomura *et al.* 2004b). To promote rice as a model for plant functional genomics and proteomics, it is important to have a better understanding of the process of meiosis and post-meiotic development in rice. Until now, few investigations have compared the process of meiosis in rice and *Arabidopsis* (Ross *et al.* 1996). This report provides a full view of male meiosis and pollen grain development in rice compared with *Arabidopsis*.

## 1 Materials and Methods

### 1.1 Plant materials and growth conditions

The plant materials used in the present study was rice (*Oryza sativa* L. cv. Zhonghua 10), which was regenerated from rice anther cultures. The anther used for regenerating rice Zhonghua 10 was from the $F_1$ progeny of a hybridization between another hybride plant (from a cross of *O. sativa* ssp. *indica* cv. Tetep and *O. sativa* ssp. *japonica* cv. South 65) and *O. sativa* ssp. *japonica* cv. Jingfeng 15 (Li *et al.* 1990). Regenerated haploid plants were planted and naturally doubled to diploidy, with the chromosome number of 2n = 24. The resulting Zhonghua 10 line can be used for efficient regeneration from tissue culture and genetic transformation. Presently, Zhonghua 10 is an important resource for functional genomics and proteomics of rice in China, including the analysis of regulators of meiosis and pollen development. The seeds were provided by Professor Mei-Fang LI (Institute of Crop Resources, Chinese Academy of Agriculture Sciences). Rice plants were grown in an isolated experimental greenhouse. *A. thaliana* plants of the Landsberg *erecta* ecotype were grown in a plant growth chamber at 20 °C under conditions of 16 h light and 8 h dark.

### 1.2 Light microscopy

Inflorescences were collected from rice shoots of plants with less than 1 cm between their flag leaf cushion and their second-to-last leaf cushion (Fig. 1a, b) and fixed in the Carnoy's fixative (ethonal : glacial acetic acid=3 : 1) at room temperature for at least 4 h before being stored in a freezer at –20 °C.

The chromosome spreads were prepared and 4',6-diamidino-2-phenylindole (DAPI) staining was performed essentially as described previously (Ross *et al.* 1996). The fixed tissue was washed twice with water and twice with 10 mmol/L citrate buffer, pH 4.5, and digested with 0.3% cytohelicase, 0.3% cellulase, and 0.3% pectolyase in citrated buffer for 30 min at 37 °C. The tissues were then washed with citrate buffer and stored at 4 °C. Four to six digested anthers were placed

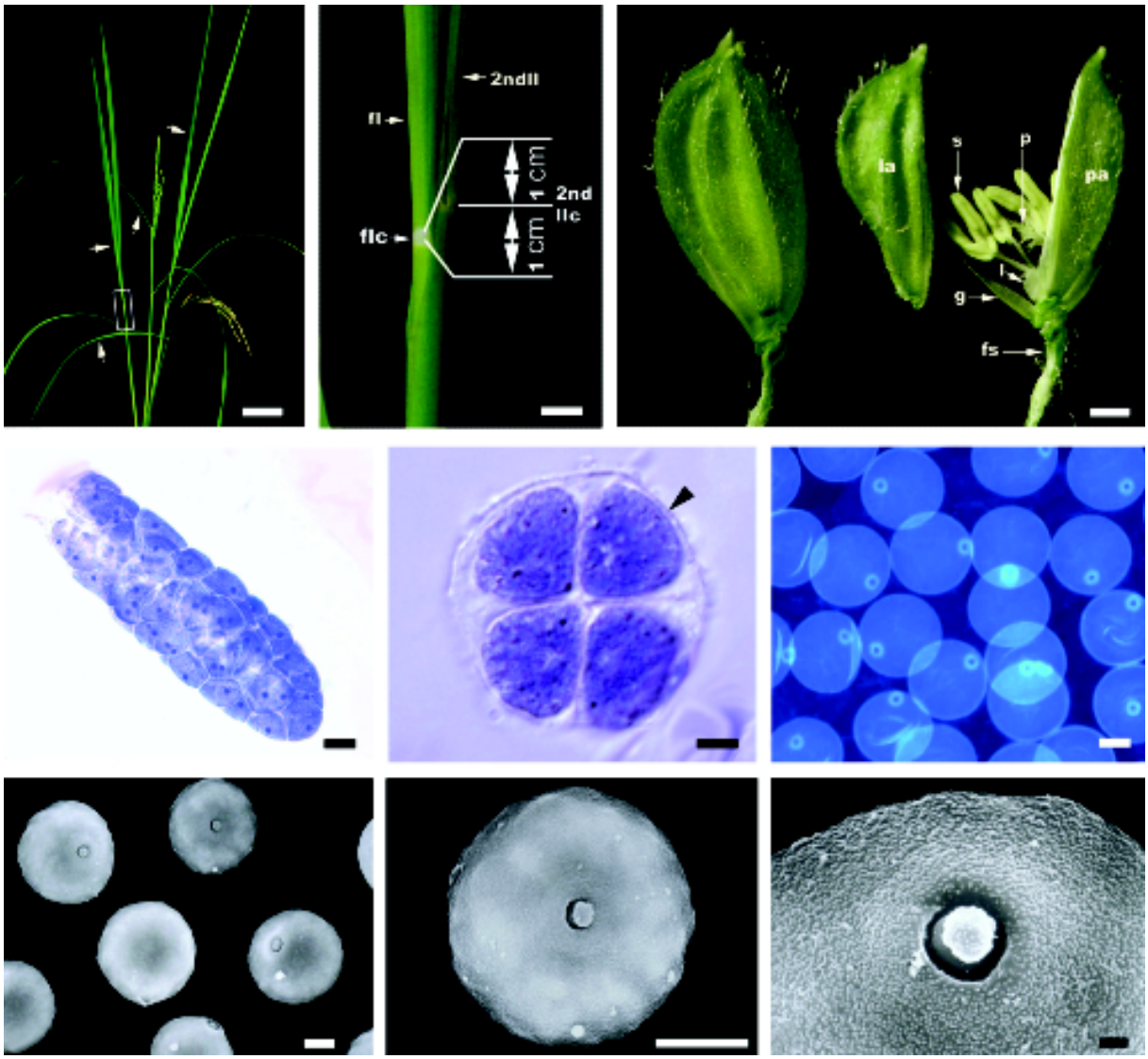



**Fig. 1.** Pollen development of rice (*Oryza sativa*). **a.** Rice plant. Each flower branch has a flag leaf (fl in (**b**)), the last leaf of the shoot (arrows). **b.** Enlargement of the framed part shown in (**a**) indicating the scale of the distance between the last leaf cushion (the flag leaf cushion (flc) and the second-to-last leaf cushion (2nd llc)). If the distance between the last leaf cushions is shorter than 1 cm, the rice flowers are in the male meiosis stage (i.e. the flag leaf cushion from the lower 1 cm to the upper 1 cm of the second leaf cushion counted backwards is the possible male meiosis stage). 2nd ll, the second-to-last leaf. **c.** Rice spikelet showing the shape of a rice spikelet (left) and dissection of the structure of a rice spikelet (right). A rice spikelet has two glumes, one lemma, one palea, two lodicules, six stamens, and two carpel gynoecium with two hairy pistils. fs, spikelet stalk; g, glume; la, lemma; pa, palea; l, lodicule; s, stamen; p, pistil. **d.** Pollen mother cells, showing a pollen mother cell cluster from one lobe of anther (further develop to a pollen sac). In each anther lobe, all pollen mother cells, derived from parietal cells by mitotic cell division, are connected to each other as one unit with a worm shape. **e.** A typical rice tetrad with four divided nuclei, packed into a transparent coat (arrow). **f.** Mature pollen grains, showing the pollen grains with a similar size, each pollen grain with one aperture. **g–i.** Morphology of pollen grains under a scanning electron microscope. **g.** A wide view, showing the pollen grains of a similar size released from one anther, all with one aperture. **h.** One pollen grain. **i.** Partial view of a pollen grain, showing the surface pattern and structure of the aperture, with a cover on the center of the aperture. Bars, 5 cm (a); 0.5 cm (b); 1 mm (c); 50 μm (d); 10 μm (e–h); 1 μm (i).

in a small drop of 60% acetic acid on a slide and pressed with another slide to release microspore mother cells (MMCs). The slides were then separated and the samples dried at room temperature for 5 min. A total of 5 μL DAPI solution (1 μg/mL DAPI in a buffer with 50% glycerol and 10 mmol/L citrate, pH 4.5) was placed onto the slide, which was then covered with a cover glass and sealed with nail polish. Slides were examined under a fluorescence microscope (Axioskop40 with HBO100; Zeiss, Oberkochen, Germany).

### 1.3 Scanning electronic microscopy

Samples were prepared as described previously (Chen *et al.* 2000). Briefly, rice spikelets were fixed in FAA (5% formaldehyde, 6% acetic acid, 62% ethanol, and 27% distilled water, v/v) at room temperature for at least 2 h (the fixed material can be stored at 4 °C for up to 6 months without changing the fixative). Fixed material was dehydrated through a graded alcohol series of 70%, 85%, 90%, and 100% ethanol for 30 min each, repeated in 100% ethanol for an additional 30 min. The spikelets were dried with liquid carbon dioxide on a Hitachi HCP-2 (Hitachi Ltd., Tokyo, Japan) critical point dryer. The anthers were carefully removed from the florets and mounted on scanning electron microscopy (SEM) stubs. Released pollen grains obtained from the anthers using a glass needle were spread on the stub. The mounted pollen grains were sputter-coated with gold SPI-MODULE Carbon Coater (Structure Probe, Inc., West Chester, PA, USA) and examined under an SEM (Hitachi S-800, Hitachi Ltd., Tokyo, Japan). Images were photographed on Shanghai 120 films (Shanghai, China). Developed films were printed on Lucky photo paper (Baoding, Hebei, China); all images were scanned using a Scanport SQ-3036 scanner (Industry, CA, USA) and edited with Adobe Photoshop v. 6.0 (Adobe Systems, Seattle, WA, USA).

## 2 Results

### 2.1 Rice meiosis and the meiotic stage of rice inflorescence

As in most flowering plants, the rice anther contains four lobes; in each lobe, the archesporial cells undergo mitotic cell division to form a cluster of microspore mother cells. Each cluster usually contains 70–90 microspore mother cells, which undergo meiosis nearly synchronously, similar to meiosis in maize. In rice and some other monocotyledons, the distance between leaf cushions seems to be correlated with the stages of flower bud development. Specifically, meiotic stages could be correlated with the distance between the leaf cushions of the flag leaf cushion and the second-to-last leaf cushion. Previously meiotic floral buds were obtained when the distance between the last two leaf cushions was shorter than 5 cm (Li and Zhang 1996). Using the knowledge of meiotic stages in other angiosperms as a guide, cells at several meiotic stages can be found in a single flower bud or a single anther because rice meiotic cells are slightly asynchronous. Our examination revealed that meiotic cells can be obtained from the rice branch when the last distance of leaf cushions is shorter than 1 cm (Fig. 1b). Rice inflorescence usually has more than 100 floral buds. The floral buds at the top portion of inflorescence develop slightly earlier than those near the base, different from the *A. thaliana* inflorescence, in which older floral buds form at the base while younger ones are generated continuously at the top. Therefore, most, if not all, meiotic stages can be obtained from a single rice inflorescence. To examine mature microspores, we collected florets before they released pollen grains.

### 2.2 Rice microspore formation and pollen development

Rice early MMCs are attached to each other and can be dissected together (Fig. 1d) from other anther tissues. Meiosis in a MMC results in the formation of a tetrad with four microspores (Fig. 1e; Yang *et al.* 2003), which are haploid. The microspore undergoes mitosis to produce a small generative cell enclosed within a large vegetative cell. In rice pollen development, a second mitosis occurs before pollen release so that the pollen is shed in a three-cell form. A mature rice pollen grain is spherical and has only one aperture (Fig. 1f–i).

### 2.3 Rice meiotic prophase I

Prophase I is the first stage of meiosis, during which

homologous chromosomes associate via pairing, synapsis and recombination. Prophase I is further divided into five different characteristic substages: leptotene, zygotene, pachytene, diplotene, and diakinesis. Preleptotene is the period of MMCs when chromosomes have just begun to condense, with only faint indications of chromosome threads (Fig. 2a).

**2.3.1 Leptotene** Leptotene is the first substage of the meiotic prophase I. At this substage, chromosomes become visible as fine threads, but homologous chromosomes have not yet associated in pairs. From leptotene through anaphase I, sister chromotids are associated along their length due to the cohesion complexes. The thread-like chromosomes are difficult to identify individually under the light microscope because of their extreme length (Fig. 2b). Many dense granules, chromomeres, resembling strings of beads, occur at irregular intervals along the length of the chromosomes (Fig. 2b). Chromomeres have their characteristic sizes and positions on each chromosome and act as crude markers for the approximate positions of genes or groups of genes and possibly function in the recognition between homologous chromosomes during pairing and synapsis.

**2.3.2 Zygotene** At zygotene, chromosomes are grouped characteristically on the side of the nucleus (Fig. 2c). The homologous chromosomes begin to align. Pairing of homologous chromosomes occurs at one or more points and gradually proceeds along the entire length in a "zipper-like" fashion. Aligned regions reveal a double structure reflecting the initial pairing of homologous chromosomes (Fig. 2c) which clearly distinguishes them from unsynapsed regions of single chromosome (Fig. 2c). At this stage, chromosomes are still very long and interwoven; it is difficult to trace entire zygotene bivalents and describe the pattern of synapses.

**2.3.3 Pachytene** At pathytene, alignment of the homologous chromosomes is complete and they are juxtaposed against each other length-wise. The homologous chromosomes should be completely synapsed. Although the synaptonemal complexes cannot be observed in these light microscopic images, they should be detected using electron microscopy. The chromosomes have contracted and thickened, allowing the twelve aligned chromosome bivalents to be traced (Fig. 2d). Each bivalent has a single extended brightly stained pericentromeric heterochromatin region, known to contain a centrally located spherical or ovoid centromere structure of less brightness (Fig. 2d). Two of the bivalents are markedly acrocentric. Both have distinct bright regions called nucleolar organizer regions (NORs) at the ends of their short arms (Fig. 2d). The two NORs are attached to nucleolus. When they are separate, one NOR was observed as larger than the other (Fig. 2d).

**2.3.4 Diplotene** The onset of the diplotene is marked by a striking change in the appearance of the bivalents (Fig. 2e). Pairing homologs begin to desynapse. In each bivalent, homologous chromosomes are no longer in close contact along their entire length. However, the homologous chromosomes in each bivalent remain in close contact at one or a few nodes, called chiasmata. A chiasma arises as a consequence of a crossover and represents an event of recombination.

**2.3.5 Diakinesis** Diakinesis is the last sub-stage of prophase I, when the bivalents are well separated from each other (Fig. 2f, g). The attached homologous chromosomes of the bivalents are highly condensed. The homologous pairs held together physically by one or more chiasmata (Fig. 2f, g). However, because of crossover interference, each bivalent has only a few (one to three) chiasmata. The highly condensed homologous chromosomes can be seen in the shape of an "X" (Fig. 2f) or an "O" (ring shape; Fig. 2f).

**2.4 Other stages of meiosis I**

**2.4.1 Metaphase I** At metaphase I, the 12 rice bivalents are aligned along the equatorial plate of the cell, and kinetochores come into contact with the microtubules of the spindle apparatus (Fig. 2h). The orientation of the centromeres among the bivalents is random with respect to the maternal and paternal chromosomes, resulting in random assortment after

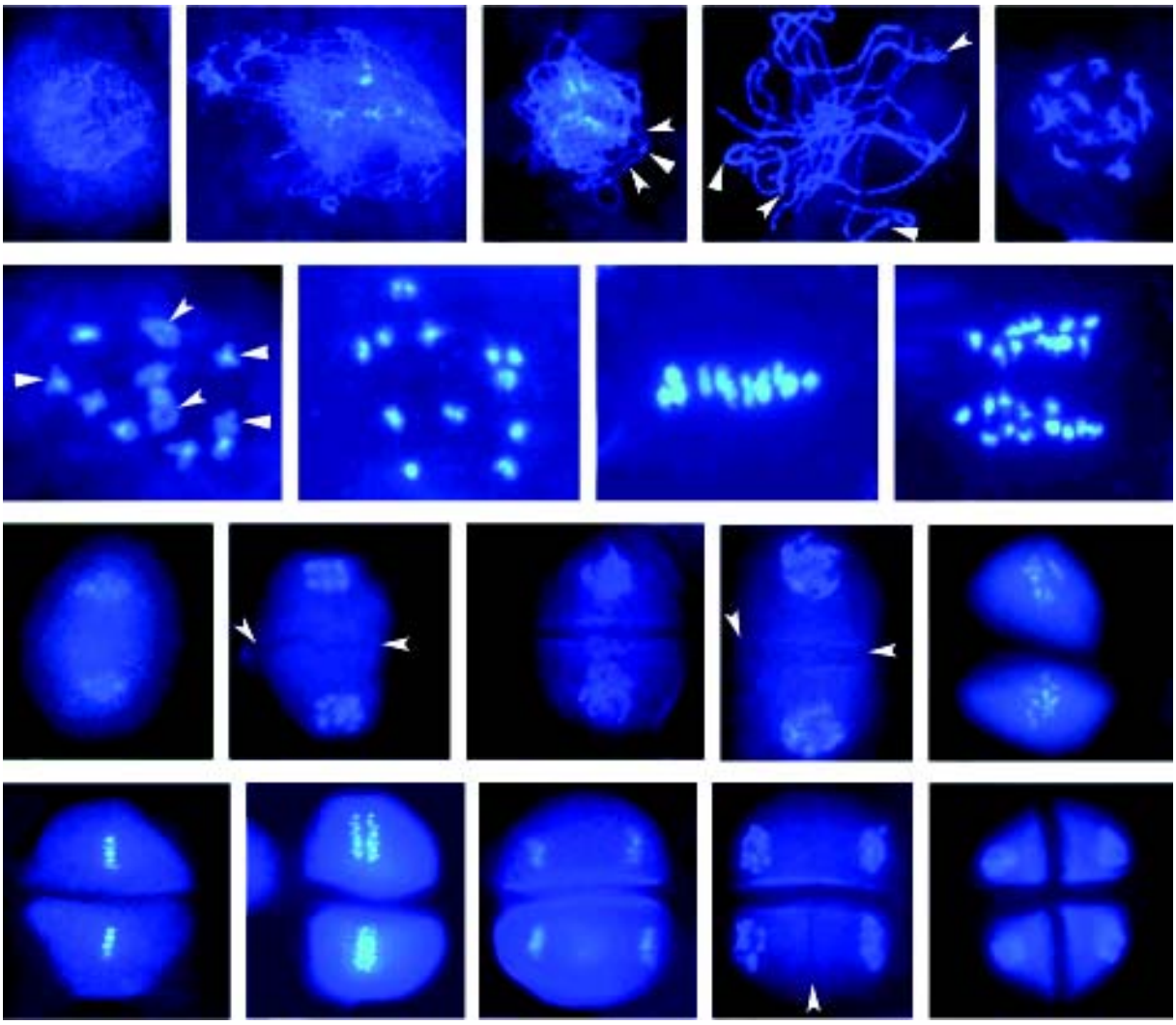


**Fig. 2.** Meiotic stages of rice (*Oryza sativa* cv. Zhonghua 10). **a.** Preleptotene, showing a relatively undifferentiated nuclear structure except for some faint indications of chromosome threads. **b.** Leptotene, showing unpaired chromosome threads (arrow). Many chromomeres are found along the chromosome like a string of beads (concave arrow). **c.** Zygotene, showing partially paired homologous chromosomes formed in a "zipper-like" fashion. Aligned regions (arrow) reveal a double-thread structure, which clearly distinguishes them from single unaligned chromosomes (concave arrow). **d.** Pachytene, showing juxtaposed homologous chromosomes into 12 bivalents. Individual bivalents are easier to identify and trace. Pericentromeric heterochromatin (arrows) and nuclear organizer region (NOR; concave arrows) are visible. **e.** Diplotene, showing partially desynapsed chromosomes. **f, g.** Diakinesis chromosomes of the bivalents are highly condensed. The bivalents are in an "X" shape (arrows) or in an "O" shape (concave arrows). **h.** Metaphase I, showing 12 maximally condensed bivalents, co-oriented at the spindle equator. **i.** Anaphase I, showing separation of the 12 chromosomes moving towards each spindle pole. **j.** Telophase I, showing two polar groups of chromosomes. **k.** Late telophase I, showing two groups of chromosomes; at the equator of the cell, a thin cell plate has formed (concave arrows). **l.** Dyad stage. **m.** Prophase II, showing two nuclei, each containing partially decondensed chromosomes. The nuclei are separated by a broad cell plate without clearly visible cytoplasmic organelles (concave arrows). **n, o.** Two views of metaphase II with two groups of condensed chromosomes at the spindle equators. **p.** Anaphase II, showing separation of chromatids towards each spindle pole. **q.** Telophase II, showing four groups of chromatids. **r.** Late telophase II, showing a thin cell plate formed (concave arrows). **s.** Tetrad of four haploid nuclei.

anaphase I.

**2.4.2 Anaphase I** Anaphase I is universally known as the stage when homologous chromosomes separate and move towards opposite poles of the spindle. The centromeres of two homologous chromosomes in each bivalent are pulled by the forces of the spindle and move away from the equator (Fig. 2i). Two groups of chromosomes, each with 12 chromosomes, are segregated from each other (Fig. 2i).

**2.4.3 Telophase I** The last stage of meiosis I begins with the formation of a nuclear membrane around each group of chromosomes that has already moved to the pole of the spindle (Fig. 2j). Chromosomes have started to decondense, but the cell plate has not formed yet. One cell has two groups of chromosomes at opposite poles.

**2.4.4 The first cytokinesis and the formation of a dyad**

By the end of telophase I, a very thin cell plate has formed and appears to divide the cell into two equal cells (Fig. 2k). The dyad stage is the result of meiosis I and cell division is complete, with a thick cell plate between the two newly formed cells (Fig. 2l).

**2.5 Meiosis II**

During prophase II, each nucleus contains 12 chromosomes, which were observed as partially separated due to condensation (Fig. 2m). The chromosomes continue to condense and reach their greatest degree of contraction at metaphase II (Fig. 2n, o), coinciding with nuclear membrane and nucleolus breakdown. The individual chromosomes align in the middle of metaphase II cells, the two chromatids are attached together at centromeric, and perhaps pericentromeric, cohesion sites.

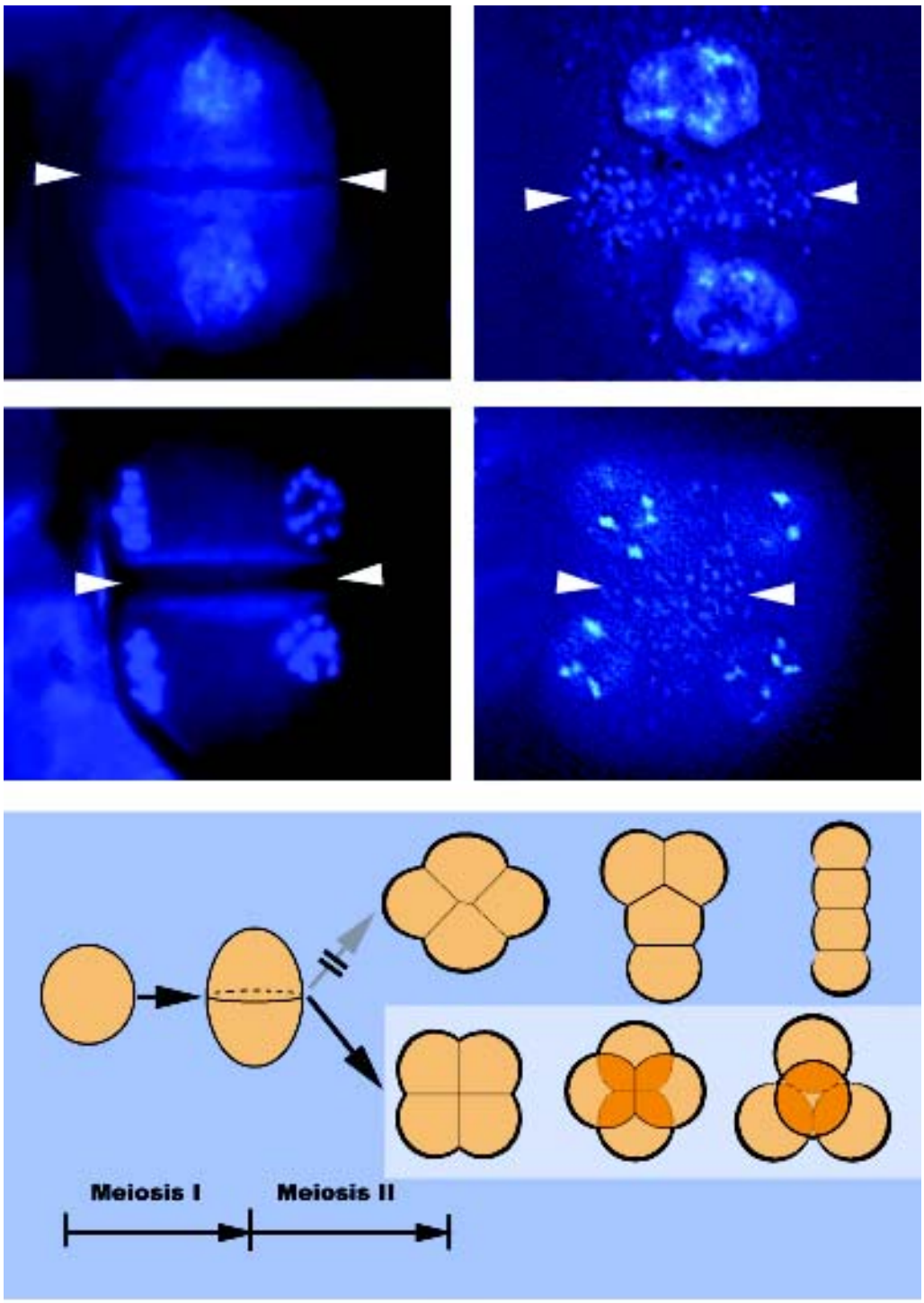
At anaphase II, sister chromotids are separated and migrate to the spindle poles (Fig. 2p), following the breakdown of chromatid cohesion at and near the centromeres. As a result, telophase II cells contain four groups of 12 newly formed chromosomes (Fig. 2q). The four groups of chromosomes decondense to form haploid interphase nuclei. The two cells often did not divide at the same moment, with one cell forming a thin cell plate slightly earlier than the other (Fig. 2r). The cytoplasm becomes partitioned by cell plates during cytokinesis, resulting in the formation of a tetrad (Fig. 2s). Subsequently, the four haploid microspores will separate from each other and continue their development into mature pollen grains.

**2.6 Comparison of rice and *A. thaliana* meiosis II**

Because *A. thaliana* is a model system for eudicotyledonous plants and has extensive genetic and genomics resource, it provides a reference for comparative studies of rice, particularly at the molecular and genomic levels. Meiosis in *A. thaliana* has been studied in recent years using a combination of cell biological and molecular genetic approaches (Caryl *et al.* 2003; Jones *et al.* 2003; Schwarzacher 2003; McCormick 2004; Ma 2005). Here, we present a brief comparison, showing some differences between these two organisms (Fig. 3). Unlike the formation of a cell plate at the end of meiosis I in rice (Fig. 3a), in *A. thaliana*, at the end of meiosis I there is a clear band of numerous organelles at the equatorial level of the dyad (Fig. 3b). The organelle band is reorganized at telophase II (Fig. 3d), before disappearing by the end of meiosis II with the formation of the tetrads. In rice, there are no obvious organelles near the cell plate (Fig. 3c). Although the formation of the cell plate requires the accumulation of membrane vesicle with cell wall materials, these vesicles are not visible because they are not likely to contain DNA.

## 3 Discussion

The purpose of presenting a morphological study of meiosis and pollen development in *O. sativa* is to provide a basic description of normal meiosis in this important experimental plant species with valuable genetic and genomic resources. *A. thaliana* serves as a model for eudicotyledonous plants; maize has a rich history of genetics and has excellent cell biology for meiosis. We believe that, with its genomic sequences and other features, rice is becoming a very good model, in addition to maize, for studying meiosis and pollen development in monocotyledonous plants.




**Figs. 3–4.** **3.** Comparison of *Oryza sativa* cv. Zhonghua 10 and *Arabidopsis thaliana*, ecotype Lansberg *erecta.* **a.** Prophase II of rice showing the broad cell plate already formed (arrows), but the cells have not divided completely. **b.** Prophase II of *Arabidopsis*, showing two nuclei separated by a band of cytoplasmic organelles (arrows). **c.** Telophase II of rice showing the cell plate (arrows). Because no organelles are visible in this area, the cell plate appears clean. **d.** Telophase II of *Arabidopsis* showing a band with cytoplasmic organelles (arrows). **4.** A model of rice meiosis process. **a.** Pollen mother cell. **b.** Dyad. **c**–**h.** Tetrads. Rice meiosis does not produce rhomboidal (c), T-shaped (d), or linear (e) tetrads. Ninety-five percent of rice tetrads are tetragonal tetrads (f) and the rest are decussate (g) or tetrahedral (h) tetrads.

Rice meiosis, like that in maize and some other plants, occurs in all flowers of the inflorescence in a short span of time, in contrast to *Arabidopsis* which has an inflorescence that produce flowers successively that undergo meiosis at different times throughout an extended period of reproductive development. The formation of cell plate in rice instead of an organelle band at the end of meiosis I may provide a way to study the reproduction and segregation of organelles. This clear organelle band occurs during meiosis in many plants, including ferns (Brown and Lemmon 2001), suggesting that it is a conserved process. The fact that rice is different from these others plants allows comparison studies of organelle regeneration and segregation.

A tetrad is a general term for a group of four united microspores. On the basis of morphology, tetrads can be divided into two types (http://www.bio.uu.nl/~palaeo/glossary/glos-tp5.htm): (i) uniplanar, with all members lying in the same plane; and (ii) multiplanar, with members in more than one plane. Uniplanar tetrads are further classified into rhomboidal (Fig. 4c), "T"-shaped (Fig. 4d), tetragonal (Fig. 4e), and linear (Fig. 4f) tetrads. Also, the multiplanar group includes a decussate (Fig. 4g) and tetrahedral (Fig. 4h) tetrad. The specific tetrad type may be related to the symmetry and/or orientation of cell division in meiosis. Up to 95% of rice tetrads are tetragonal; the remaining 5% seem to be decussate or tetrahedral tetrads. In meiosis II, each of the two dyad cells went through one cell division. Most rice yield tetragonal tetrads, with a concurrent cell separation (Fig. 4f), or a small portion of them are vertical to each other (Fig. 4g). There are no other types of tetrads generated in rice.

A mature rice pollen grain has only one germination aperture. It was reported that rice plants regenerated from tissue cultures may produce pollen grains that have two apertures (Chen 1988). In addition, a pollen grain derived from an irregular meiotic process or cell division may have two or more apertures (Chen 1988). The surface pattern and aperture number of pollen grains are formed at post-meiotic development and may be regulated by plant hormones and/or other unknown factors. The fully developed surface structure of pollen grain affects the orientation of the growing pollen tube and possibly fertility.

With the extensive recourses of genomics, rice is emerging as an excellent system for functional studies of meiosis, as *Arabidopsis* and maize have been. Further study may also be able to discover the different mechanisms of organelle biogenesis between monocots and eudicots, the two groups of flowering plants that rice and *Arabidopsis* represent.

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