Global Gene Profiling of Laser-Captured Pollen Mother Cells Indicates Molecular Pathways and Gene Subfamilies Involved in Rice Meiosis^{1[W][OA]}

Xiang Tang², Zhi-Yong Zhang², Wen-Juan Zhang, Xing-Ming Zhao, Xuan Li, Dong Zhang, Qiao-Quan Liu, and Wei-Hua Tang^{*}

National Key Laboratory of Plant Molecular Genetics (X.T., Z.-Y.Z., D.Z., W.-H.T.) and Key Laboratory of Synthetic Biology (X.L.), Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China; Key Laboratory of Plant Functional Genomics of the Ministry of Education, Yangzhou University, Yangzhou 225009, China (X.T., Q.-Q.L.); and Institute of Systems Biology, Shanghai University, Shanghai 200444, China (W.-J.Z., X.-M.Z.)

Pollen mother cells (PMCs) represent a critical early stage in plant sexual reproduction in which the stage is set for male gamete formation. Understanding the global molecular genetics of this early meiotic stage has so far been limited to whole stamen or floret transcriptome studies, but since PMCs are a discrete population of cells in developmental synchrony, they provide the potential for precise transcriptome analysis and for enhancing our understanding of the transition to meiosis. As a step toward identifying the premeiotic transcriptome, we performed microarray analysis on a homogenous population of rice (*Oryza sativa*) PMCs isolated by laser microdissection and compared them with those of tricellular pollen and seedling. Known meiotic genes, including *OsSPO11-1*, *PAIR1*, *PAIR2*, *PAIR3*, *OsDMC1*, *OsMEL1*, *OsRAD21-4*, *OsSDS*, and *ZEP1*, all showed preferential expression in PMCs. The Kyoto Encyclopedia of Genes and Genomes pathways significantly enriched in PMC-preferential genes are DNA replication and repair pathways. Our genome-wide survey showed that, in the buildup to meiosis, PMCs accumulate the molecular machinery for meiosis at the mRNA level. We identified 1,158 PMC-preferential genes and suggested candidate genes and pathways involved in meiotic recombination and meiotic cell cycle control. Regarding the developmental context for meiosis, the DEF-like, AGL2-like, and AGL6-like subclades of MADS box transcription factors are PMC-preferentially expressed, the trans-zeatin type of cytokinin might be preferentially synthesized, and the gibberellin signaling pathway is likely active in PMCs. The ubiquitin-mediated proteolysis pathway is enriched in the 127 genes that are expressed in PMCs but not in tricellular pollen or seedling.

In flowering plants, male reproductive cells develop in anthers. After differentiation from progenitor cells in the anther primordium, the sporogenous cells divide to generate a population of pollen mother cells (PMCs). More or less synchronously, PMCs enter meiosis (then called meiocytes) to produce four connected haploid microspores (tetrad), which then separate as unicellular microspores. Each microspore undergoes an asymmetric mitosis to produce the bicellular pollen, which comprises a vegetative cell and an embedded generative cell. The generative cell divides to form two sperm, and the resulting male gametophyte is called the tricellular pollen (McCormick, 1993). The male reproductive cells are surrounded by several different layers of sporophytic cells of the anther throughout their development.

Complex gene networks in both gametophytic and sporophytic tissues of the anther regulate male reproductive development. Genome-wide gene expression data in a cell type- and stage-specific manner should provide a detailed framework for modeling cell fate and male reproductive development. Previous transcriptomic studies using stamens or florets, which are composed of mixed types of cells, may identify some relevant genes based on the expressional changes among different anther development stages. For example, Lu et al. (2006) dissected the rice (Oryza sativa) stamens at various stages for microarray analysis and reported 26 genes to be preferentially up-regulated during early stamen development and therefore suggested to participate in the regulation of rice stamen development at premeiotic stages. Ma et al. (2008) performed microarray analysis on maize (Zea mays) anthers of seven developmental stages (from premeiotic to mature pollen) and reported 234 genes as premeiosis

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² These authors contributed equally to the article.

^{*} Corresponding author; e-mail whtang@sibs.ac.cn.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Wei-Hua Tang (whtang@sibs.ac.cn).

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related and 674 genes as persistent through meiosis. However, such studies cannot provide genome-wide gene expression information of a single cell type.

Laser microdissection combined with microarray enables cell type-specific expression profiling. With this tool, Suwabe et al. (2008) reported the separated transcriptomes of the male gametophyte and their surrounding tapetum during and after meiosis, but expression data from premeiotic PMCs are still lacking.

PMCs represent a key stage for transcriptome research, since only PMCs and megaspore mother cells will enter meiosis, while all other cell divisions are mitotic. A complete set of RNA transcripts in a homogenous population of cells poised to undergo meiosis holds tremendous information on how a cell prepares for meiosis.

The process of meiosis is highly conserved in all eukaryotes, but comparison of meiosis-related pathways in yeast and mouse indicates that significant diversity exists in the signal cascades that control the process of meiosis (Chu et al., 1998; Marston and Amon, 2004; Baltus et al., 2006). In maize, the requirement of a plant-specific protein, Ameiotic1 (Am1), for meiosis initiation (Pawlowski et al., 2009) also supports this idea. Thus, obtaining the premeiotic transcriptome in plants is likely to uncover novel genes and pathways. In addition, rice is an important crop, and crop traits are reassorted during meiosis. Directly studying molecular mechanisms of meiosis in rice can reduce the distance from biological knowledge to application in rice breeding.

With this goal in mind, we used laser-capture microdissection of rice (subsp. japonica) stamens to isolate PMCs and their transcripts, followed by transcriptome analysis using microarray hybridization. Using twocolor probe hybridization with Agilent 60-mer oligomicroarrays, PMC transcripts were compared with transcripts from two tissues, tricellular pollen (TCP), which comprises three nondividing cells, and seedling, which contains many mitotic dividing cells. Known meiotic genes show significantly higher expression in PMCs than in TCP or seedling. Based on the selective expression patterns and protein sequence homology, we sort out genes encoding core proteins involved in meiotic double-strand DNA break (DSB) formation and repairs, genes controlling meiotic cell cycle progression, as well as the molecular context for meiosis.

RESULTS AND DISCUSSION

Laser Microdissection and Microarray Analysis of Rice PMCs

Stamens in immature panicles of rice cv Nipponbare harbor PMCs just before entering meiosis (Fig. 1, A and B). Chromosomal staining, callose staining, and the morphology of surrounding cell layers in the anther helped to identify this specific developmental stage (Fig. 1, C–E; Supplemental Fig. S1). Using a



Figure 1. Laser microdissection of rice PMCs. A to F, 4',6-Diamino-2phenylindole solution-stained anthers and microspore/pollen of rice at various stages. A and B, Cross sections of anthers at stage 5 collected from approximately 2-mm florets, used as a sample for laser microdissection isolation. C and D, Microspore during meiosis released from anthers collected from 3- to 4-mm florets. E, Microspore at the tetrad stage released from anthers collected from approximately 5-mm florets. F, TCP from approximately 6-mm florets. G to I, Isolation of PMCs from cross sections of anthers at stage 5 by the Veritas Microdissection System. G and H, A cross section of an anther on the slide before (G) and after (H) laser capture. The orange arrow points to the cell targeted for capture. I, PMCs captured in the cap from the area shown in G. Bars = 50 μ m.

tissue preparation method optimized for the preservation of both mRNA and cytological features (Tang et al., 2006), we isolated a homogenous population of PMCs by laser-capture microdissection (Fig. 1, G-I) from cross sections of these panicles. Total RNAs of high quality were extracted from two independent biological samples of PMCs (approximately 800 per isolation; Supplemental Fig. S2A). The $poly(A^+)$ mRNA was then linearly amplified (Supplemental Fig. S2B) for microarray hybridization. We used the Agilent 44K rice genome microarray (GPL6864) to profile PMCs using TCP (Fig. 1F) and 1-week-old seedling for comparisons. The microarray covers about 95% of the currently annotated rice genes and loci. A total of 29,008 distinct rice genes have representative probes (60-mers) on the array, including 28,840 putative protein-coding genes and 168 micro-RNA (miRNA)-coding genes. The microarray data were processed based on sufficient considerations for cell type-specific transcriptome analysis to avoid data distortion (for details, see "Materials and Methods").

Verification of Microarray Data

The variation range of mRNA copy numbers of individual genes within a cell was estimated as 5 orders of magnitude (Patanjali et al., 1991). For 60-mer oligomicroarray hybridization, the mRNA concentration (which represents expression level) linearly correlates with signal intensity spanning more than 5 orders of magnitude until intensity values approach saturation (Hughes et al., 2001; Shi et al., 2006). The detection depth allows us to further group gene expression levels as low, medium, or high based on hybridization intensities. In PMCs, there are about 25% expressed genes whose log₂ intensity values are below 11 and another 25% whose log₂ intensity values are above 15. Therefore, we grouped the expression levels as low, medium, and high for the log₂ intensity value ranges 9 to 11, 11 to 15, and above 15.

We performed quantitative reverse transcription (RT)-PCR to test the expression of 13 genes using as a reference Os03g0268000, which encodes a putative protein phosphatase that is highly expressed in PMCs, TCP, and seedling. Expression levels were consistent with microarray data (Fig. 2) for medium to highly expressed genes, but some differences were seen for weakly expressed genes.

We also performed in situ hybridization for three PMC-expressed genes: *SKP1-like* (Os07g0409500), *Am1-like* (Os03g0650400), and *SPL-like* (Os01g0212500). The results confirmed their expression in PMCs (Fig. 3). *SKP1-like* (Os07g0409500) is specifically expressed in PMCs, while *Am1-like* and *SPL-like* are expressed in PMCs as well as in surrounding somatic cells.

Twenty-six genes that were preferentially up-regulated during early stamen development were suggested to participate in the regulation of rice stamen development at premeiotic stages (Lu et al., 2006). Our microarray data (Supplemental Table S1) show that all 26 genes are expressed in PMCs, and many of them (such as U78891 and AK070863) are expressed higher in PMCs than in TCP. The expression patterns of these genes from our microarray are consistent with Lu et al. (2006) and their suggested role in meiosis.

In order to estimate the absolute copy number of mRNA per cell for individual genes, we used laser microdissection to capture various numbers of PMCs (seven, 16, 36, and 41) in separate pools. Then, using plasmid DNA-containing fragments of two rice genes, Os09g0480300 and Am1-like, as a reference, we performed real-time PCR to measure the mRNA copy number per captured PMC. The average mRNA numbers were approximately 600 and 1,000, respectively. Correspondingly, the microarray intensity values for PMCs were 79,423 and 114,355 for these respective genes. Given the intensities, the sum of all PMCexpressed genes on the microarray of 455,220,997, and the linear correlation between 60-mer probe intensities and mRNA concentration, we estimated the total mRNA copy number in a single PMC to be about 3,650,000 (Supplemental Fig. S3).

Expression Patterns of PMCs Are More Similar to That of Seedling Than to That of TCP

The 60-mer oligomicroarrays can resolve gene expressional differences over 5 orders of magnitude (signal distribution ranges from 47 to 661,955 in our case, corresponding to 5.57-19.34 after log₂ transformation in Fig. 4A). The density plots of all the hybridization signals from two biological replicates of PMCs as well as the signal distribution of negative control probes show that the hybridization intensities of expressed genes and nonexpressed genes resolved to two peaks (Fig. 4, A and B; see "Materials and Methods"). At least 59% (17,196 of 29,008) of rice genes are expressed in PMCs. This percentage is higher than that in TCP (41%, 11,867 genes) but lower than that in seedling (73%, 21,310 genes). The number of expressed rice genes in TCP is slightly higher than that of expressed maize genes in mature pollen (10,539; Ma et al., 2008).

In addition, PMCs share more expressed genes with seedling (16,128 of 17,196, 94%) than with TCP (10,202 of 17,196, 59%; Fig. 4C). Combining transcriptomic data from the meiosis microspore, tetrad, unicellular pollen,

Figure 2. Validation of microarray results by real-time PCR. The values indicate the logarithm of expression ratio to the reference gene (Os03g0268000). Each value represents the mean of three biological replicates. Error bars represent st. Asterisks signify a significant difference between real-time PCR results and microarray data at the level of P < 0.05. The two lines divide the gene expression levels into three groups: high, median, and low levels.



PMC(RT-PCR) PMC(microarray) TCP(RT-PCR) TCP(microarray) seedling(RT-PCR) seedling(microarray)

Figure 3. In situ hybridization of SKP1-like (Os07g0409500), Am1like (Os03g0650400), and SPL-like (Os01g0212500) in stage 5 anthers. A, Longitudinal section of rice florets hybridized with SKP1-like antisense RNA probe. B, Cross section of rice anthers hybridized with SKP1-like antisense RNA probe. C, Longitudinal section of rice florets hybridized with SKP1-like sense RNA probe. D, A cross section hybridized with AM1-like antisense RNA probe. E, A cross section hybridized with AM1-like sense RNA probe. F, A cross section hybridized with SPL-like antisense RNA probe. G, A cross section hybridized with SPL*like* sense RNA probe. Bars = 50 μ m.



bicellular pollen, TCP, and tapetum cells at meiosis, tetrad, and unicellular stages (Hobo et al., 2008; Suwabe et al., 2008) with our microarray data, our principal component analysis shows that the transcriptome of PMCs is more similar to that of seedling than to that of pollen/microspores after meiosis (Fig. 5A). The hierarchical clustering dendrogram (Fig. 5B) shows that the seedling groups together with the premeiotic PMC, the male meiocytes, and the finishing stage of meiosis, tetrads, while the later stages of unicellular pollen, bicellular pollen, and TCP group together. These results further suggest that the cellular transcriptome is significantly changed at the end of meiosis.

Expression of DNA Replication and Repair Pathways Is Dominant in PMCs

Because PMCs are a relatively uniform cell type, it is reasonable to consider that our transcript data represent pathways operating within the same cell. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment assays can help in interpreting microarray data (Kanehisa and Goto, 2000). Among all genes present on the microarray, 1,618 have been annotated to 133 KEGG pathways. Ninety-one pathways have 10 or more genes on this microarray, a threshold we selected as containing enough pathway steps to be meaningful. While the significantly enriched pathways in PMC-expressed genes and in TCP-expressed genes are scattered among different categories, and in contrast to the fact that the enriched pathways in TCP- and seedling-preferentially expressed genes belong to metabolism, the five enriched pathways in PMC-preferentially expressed genes all belong to DNA replication and repair in the major category of genetic information processing (Table I; Supplemental Table S2). It is a reasonable conjecture that PMC would activate genes directly responsible for DNA replication and recombination in order to prepare for meiosis.



Figure 4. Summary of global gene expression levels. A, Density plot of the expression of 29,008 distinct genes in PMCs. The broken lines roughly indicate the intensity (log-transformed) ranges of the genes not expressed (below 7.5), not or very low expressed (7.5–9), low expressed (9–11), medium expressed (11–15), and high expressed (above 15). See "Materials and Methods" for detailed statistical analysis. B, Percentages of genes expressed or not expressed in PMCs, TCP, and seedling mixed tissues. C, Venn diagram illustrating the overlap in gene expression among PMCs, TCP, and seedling.



Figure 5. Principal component analysis for gene expression data of nine different cell types and corresponding hierarchical clustering for these cell types. A, Spatial distribution of the first three principal components (PCs). B, Hierarchical clustering dendrogram using Pearson correlation coefficients and the single linkage method.

Meiotic Genes Show Preferential Expression in PMCs

Then we checked the gene expression for all 12 rice meiotic genes. Figure 6 (left panel) shows their involved steps and their expression data from our microarray. Supplemental Table S3 provides the description of these genes and their expression fold changes. All, except for two, of the 12 rice meiotic genes showed higher expression in PMCs than in TCP and seedling. For example, OsSPO11-1 is thought to catalyze DSB, which initiates homologous chromosome recombination (Yu et al., 2010). OsSPO11-1 showed more than 10-fold higher expression in PMCs than in TCP or seedling. PAIR1, a coiled-coil protein, has been reported to perform an unknown function during DSB formation (Nonomura et al., 2004). PAIR1 was only expressed in PMCs, not in TCP or seedling. From a first impression, OsRAD51 (Rajanikant et al., 2008) and OsMER3/ OsRCK (Chang et al., 2009a; Wang et al., 2009) were exceptions. But RAD51 is a recombinase that functions in both meiosis and mitosis in budding yeast (Masson and West, 2001); therefore, the high expression of OsRAD51 in both PMCs and seedling is consistent with its role. OsMER3/OsRCK functions in the formation of interfering crossovers. OsMER3/OsRCK has been reported to be preferentially expressed in young flowers by RT-PCR (Chang et al., 2009a), while our microarray data based on the Os02g0617500 probe showed no expression in any tested samples. It turned out that the 60-mer probe of Os02g0617500 was not able to represent OsMER3/OsRCK because it was designed based on a previous incorrect annotation (for details, see Supplemental Table S3). The PMC-preferential expression patterns of the 10 genes are consistent with their expected role in meiosis.

At the mRNA Level, the Molecular Machinery for Meiosis Presents Prior to the Cellular Behavior of Meiosis

We define PMCs as sporogenous cells just before meiosis. The callose deposition, which blocks cytoplasmic connections between PMCs, could be considered as a landmark for meiosis initiation (McCormick, 1993). The PMCs we isolated for microarray analysis had not initiated meiosis based on their nuclear morphology (Fig. 1) and lack of callose deposition (Supplemental Fig. S1). However, DNA replication and repair pathways are enriched in genes that were preferentially expressed in PMCs, and known meiotic genes showed selective expression in PMCs. This result nicely demonstrated a long-held theory that the molecular machinery for cell behavior is put in place before the cell actually carries out the behavior.

Meiosis can be considered as a modification of mitosis with meiosis-specific additions. Many cells in growing seedling are undergoing mitosis, while TCP comprise cells that have finished cell division and probably have shut down the cell division machinery and been reprogrammed for cell fusion in fertilization. From the meiotic/mitotic point of view, it is reasonable that PMCs had more in common with seedling than with TCP at the transcriptome level (Figs. 4C and 5), although morphologically, PMCs are less similar to seedling than to TCP.

Candidate Genes Involved in DSB Formation and Repairs

The conservation of meiosis among eukaryotes allows the identification of rice meiotic genes to be initiated from candidate genes with sequence homology to characterized meiotic genes in other eukaryotes (yeast, mammals, Arabidopsis [*Arabidopsis thaliana*], and maize). However, sequence homology does not guarantee functional conservation, and in the case in which multiple rice homologs can be found for one meiotic gene, it might be difficult to pinpoint the ortholog. The expression pattern that resulted from our microarray analysis can provide another piece of information in functional characterization. The right panel of Figure 6 provides 34 candidate meiotic genes identified based on their sequence homology and PMC-preferential expression pattern from our microarray data.

For example, in the process of DSB formation and repair, maize POOR HOMOLOGOUS SYNAPSIS1 (PHS1) functions in loading the recombination machinery onto chromosomes (Pawlowski et al., 2004). The putative *OsPHS1* showed more than 30-fold higher expression in PMCs than in TCP or in seedling, which

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Theory propariod biosynthesis 40 13 5 52 2 0.45 1 20 <0.0001 1.11 Xenobiotics biodegradution (7) 2. Genetic information processing 2.1 Transcription (2) Basal transcription factors 21 21 0.066 20 0.0030 21 0.20 2 0.08 0 0 2.2 Translation (2) Ribosome 189 184 <0.0001	Carolenoid biosynthesis	17	15	0.80	0		10	0.82	2	0.03	1	0.44	20	<0.0001
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2.2 Translation (2) 189 184 <0.0001	Basal transcription factors	21	21	0.066	20	0 0030	21	0.20	2	0.08	0		0	
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DNA replication 37 36 0.050 25 0.67 36 0.28 4 0.0049 0 0 Homologous recombination 23 23 0.055 14 22 0.59 3 0.0044 0 0 Base excision repair 34 31 0.40 18 31 6 <0.0001	2.4 Replication and repair (5)			0.002		010010	15	0.10	-	0.50	0			
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Base excision repair 34 31 0.40 18 31 6 <0.0001 0 2 Mismatch repair 26 25 0.14 18 0.59 26 0.15 4 0.0002 0 0 Nucleotide excision repair 38 36 0.13 31 0.026 37 0.27 4 0.0060 0 0 3. Environmental information processing .1 .1 0.026 37 0.27 4 0.0060 0 0 3. Environmental information processing .1<	Homologous recombination	23	23	0.055	14		22	0.59	3	0.0044	0		0	
Mismatch repair 26 25 0.14 18 0.59 26 0.15 4 0.0002 0 0 Nucleotide excision repair 38 36 0.13 31 0.026 37 0.27 4 0.0060 0 0 3. Environmental information processing 3.1 Membrane transport (1)	Base excision repair	34	31	0.40	18		31		6	< 0.0001	0		2	
Nucleotide excision repair 38 36 0.13 31 0.026 37 0.27 4 0.0060 0 0 3. Environmental information processing 3.1 Membrane transport (1) 3.2 Signal transaction (1) Total in 133 KEGG pathways 1,618 1,394 1,039 1,500 48 45 255	Mismatch repair	26	25	0.14	18	0.59	26	0.15	4	0.0002	0		0	
3. Environmental information processing 3.1 Membrane transport (1) 3.2 Signal transaction (1) Total in 133 KEGG pathways 1,618 1,394 1,039 1,500 48 45 255	Nucleotide excision repair	38	36	0.13	31	0.026	37	0.27	4	0.0060	0		0	
3.1 Membrane transport (1) 3.2 Signal transaction (1) Total in 133 KEGG pathways 1,618 1,394 1,039 1,500 48 45 255	3. Environmental information processing													
3.2 Signal transaction (1) Total in 133 KEGG pathways 1,618 1,394 1,039 1,500 48 45 255	3.1 Membrane transport (1)													
Total in 133 KEGG pathways 1,618 1,394 1,039 1,500 48 45 255	3.2 Signal transaction (1)													
	Total in 133 KEGG pathways	1,618	1,394		1,039		1,500		48		45		255	

Tang et al.

Table I. KEGG pathways enriched in PMC-, TCP-, seedling-expressed/preferentially expressed genes

^a"Preferentially expressed" is defined as the intensity in the target sample is significantly higher than in any other samples (P < 0.001) and the fold change is greater than 4. ^bThe *P* values of the χ^2 test for enriched pathways are listed; those for depleted pathways are omitted. P < 0.01 is in boldface, considered as significantly enriched. ^cThe number in parentheses is the number of pathways included in this category, while names of the pathway are provided only if there is at least one enriched case. See Supplemental Table S2 for a full list.

suggests its functional conservation with maize *PHS1*. For another example, the heterotrimeric complex of replication protein A (RPA) is highly conserved and is required for multiple processes, including DNA rep-

lication, repair, and homologous recombination. Rice has three paralogs for the RPA1 subunit, three for the RPA2 subunit, one for the RPA3 subunit, and two other RPA1-like proteins. Besides the characterized

Known meiotic genes in rice				Putative meiotic genes								
1. Meiotic recombin	nation (DSB formati	on and repa	air)									
Double-strand break formatio	Gene name RAP ID OSSP011-1 OS03g0752200 (Yu et al., 2010) PAIR1 OS03g0106300 (Nonomura et al., 2004)	PMC1 PMC2 seedling1 seedling2 24,413 58,176 2,483 2,298 11,196 4,682 278 179	sity TCP1 TCP2 seedling3 seedling3 3,991 2,7 2,337 2,4 150 1 198 1	TCP2 recting4 2.736 2.439 196 187 Excision repair (BER)								
	g 			putative Os/RAD50 Os/02g0497500 9.774 18.817 98 85 putative Os/RAD50 0.502g0497500 9.774 18.817 98 85 putative Os/RAD50 0.502g0497500 9.774 18.817 98 85 putative Os/RAE11 0.504g0635900 19.629 20.527 1.431 365 putative Os/RAE11 0.504g0635900 19.629 20.527 1.431 365 putative Os/RAE11 0.504g0635900 12.250 5.194 8.99 715 putative Os/RAE11 0.510g0487300 12.250 5.194 8.99 715 methyladenine glycosylase 2.776 12.402 methyladenine glycosylase 2.778 putative Usal/S1 0.510g0487300 4.800 4.412 4.582 4.585	39,941 94 78 1,841 1,686 1,737 17,842 111 103 10,686 11,536 11,176 8,688 418 108 2,412 2,481 2,500 34,661 1,429 2,342							
Loading the recombination machinery				outative OsBRCA2 Os01g0154800 21.844 32.270 1.490 1.082 putative single- 0.01g0164200 4.373 4.004 4.381 4.529 putative single- 0.01g064200 4.380 1.590 1.590 putative single- 0.01g0642000 4.331 3.501 1.590 1.590 putative of single- 0.001g0642000 4.2331 1.385 11.301 11.807 putative of single- 0.001g08/10100 36.231 putative OsPH51 Os06g0473000 42.333 26.724 79 166 codexyribonuclease III 2.011	7,887 8,329 8,301 6,045 1,798 701 485 499 530 29,907 261 515 1,762 1,725 1,717							
Strand invasio	OsDMC1 Os11g0146800 (Kant et al., 2005) OsRAD51 Os11g0515800 (Rajanikant et al., 2008) PAIR2 Os09g0506800 (Nonomura et al., 2006) OsRPA1a Os02g0776800	66.214 50.248 1,239 974 1,502 17.544 8,323 7,924 511.402 348.628 26,109 17,730 97.733 75,841	368 2 897 9 18,074 18,4 7,966 8,4 5,370 2,1 11,321 11,2 47,426 26	286 919 (RAD51 paralog) 0s05g0121700 2.893 3.156 139 142 783 143 783 <th143< th=""></th143<>	r 34,918 1,292 1,884 6,873 7,107 8,054 123 99							
Interferin		30,631 27,614 7,447 8,770 285 257 tte) 128 121 ta) 97 98	28,710 27,2 134 1 164 1 114 1 93	27.249 11.287 11.287 10.66 homolog of AUMSHE Os05g0499300 3.876 4.645 139 97 RPA1-like Os05g0244600 111.47.4 6.13 1066 780 6.68 716 6.97 0.56 0.876.4 4.13 156 putative MSH3 Os05g024800 10.456 27.322 1.527 2.178 0.56 25.95 1.553 1.19 121 homolog of AUPTD Os05g0588200 2.055 1.777 2.303 2.811 1 </td <td>06 11,313 11,138 10 5,677 3,375 40 25,254 28,048 79 4,124 3,005 51 1,333 1,417</td>	06 11,313 11,138 10 5,677 3,375 40 25,254 28,048 79 4,124 3,005 51 1,333 1,417							
Non-crossovers Noninterfering				putative MUS81 0s01g0948100 15,632 21,288 5,316 1,993 similar to SLX4 0s11g0916300 12,681 22,685 91 75 576 588 537 501 1983 1983	BER, 1 44 1,175 1,087 04 8,974 9,820							
2. Meiotic chromo Sister chromatid cohesi Homologous chromoso pairing & synapsis	SOM <u>e structure con</u> on OsRAD21-4 Os05g0580500 (Zhang et al., 2006) PAIR3 Os10g0405500 (Yuan et al., 2009)	trol 16,247 10,715 479 457 20,266 23,277 1,816 1,743	325 83 463 44 457 33 1,160 1,1	836 440 321 1.168								
3. Meiotic cell cyc	le control OsSDS Oc03g0225200 (Chang et al., 2009a) OsMEL 1 Os03g0800200 (Nonomura et al., 2007)	11.494 4.304 246 210 323.612 214.964 4,377 2.629	99 23 174 18 1.823 1,11 1,476 1,6	Nomolog of maize Ameiotic 1 0.803g0650400 140 142 8.564 13.356 5.872 Os01g0243900 46. (Pawlowski et al.,2009) 13.255 12.018 12.250 12.888 0.507 0.504g0208400 22 183 1.164 homologs of Arabidospi 0.507g0409500 208.270 10.853 2016 620 cyclin-like F-box proteins 0.504g0208400 22 5KP1-like (Yang et al., 1999a) 0.507g0624900 1.177 1.476 97 88 (note: more in supplemental Os01g0595600 14 1.630 0.507g0625400 292.372 27.107 27.108 38.911 Os06g0685200 10 1.999a) 0.507g0625400 292.472 27.137 38.911 Os06g0685200 10 0.207g0625400 292.480 22.480 4.846 8.747 4.882 putative RPN10 (26S Os01g0141400 2 0.509g063950 32.480 14.846 8.747 4.882 putative RPN10 (26S Os01g0141400 2	579 23,978 89 81 344 170 72 71 769 16,936 86 81 220 157 102 100 107 9,093 69 65 265 172 66 60 476 7,801 112 100 149 122 100 69 387 1,930 96 177 102 75 67 68							
4. Anther develop	Ment regulation (co OsMADS16/Spw1 Os06g0712700 (Nagasawa et al., 2003) OsMADS58 Os05g0203800 (Argument at al., 2008)	ntext for me 134,498 165,622 12,694 11,654 1,436 2,260 207 200	Biosis) 2,057 43 11,405 11,9 141 1	similar to Arabidopsis Os01g0212500 6,976 3,691 769 299 432 SPLNZZ (Yang et al., 1999b) 104 82 93 83 11.922 152	32.313 1,436 79 690 285 297							
Transcription factors	(Yamaguchi et al., 2006) OsMADS3 Os01g0201700 (Yamaguchi et al., 2006) OsMADS2 Os01g088310 (Yao et al., 2008) OsMADS4 Os05g042340/ (Yao et al., 2008)	307 289 235 945 216 115 38,686 90.502 4,730 4,516 22,204 10,669 532 449	370 33 736 10 1,436 1 1,113 60 4,637 4,5 9,400 15,0 574 5	351 M α clade OsMADS72 Os03g0253400 2.082 1.675 182 606 4.594 160 169 185 15003 550 550 160 169 185	143 138							
AGL6-like clade AGL2-like clade	LTab et al., 2008) OsMADS6 (MFO) Os02g0682200 Li et al., 2010) OsMADS1 (AFO) Os03g0215400 (Prasad et al., 2005) OsMADS34 OsM390534 Os03g0753100 (Gao et al., 2010)	332 449 1,265 6,972 423 471 421 7,703 76 83 1,225 3,748 71 78	574 58 296 26 449 45 105 9 125 85 71 10	350 264 454 OsMADS17 Os04g0580700 988 2.930 76 72 454 OsMADS17 Os04g0580700 447 451 427 465 93 OsMADS5 Os06g0162800 6135 13.865 86 81 76 70 OsMADS5 Os06g0162800 138 137 136 66 OsMADS7/45 Os08g0531700 6491 174 526 1.017 1.499 224 335 64 69 64 69 66	158 185 206 211							
Receptor kinases Ligand for receptor kinases	MSP1 Os01g0917500 (Nonomura et al., 2003) OsTDL1A Os12g0472500 (Zhao et al., 2008)	54,829 40,829 3,571 3,105 80,187 36,956 1,147 705	180 18 3,109 3,20 11,189 6,5 526 5	158 colc 3.207 scal 6.521 inte	316 pr 1,000 low ile for 3,162 ansity 10,000 med 31,623 100,000 high							

Figure 6. A framework of meiotic recombination pathway genes in rice with expression data in PMCs, TCP, and seedling. The microarray data for all the listed genes were organized the same as the top gene in the left panel. The left panel lists the genes that have been functionally characterized in rice. The right panel lists the putative meiosis-related genes supported/suggested by our microarray data. References cited in Figure 6: Yang et al. (1999a, 1999b); Nagasawa et al. (2003); Nonomura et al. (2003,

RPA1a (Chang et al., 2009b), one of the RPA2 paralogs (Os06g0693300) and another RPA1-like protein (Os06g0103400) are more likely to function in meiotic recombination based on their PMC-preferential expression patterns.

The base excision repair (BER) pathway is enriched in PMC-preferential genes. BER is thought to be responsible for repairing damaged DNA with singlestrand breaks. Single-strand breaks of DNA can be processed by either short-patch (where a single nucleotide is replaced) or long-patch (where two to 10 new nucleotides are synthesized) BER. Figure 6 (right) and Supplemental Figure S4 show that of the seven genes that are significantly elevated in PMCs over TČP or seedling, six are of the long-patch type exclusively and one is common to both short- and long-patch BER. This suggests that the long-patch BER might share common molecules with meiotic recombination, such as DNA glycosylases, methyl-CpG-binding domain protein, and exodeoxyribonuclease III for DSB end processing and poly(ADP)-Rib polymerase and DNA ligase I for DSB repair.

Candidate Genes Function in Control of the Meiotic Cell Cycle

Maize Am1 has been reported to control the initiation of meiosis (Pawlowski et al., 2009). However, *Am1* has been found to be expressed highly in many tissues at the mRNA level. Given its specific function in meiosis only, one may wonder whether *Am1* transcripts are really equally abundant in all cell types whether meiotic or nonmeiotic. Os03g0650400 is a rice homolog of Am1 (overall amino acid identity of 62%; Supplemental Fig. S5A). Figure 6 (right) showed that Os03g0650400 was expressed about 8-fold higher in PMCs than in TCP or seedling. In situ hybridization results (Fig. 3D) also show that Os03g0650400 transcripts are more abundant in PMCs than in surrounding cells at anther stage 5. The expression pattern for this gene is consistent with its premeiotic function.

Cyclins appear to play a major role regulating meiosis progression (Hamant et al., 2006). There are 33 cyclin-like genes present on the microarray, and our data suggest that, besides OsSDS, which has been reported (Chang et al., 2009a), only Os03g0203800 (*OsCycD2;3*) showed significantly higher expression in PMCs than in both TCP and seedling (Supplemental Table S4).

The S-phase kinase-associated protein1 (SKP1) is an adaptor protein of the Skp1-Rbx1-Cul1-F-box protein (SCF) complex in the ubiquitin-mediated proteolysis pathway. Arabidopsis SKP1-like1 (ASK1) functions in chiasmata resolution and sister chromatid cohesion release (Yang et al., 1999b) by promoting the degradation of cyclins with the F-box (Bai et al., 1996). There are 17 homologs of Arabidopsis SKP1-like genes in rice. Our expression data show that several of them were preferentially expressed in PMCs (Fig. 6; Supplemental Table S4), and in situ hybridization results (Figs. 3, A and B) show that one SKP1-like gene (Os07g0409500) was specifically expressed in PMCs, not in surrounding somatic cells. Another SKP1-like gene (Os07g0624900) is also among the 127 genes that we identified as only expressed in PMCs and not in TCP or seedling (Supplemental Table S5). Furthermore, F-box-like proteins (18) are the most significantly enriched group within the 127 genes. Ten of these F-box-like proteins were included in the list of the rice F-box protein superfamily (Xu et al., 2009), and four of the 10 contain a FBD domain (pfam:cl11661) in addition to the N-terminal conserved F-box. Another F-box protein contains a DUF295 domain, while two more DUF295-containing proteins are expressed in PMCs but not in TCP or seedling. F-box proteins are substrate-recognition components of the SCF ubiquitin ligases.

The 127-gene list also includes two other components in the ubiquitin-mediated proteolysis pathway, Os05g0352700 (a RING domain-containing protein) and Os10g0141400 (a putative RPN10, 26S proteasome non-ATPase regulatory subunit, responsible for polyubiquitin chain binding). The deviation of a conserved polyubiquitin chain-binding motif (LAM/LALRL/V; Fu et al., 1998) in Os10g0141400 (LAETFRLA) might suggest different substrates or binding mechanisms in meiosis. The enrichment of ubiquitin-mediated protein degradation pathway genes in the 127-gene list suggests that proteolysis might also be a key mechanism that drives the events of meiosis, as it is of mitosis.

Specific Subfamilies of Transcription Factors That Were Preferentially Expressed in PMCs Might Help Provide Developmental Context for Meiosis

There are 2,384 transcription factors in 63 subfamilies in *japonica* rice according to the Database of Rice Transcription Factors (Gao et al., 2006): 1,541 (65%) of these are present on the microarray, and 908 (59%) are expressed in PMCs (Supplemental Table S7). Among PMC-expressed transcription factors, there are more highly expressed ones than weakly expressed ones (Supplemental Fig. S7).

The developmental context is required for successful completion of meiosis. For example, MADS box transcription factors, including ABC-class proteins, are important in setting up the identity of floral organs, including anther. Two transcription factors, OsMADS3 and OsMADS58, might function similarly to AGAMOUS (Yamaguchi et al., 2006). Supplemental Figure S6 shows detailed expression data of MADS

Figure 6. (Continued.)

^{2004, 2006, 2007);} Kant et al. (2005); Prasad et al. (2005); Yamaguchi et al. (2006); Zhang et al. (2006); Rajanikant et al. (2008); Yao et al. (2008); Zhao et al. (2008); Chang et al. (2009a, 2009b); Pawlowski et al. (2009); Wang et al. (2009, 2010); Yuan et al. (2009); Gao et al. (2010); Li et al. (2010); Yu et al. (2010).

transcription factors from a phylogenetic perspective. Among the 75 rice MADS proteins, 41 are present on our microarray. In the MIKC clade, members of the DEF-like, AGL2-like, and AGL6-like subclades all show higher expression in PMCs than in TCP and seedling (Fig. 6). Within the MIKC clade, OsMADS2, a member of the GLO-like subclade, and OsMADS56, a member of the TM3-like subclade, also showed PMCpreferential expression. OsMADS72, the only member of M α present on the microarray, is expressed in PMCs but not in TCP or seedling.

The Polycomb-group (Pc-G) transcription regulator family, the PHD family, the Alfin family, and C3H family transcription factors are significantly enriched in genes highly expressed in PMCs but not enriched in genes highly expressed in TCP or seedling (Supplemental Fig. S7). All 29 Pc-G proteins on the microarray are expressed in PMCs, a uniquely high level of representation among the 38 transcription factor subfamilies having 10 or more members. PMC-highly expressed Pc-G genes include Os06g0275500 (homolog of maize enhancer of zeste1) and Os12g0613200 (trithorax-like). Interestingly, the two antagonistic groups of Pc-G (repressors and activators) both are highly expressed in PMCs. Pc-G genes contain the SET domain, the catalytic domain of protein methyltransferase. All 34 genes encoding SET domain-containing proteins present on the microarray (Supplemental Table S6) are expressed in PMCs, and six of them are preferentially expressed in PMCs. Five out of the six belong to the A subclade.

Protein kinases are common components of signaling pathways that function in anther development. Among the 1,108 protein kinases on the microarray (representing 77% of the 1,429 annotated in the rice genome, based on Dardick et al. [2007]), 572 (52%) are expressed in PMCs (including 205 putative receptor kinases), and 27 (including 13 putative receptor kinases) are expressed more than 4-fold higher in PMCs than in TCP or seedling (Supplemental Table S14). A putative mitogen-activated protein kinase, Os02g0135200, was expressed more than 20-fold higher in PMCs than in TCP and seedling. From a phylogenic standpoint, the expression pattern for protein kinases varies widely among members within subclades.

miRNA-Level Control of Gene Expression Might Be Active in PMCs

OsMEL1, a germ cell-specific member of the AR-GONAUTE family, has been reported to be essential for meiosis progression (Nonomura et al., 2007). Our microarray results show that major components of miRNA biosynthesis pathways, including Argonautes, DICER-like, and RDRP, are expressed in PMCs, suggesting that miRNA-related control of gene expression might be active in PMCs (Supplemental Fig. S8A). Some 60-mer probes in our microarray (such as miR167h in Supplemental Fig. S8D) were designed to bind the stem-loop region of miRNA-encoding genes. The linear amplification pro-

cedure in our RNA preparation amplifies exclusively poly(A⁺) mRNA, so our microarray can also provide expression data for primary miRNA (pri-miRNA) but not for pre-miRNA or miRNA [which lack poly(A) tails]. pri-miRNA levels are thought to correlate well with miRNA levels. Supplemental Figure 8B lists the nine miRNAs that might be expressed in PMCs, among which miR167h is expressed in PMCs, exclusive of TCP or seedling.

A Global Snapshot of Plant Hormone Biosynthesis Pathways in PMCs

Hormone control is a key for male germ cell differentiation in mammals (Berruti, 2006). However, while plant hormones are known to regulate almost every aspect of plant development, including anther/pollen development, our knowledge on hormone regulation of meiosis is limited. Seven types of plant hormone have been implicated to function in anther/pollen development (for review, see Supplemental Table S16). Because hormones can function as both local (paracrine) and long-distance signals (Faiss et al., 1997; Blakeslee et al., 2005), it is difficult to distinguish local signal from long-distance signal in whole organ studies. Recently, Hirano et al. (2008) reported cell type-specific expression profiles of phytohormone biosynthesis and signaling genes in tapetum and microspore at meiosis and later stages. This enabled the analysis of endogenous phytohormone biosynthesis and phytohormone response in a given cell type independently of other cells. Their results indicated that the sets of genes required for the synthesis of auxin (indole-3-acetic acid) and GA (GA₄) are highly expressed in postmeiotic microspore/pollen, while genes for GA signaling are preferentially expressed in meiotic microspore and tapetum. Our microarray data allowed us to survey expression profiles of phytohormone-related genes at premeiotic PMCs. Because biosynthesis of the seven phytohormones is interconnected at the metabolism level within a cell, we show global expression patterns of genes in seven hormone biosynthetic pathways in PMCs and TCP (Fig. 7; Supplemental Fig. S9) based on the KEGG pathway map. The arrow colors indicate expression levels of the genes encoding enzymes responsible for this reaction. Among the eight hormones, the pathway genes of cytokinin show the dominant expression pattern in PMCs. The genes encoding enzymes responsible for each step for biosynthesis from the TCA cycle to cytokinins (mainly trans-zeatin type) are all expressed in PMCs. Furthermore, genes encoding proteins in the pathway that leads to the synthesis of trans-zeatin are mostly expressed higher in PMCs than in the other two samples, indicating that PMCs might synthesize trans-zeatin. Cytokinins are capable of promoting mitotic cell division. Our results suggest a localized biosynthesis of trans-zeatin during meiotic cell division.

Combining microarray data from the microspore and the surrounding tapetum cells after PMC stages



Figure 7. Plant hormone biosynthesis pathways in rice from the KEGG Web site, with colored arrows indicating the gene expression in PMCs of responsible enzymes.

(Suwabe et al., 2008) with our data, we observed that the expression of genes responsible for the last two steps of trans-zeatin synthesis (Os05g0551700 and Os07g0693500) was significantly reduced in later stages and in tapetum cells (Supplemental Fig. S9). Particularly, Os05g0551700 showed no expression or low expression in any cell type other than PMC, according to Suwabe et al. (2008) and Jiao et al. (2009). Os07g0693500 showed gradually reduced expression in rice anthers from stage 2 to stage 7, according to Lu et al. (2006). These data suggest that trans-zeatin biosynthesis might be reduced in later stages and in the surrounding tapetum.

The green arrows in Figure 7 indicate genes responsible for this reaction that are not expressed in PMCs. Expression data show that the genes encoding enzymes catalyzing the final steps in the biosynthesis of ethylene and brassinosteroids (such as OsACOs and OsDWARF/CYP85A1; D2) are not expressed in PMCs, suggesting that ethylene and brassinosteroids may not be synthesized in PMCs. However, genes encoding enzymes deactivating brassinosteroids (such as *OsBAS1L1* and *OsBAS1L2*) are expressed in PMCs, suggesting that PMCs might inactivate the brassinosteroids produced in surrounding tissues. In addition, the brassinosteroid receptors OsBRI1 and OsBAK1 as well as ethylene signaling genes (*OsETR1*, *OsEIN4*, *OsCTR1*, etc.) are highly expressed in PMCs (Supplemental Table S8), indicating that PMCs might be able to respond to environmental brassinosteroids and ethylene. In TCP, ethylene signaling genes (*OsETR1*, *OsEIN4*, *OsCTR1*, etc.) are no longer expressed, suggesting that TCP may not respond to ethylene.

The GA Signaling Pathway Might Be Active in PMCs

Figure 8 shows that genes encoding enzymes for the final cytosolic steps in GA biosynthesis (OsGA20ox and OsGA3ox) are expressed in PMCs, and some of the genes for earlier synthesis pathways (such as KAO in ER steps and CPS in plastid steps) as well as the genes encoding GA-deactivating enzymes are not expressed in PMCs. Thus, GA might be synthesized in PMCs from intermediate products. Figure 8 also shows that all the genes in the GA signaling pathway are expressed at medium to high levels in PMCs,



Figure 8. Expression of genes in the GA biosynthetic and signaling pathways. The biosynthetic pathway is modified from Yamaguchi (2008). The signaling pathway is modified from Ueguchi-Tanaka et al. (2007). The intensities are directly labeled besides the RAP identifiers in order as labeled for *GID2*. The color scale for intensities is shown at bottom left

including *SLR1*, encoding the only DELLA protein in rice, *GID1*, encoding the GA receptor, *GID2*, which encodes an F-box subunit of the SCF E3 complex that can specifically interact with phosphorylated SLR1, and *EL1*, encoding the casein kinase I that phosphorylates SLR1 (Dai and Xue, 2010). But in TCP, *SLR1* and *GID1* are no longer expressed. These expression patterns suggest that turnover of SLR1 might be active in PMCs but not in TCP. A *SLR*-like gene, *SLRL2* (Os05g0574900), was expressed in PMCs but not in tapetum (Hirano et al., 2008), TCP, or seedling (Sup-

Plant Physiol. Vol. 154, 2010

plemental Table S8). SLR1 belongs to the GRAS transcription factor family. Another GRAS family transcription factor, Os11g0139600, was also expressed in PMCs but not in TCP or seedling (Supplemental Table S5). In addition, another gene that can suppress SLR1, OsSPY, is also highly expressed in PMCs. Therefore, suppression of SLR1 to OsGAMYB is likely to be released, consistent with the high expression of OsGAMYB and two OsGAMYB-like genes in PMCs. GAMYB has been reported to regulate rice anther development (Aya et al., 2009). Combining data from Aya et al. (2009) and our microarray data, we also provide a list of 29 PMC-expressed genes (Supplemental Table S9) that contain putative MYB transcription factor recognition sites in their promoter regions. These genes might be downstream genes regulated by OsGAMYBs. In conclusion, our microarray analysis suggests that GA signaling may function in PMCs, consistent with a previous report that a *gid1* mutant showed abnormal PMCs and failed to complete meiosis to form tetrads (Aya et al., 2009).

Combining microarray data from the microspore and the surrounding tapetum cells after PMC stages (Hirano et al., 2008; Suwabe et al., 2008), we can see that *SLR1* is always expressed in tapetum, is highly to medially expressed in PMCs and tetrad, but is very lowly expressed or not expressed in unicellular microspore, bicellular microspore, or TCP. We have shown that transcriptomes of PMCs group together with tetrads but that unicellular pollen to TCP form a distinct group (Fig. 5B). Since DELLA proteins have been considered as integrators of response to multiple growth-regulatory signals (Alvey and Harberd, 2005), it might be interesting to determine whether the reduction of rice *DELLA* expression after meiosis can be responsible for the transcriptome shift at the end of meiosis.

The 1,158 PMC-Preferential Genes and 127 Genes in PMCs But Not in TCP or Seedling Are Candidates for Meiosis Regulators

The cell is the basic unit of life, not only structurally but also functionally. The entire transcriptome of a cell is a key determinant of the identity and behavior of the cell. Here, we reported the high-resolution transcriptome of an important type of cell, PMC, moving forward to comprehensively illustrate the molecular identity of PMCs and explain the cellular behavior of PMCs at the molecular level.

Our experiment provides three very different sets of microarray data, one for a cell just about to enter meiosis (PMC), one for an organism composed of three cells (a vegetative cell and two sperm) that all have stopped cell division in preparing for double fertilization (TCP), and one for a mixed tissue containing many types of cells in various stages of mitotic cell division (seedling). Although an overstatement, comparing PMCs with seedling might be considered as meiosis versus mitosis, and comparing PMCs and seedling with TCP might be considered as dividing cells versus nondividing cells. The meiosis-related genes should be enriched in the genes expressed significantly higher in PMCs than in seedling and TCP. Supplemental Table S13 provides the whole set of genes (1,158) with at least 4-fold higher expression in PMCs than in either TCP or seedling, which we defined as PMC-preferential core transcripts. The PMC-preferential core transcripts should also contain genes that have not been implicated in meiosis, which can be candidates for further functional research.

Among the 1,158 core genes, 127 genes that were expressed in PMCs, but not in seedling or TCP (Supplemental Table S5), could be more specifically meiosis related. Besides the 21 genes in the ubiquitin-mediated proteolysis pathway we already mentioned, there are five pentatricopeptide repeat motif-containing proteins (3.9%), while this family only comprises 0.88% of the rice genome. A putative histone deacetylase (HDAC; Os02g0214900) is worth mentioning. It is also known as HDA703, a class I RPD3/HDA1 family HDAC, whose expression was not detected in the leaves, stems, or roots of 7-d-old seedlings (Fu et al., 2007). Downregulation of HDA703 by artificial miRNA reduced rice fertility (Hu et al., 2009). In mammals, HDAC inhibition down-regulates homologous recombination DNA repair pathways (Kachhap et al., 2010).

Our microarray data cannot tell whether these genes are expressed in tapetum cells or other cell types during vegetative growth. For 59 out of 127 genes, the expression patterns in tapetum cells (Suwabe et al., 2008) and in 40 vegetative cell types (Jiao et al., 2009) were reported. Supplemental Table S5 also shows that nine of them are not expressed or are expressed at low levels in tapetum cells or the 40 vegetative cell types.

We also randomly selected five of 127 genes to check their expression pattern in rice immature panicles at anther stages 3, 5, and 7 and in panicles at the TCP stage, along with other organs (leaf, stem, and root) by a conventional RT-PCR assay. Our results show that none of them were expressed in young leaf, root, or stem (Supplemental Fig. S10). The expression of Os05g0484000 (a cyclin-like F-box family gene) can only be detected in panicles at anther stage 5 (harboring PMCs). The expression of Os08g0164000 and Os10g0484800 was not detected in immature panicles at anther stage 3 or in mature panicles at the TCP stage.

In sexual reproduction, premeiotic PMCs in plants are roughly equivalent to differentiating spermatagonia in mammals. Probably due to the asynchrony in meiosis entry of spermatagonia (Pellegrini et al., 2010), global transcriptome data of a single type of male germ cells at the specific stage just before entering meiosis in mammals are also lacking (Chalmel et al., 2007). The process of meiosis is highly conserved in all eukaryotes. Thus, obtaining the premeiotic transcriptome in plants might also provide hints for mammal studies.

MATERIALS AND METHODS

Plant Materials and Tissue Collection

Rice (*Oryza sativa* subsp. *japonica* 'Nipponbare') plants were grown in the greenhouse at $28^{\circ}C \pm 1^{\circ}C$ and a 13-h-day/11-h-night cycle.

According to the descriptions by Lu et al. (2006) and Chen et al. (2005) and our own observations, we collected immature panicles at developmental stage 5 for isolation of PMCs. Features used to distinguish this stage are as follows: (1) rice was grown for about 2 months, not flowering yet; (2) the distance between the last two leaf collars was within 2 cm; (3) the immature panicle length was about 2.5 cm; (4) the floret length was about 2 mm. The stage of rice anthers was further confirmed by microscopic examination of the cross sections. The cross section of stamens at this stage start to show a "butterfly" shape, and four layers of cells are visible. Staining with 4',6-diamino-2phenylindole solution (at 1 μ g mL⁻¹ concentration with 0.2% [v/v] Tween 20) of PMCs showed one nucleus per cell without visible chromosomes (Fig. 1, A and B), while in a slightly later stage (judged by floret length of 2.5–3.5 mm), these cells enter meiosis, with chromosomes visible (Fig. 1, C and D). Cell walls of PMCs have little visible callose, while meiocytes deposit callose in cell walls (Chen et al., 2007). We stained stamen sections selected with decolorized aniline blue (0.1%) and found that the majority of developing microspores had little visible callose, only a small proportion showing callose between meiocytes (Supplemental Fig. S1). With all the above, we concluded that our target cells were in the premeiotic stage.

To isolate TCP, we collected anthers from flowering panicles and released pollen by incubation and shaking in 40% (w/v) Suc. The TCP were then precipitated after centrifuging (300g, 4° C).

Whole seedlings were collected 1 week after planting in water and grown at $26^{\circ}C \pm 1^{\circ}C$ with a 14-h-day/10-h-night cycle.

Sample Preparation for Laser Microdissection

Immature panicles harboring PMCs were prepared by microwave-accelerated acetone fixation and paraffin embedding as described by Tang et al. (2006). Cross sections (8–10 µm thickness) of florets were then cut on a rotary microtome (Leica Microtome). A paraffin-tape transfer system (Instrumedics) was used to mount paraffin sections onto slides according to the manufacturer's protocol. The slides were deparaffinized twice for 5 min each in pure Histoclear II and air dried, and they were ready for laser microdissection within 1 d.

Laser-Capture Microdissection

Homogenous PMCs were isolated using a Veritas Microdissection System (Arcturus/Molecular Devices). PMCs can be distinguished from their surrounding cells in cross sections of rice florets at the selected stage. After labeling the target cells on the monitor, a fine UV laser beam was used to cut around the target PMCs to disconnect from surrounding cells, and then an infrared laser beam was targeted to the specific area of the thermoplastic polymer film of the transfer cap just above the PMCs, activating the film to fuse with the target cells. By this means, the target cells were captured by the cap and could then be transferred to RNA extraction buffer. Two biological replicates of PMCs, composed of around 800 cells each, were captured for RNA isolation.

RNA Extraction and Amplification

Total RNAs from laser-capture microdissection-derived PMCs and collected TCP were extracted with the PicoPure RNA isolation kit (Arcturus/ Molecular Devices). The integrity of the total RNA sample was evaluated on an Agilent 2100 Bioanalyzer using RNA-6000 Pico LabChips (Agilent Technologies). Only those RNA samples with a 28S:18S ribosomal RNA ratio of greater than 1 were used for further amplification (Supplemental Fig. S2A). Total RNA of seedlings was extracted directly by the Trizol reagent (Chomczynski and Sacchi, 1987).

All total RNA samples were linearly amplified using a TargetAmp tworound amino-allyl antisense RNA (aRNA) amplification kit (Epicentre Biotechnologies) with SuperScript III and SuperScript II reverse transcriptases (Invitrogen). For each amplification, approximately 1 ng of total RNA was used as starting material and about 8 μ g of amino-allyl aRNA was recovered. The quality of amplified RNA was also assessed using an Agilent 2100 Bioanalyzer, and only those with a "bell-shaped" curve with peak size above 300 nucleotides (Supplemental Fig. S2B) were used for probe labeling.

Amino-Allyl aRNA Labeling and Microarray Hybridization

Amino-allyl aRNAs were coupled with Cy3 or Cy5 monoreactive *N*-hydroxysuccinimide esters for 30 min in the dark according to the Ambion dye-coupling protocol (Amino-Allyl MessageAmp II Kit; Ambion). After the reactions were quenched with 4 \mbox{M} hydroxylamine HCl, RNeasy columns (Qiagen) were used to separate labeled RNAs from uncoupled dye molecules. Samples were eluted with water and quantitated on a spectrophotometer to measure sample concentration and dye incorporation. Cy3- and Cy5-labeled aRNAs (1 $\mbox{\mu}$ g ach) were combined and hybridized for 17 h at 65°C in a rotating hybridization oven (60-mer Oligo Microarray Processing Protocol version 4.1; Agilent Technologies). The slides were washed at room temper-

ature and scanned on the Agilent Microarray Scanner, with scan resolution of 5 μm and photomultiplier tube of 100% and 10%.

Microarray and Experimental Design

The rice 4 × 44K oligomicroarray (chip code 251524111447; Agilent Technologies) consists of 45,152 spots, including 1,283 representing negative controls and 43,734 oligonucleotides synthesized based on the nucleotide sequence and full-length cDNA data of the Rice Annotation Project (RAP). The same microarray chip was used by Shimono et al. (2007) and Suwabe et al. (2008). A total of 43,734 60-mer probes represent 29,008 distinct rice genes. Among the 28,840 protein-coding genes, 22,532 have single sequence probes each, 4,619 have two sequence probes each, 1,235 have three sequence probes each, 330 have four sequences each, 92 have five sequences each, 16 have six sequences each, 11 have seven sequences each, three have eight sequences each, and two have nine sequences each.

Four microarrays of two colors were hybridized by ShanghaiBio Corp. Two independent biological replicates for each cell type, PMC and TCP, were labeled with Cy3. A mixture of two biological samples of seedlings was labeled with Cy5 and used as a common reference in all the hybridizations. The common reference was prepared once and divided into identical aliquots for all experiments. An additional independent biological sample of seedling was also labeled with Cy5 and hybridized with the same array.

Microarray Feature Extraction and Preprocessing

TIFF images were inspected visually to ensure that the number of saturated spots on each array ranged from two to 10, and probe features were extracted with Agilent Feature Extraction Software (version 9.5.3) using the configuration suggested by Agilent (Protocol for Use with Agilent Gene Expression Oligomicroarrays, version 5.7, March 2008).

The quality of the microarray data was ensured by the following criteria: (1) biological replicates must have a correlation $r \ge 0.9$ (the correlation r between TCP1 and TCP2 is 0.9698, that between PMC1 and PMC2 is 0.9037, and that between additional biological repetitions of seedling and the common reference seedling is 0.9443); and (2) the irregular spots were further removed when their median intensities were below the local background or replicated probes had a large variation (i.e. median intensities between replicated probes having a greater than 5-fold difference).

Among different probes representing the same gene, the hybridization intensities of probes whose target sequence positions are within zero to 300 nucleotides downstream of the end of the open reading frame were similar (less than 2-fold difference), while intensities of probes targeted to other regions were significantly reduced (Supplemental Fig. S11). The intensity reduction is probably because mRNA fragments far upstream from the poly (A) tail were not preserved as well after RNA amplification with oligo(dT) primers. For genes with multiple sequence probes, we manually chose the probe with targeted sequence position closest to 200 nucleotides downstream of the translation termination codon to represent the gene. We surveyed genes with single probes; roughly 90% of them are targeted with zero to 300 nucleotides downstream of the translation termination codon. Because we chose a 4-fold difference of intensity as a threshold for significantly different expression in addition to statistical tests, the intensity difference caused by probe position difference (less than 2-fold) should not affect our results.

Data Normalization and Statistical Analysis

Data normalization is performed without a background subtraction step as well as with background subtraction. In general, the results are similar either with or without background subtraction. We chose to present the results without background in the tables and figures, and we present the results with background subtraction in the supplemental tables. Following the recommendation by Zahurak et al. (2007), we prefer not to subtract local background for two reasons: (1) we observed a slightly larger noise-to-signal ratio for low-intensity probes when local background was subtracted; and (2) the correlation *r* between biological replicates was decreased after background subtraction.

Although Loess normalization performs well in many cases using twocolor microarray, it is not appropriate for processing microarray data from laser-microdissected cells. Intensity-based normalization is more suitable ('t Hoen et al., 2004), because we found data distortion after using Loess normalization. Jiao et al. (2009) also did not use Loess normalization in analyzing the cell type-specific transcriptome data for 40 cell types. Loess normalization requires the assumption that most of the genes are constantly expressed over the range of intensities or that the numbers of up- and downregulated genes over the intensity range are equal (Yang et al., 2002; Zahurak et al., 2007). Because only a small proportion of genes are expressed at similar levels between PMC, TCP, and seedling, and a significant number of genes are differentially expressed between them, an alternative normalization is performed in our study. We here give rationale to a simple method basically using median signal (absolute intensity value) to represent the individual gene expression level given the baseline of the microarray (provided by more than 1,000 negative control probe intensity values) and the maximum distribution of the intensity ratio close to 1:1 for microarrays using identical amounts of aRNA for hybridization. The maximum distribution of the intensity ratio close to 1:1 indicates that although there are significant differences of gene expression among laser-capture microdissection samples, still the most abundant distribution of gene expression is for genes that are not changed (Supplemental Fig. S12).

To meet the needs for profiling differentially expressed gene among the tissues, we performed a two-step array normalization process: (1) baseline transformation based on the negative control probes; (2) scale normalization based on the positive "housekeeping" genes (Supplemental Table S10) that were constantly expressed among the tissues. The baselines for PMC, TCP, and seedling are similar (Supplemental Fig. S13). The saturated intensities and the number of probes at the saturation level for PMC, TCP, and seedling are similar (Supplemental Table S11). The routine housekeeping genes such as actin, tubulin, or histone are different between PMC, TCP, and seedling, but the total intensities for the 101 genes encoding enzymes in the oxidative phosphorylation pathway (energy metabolism) are similar (Supplemental Table S15). This supports the notion that the core energy supply is maintained at a similar level among different types of living cells. To assess positively expressed genes, we defined the 95th percentile of the intensities from the negative controls as the threshold for significant gene expression. This cutoff is a result of balancing the number of false positives and missing truly expressed genes. Gene replicates with at least one above the threshold were examined with Student's *t* test, and P < 0.001 was considered significantly expressed. Among significantly expressed genes, the top 25th percentile are considered highly expressed, the middle 50th percentile are considered intermediately expressed, and the bottom 25th percentile are considered lowly expressed.

We performed a second analysis using a method similar to that of Nakazono et al. (2003). We found that the second method produced almost identical results as our original method.

GeneSpring GX9.0 (Agilent) was used for microarray analysis. Microsoft Excel 2003 and software R were used for statistical tests. Online genomic analysis tools of the KEGG pathway database were used for finding overlapping genes, and enrichment analysis were done by χ^2 test. Our data were deposited in the Gene Expression Omnibus under accession number GSE17098.

Validation of Microarray Data by Quantitative RT-PCR, in Situ Hybridization, and Conventional RT-PCR

We performed quantitative real-time PCR of reverse-transcribed RNA with SYBR Green I detection on an iCycler (Bio-Rad) as described by Tang et al. (2006).

To measure the absolute mRNA copy number in each laser-captured PMC, we inserted the genomic DNA fragments of Os09g0480300 and Os03g0650400 (*AM1-like*) into pTG-19 vector separately.

For in situ hybridization, a 563-bp AM1-like (Os03g0650400) DNA fragment, a 494-bp SKP1-like (Os07g0409500) DNA fragment, and a 561-bp SPL-like (Os01g0212500) DNA fragment were separately inserted into pBluescript SK+ vector for RNA probe synthesis. The antisense and sense RNA probes were synthesized by in vitro transcription using T7 and T3 RNA polymerase, respectively, using DIG RNA Labeling Mixture (Roche). We used a microwave tissue-processing technique for the fixation and embedding of stage 5 anthers (Supplemental Table S12). In situ hybridization experiments using 10μ m sections were carried out as described (Cox and Goldberg, 1988; Langdale, 1993).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Aniline blue staining of rice anthers at the stage for PMC isolation.

Supplemental Figure S2. RNA quality assessment.

Supplemental Figure S3. Calculation of mRNA copy number.

Supplemental Figure S4. Rice genes in base excision repair pathways with expression data.

Supplemental Figure S5. Protein sequence alignments for Am1 and SPL.

- Supplemental Figure S6. Expression data of rice MADS transcription factors with a phylogenic view.
- Supplemental Figure S7. Percentage of highly expressed transcription factors.
- Supplemental Figure S8. miRNA pathways in PMCs.
- Supplemental Figure S9. Overview of plant hormone biosynthesis pathways in rice TCP (A), seedling (B), and tapetum (C).
- Supplemental Figure S10. RT-PCR results for five genes in rice panicles at different developmental stages and other organs (young rice leaf, stem, and root).
- Supplemental Figure S11. Probe position effects on hybridization intensities.
- **Supplemental Figure S12.** Density plot of log₂ intensity ratios of seedling versus PMC and TCP versus PMC.
- Supplemental Figure S13. Density plot of \log_2 intensity for all the negative probes as the baseline.
- Supplemental Table S1. Microarray data for the 26 genes involved in rice early stamen development (Lu et al., 2006).
- Supplemental Table S2. Detailed results of KEGG pathway enrichment analysis.
- Supplemental Table S3. Description of 12 known meiotic genes.
- Supplemental Table S4. Microarray data for the genes similar to meiotic genes.
- **Supplemental Table S5.** Microarray data of 127 genes that were expressed in PMCs but not in TCP or seedling.
- Supplemental Table S6. Microarray data for 34 rice SET domain-containing proteins.
- Supplemental Table S7. Transcription factor and regulator expression summary.
- Supplemental Table S8. Microarray data for 288 genes in plant hormone biosynthesis and signaling pathways.
- **Supplemental Table S9.** Twenty-nine PMC-expressed genes containing a putative MYB-binding motif in the promoter region.
- Supplemental Table S10. Twenty housekeeping genes used for normalization.
- **Supplemental Table S11.** Saturated intensity values and the number of probes at the saturation level.
- Supplemental Table S12. Primer sequences.
- Supplemental Table S13. Microarray data for 1,158 PMC-preferential genes.
- **Supplemental Table S14.** Microarray data for 1,108 rice kinase genes (genes that were expressed more than 4-fold higher in PMC than in TCP and seedling are indicated by asterisks after the RAP identifier).
- Supplemental Table S15. Microarray data for genes in the oxidative phosphorylation pathway.
- Supplemental Table S16. Articles related to plant hormone function in anther or pollen development.

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