

Separated Transcriptomes of Male Gametophyte and Tapetum in Rice: Validity of a Laser Microdissection (LM) Microarray

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In flowering plants, the male gametophyte, the pollen, develops in the anther. Complex patterns of gene expression in both the gametophytic and sporophytic tissues of the anther regulate this process. The gene expression profiles of the microspore/pollen and the sporophytic tapetum are of particular interest. In this study, a microarray technique combined with laser microdissection (44K LM-microarray) was developed and used to characterize separately the transcriptomes of the microspore/pollen and tapetum in rice. Expression profiles of 11 known tapetum specific-genes were consistent with previous reports. Based on their spatial and temporal expression patterns, 140 genes which had been previously defined as anther specific were further classified as male gametophyte specific (71 genes, 51%), tapetum-specific (seven genes, 5%) or expressed in both male gametophyte and tapetum (62 genes, 44%). These results indicate that the 44K LM-microarray is a reliable tool to analyze the gene expression profiles of two important cell types in the anther, the microspore/pollen and tapetum.

Keywords: Anther • Laser microdissection • Microarray • *Oryza sativa* • L. Microspore/pollen • Tapetum.

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Abbreviations: DIG, digoxigenin; GUS, β -glucuronidase; IR, infrared; LM, laser microdissection.

Introduction

In flowering plants, the male gametophyte, the pollen, develops in the anther. The free pollen cells are released in the anther locule, whose wall consists of the tapetum, middle layer, endothecium and epidermis (Fig. 1). Development of pollen cells in the locule is synchronized with functional anther development (Goldberg et al. 1993, Scott et al. 2004). After differentiation of the male germline, pollen mother cells undergo meiosis to form tetrads of haploid microspores in the anther locule. These microspores then mature into pollen grains through cell division and formation of the complex pollen wall. Through an asymmetric mitosis, the uninuclear microspore develops into bicellular pollen comprising a larger vegetative cell and a smaller generative cell (McCormick 1993, McCormick 2004). In plant species with tricellular pollen (including *Arabidopsis* and rice), the generative cell then undergoes a second mitosis to form two sperm cells. During pollen maturation, the tapetum acts as a nutritive tissue, providing materials for pollen wall formation, and subsequently disintegrates in the later

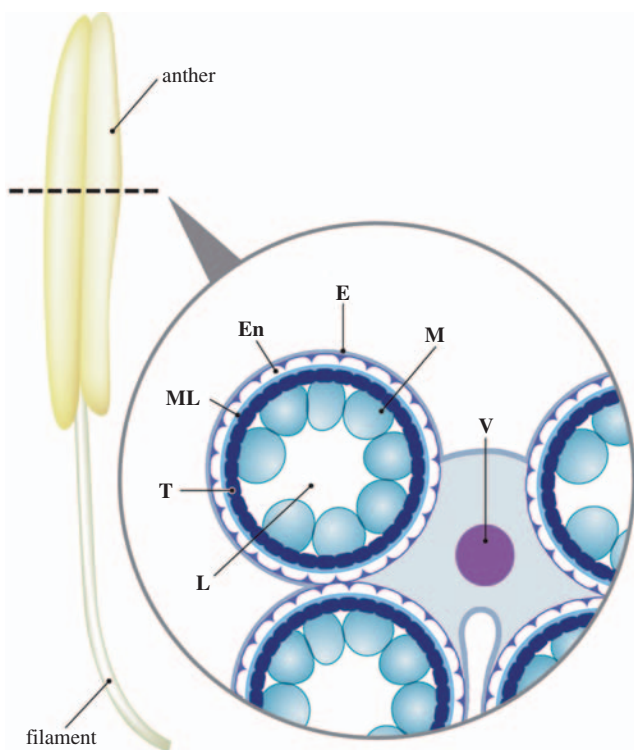


Fig. 1 Scheme of a cross-section of a rice anther containing immature microspores. L, locule; T, tapetum; ML, middle layer; En, endothecium; E, epidermis; M, microspores; V, vascular bundle.

stages of pollen development (Goldberg et al. 1993, Scott et al. 2004).

The important biological events described above are regulated by complex patterns of gene expression in both the gametophytic and sporophytic tissues during anther and male gametophyte development (McCormick 2004, Scott et al. 2004). Gene expression in the anther has been studied intensively in important crops and model plants by using conventional cDNA cloning, promoter analysis and microarrays (Koltunow et al. 1990, Scott et al. 1991, Tsuchiya et al. 1994, Hihara et al. 1996, Rubinelli et al. 1998, Jeon et al. 1999, Endo et al. 2002, Amagai et al. 2003, Masuko et al. 2006). In particular, genes specifically expressed in the tapetum have attracted the attention of phytologists because the tapetum exhibits high metabolic activity (Scott et al. 2004) and is related to both male sterility (Wilson et al. 2001, Sorensen et al. 2003, Yui et al. 2003, Ariizumi et al. 2004, Yang et al. 2007) and sporophytic self-incompatibility (Takayama et al. 2000, Watanabe et al. 2001), indicating a critical role for the tapetum in pollen development and maturation.

Anther transcriptomes analyzed by microarrays offer important information for understanding the genetic regulation of anther and pollen development. Of particular scientific interest are the gene expression profiles of two important

cell types within the anther: the microspore/pollen and tapetum. However, most of the gene expression analyses conducted to date have employed whole anther tissue taken at different developmental stages (Endo et al. 2002, Mandaokar et al. 2003, Endo et al. 2004, Lan et al. 2004, Yamaguchi et al. 2004, Jung et al. 2005, Wang et al. 2005, Ma et al. 2006) or mature pollen grains (Honys and Twell 2003), and have not distinguished the cell types within the anther. Microarray technology is able to explore a large number of genes whose transcript levels change between states or development stages. However, sufficient specific tissue must be available in order to isolate enough mRNA to conduct the hybridization, typically 1–2 μg (Duggan et al. 1999, Richmond and Somerville 2000). Furthermore, it can be technically difficult to isolate single cell types from plant organs. Thus, most microarray studies have been conducted using RNAs extracted from a mixture of tissues and/or cell types (Girke et al. 2000). Together these technical challenges have made it impossible to analyze a large number of gene expression profiles in precisely separated cell types with high resolution. Honys and Twell (2004) established stage-separated spore isolation procedures for *Arabidopsis*, and performed transcriptome analyses of male gametophyte development. In brief, inflorescences were ground in mannitol, and the mixture filtered through nylon mesh. By centrifugation of the filtered microspores in a Percoll step gradient, uninucleate microspores, bicellular pollen and immature tricellular pollen were discriminated and isolated. The haploid male gametophyte-specific transcriptome revealed large-scale repression of various genes and an increase in the proportion of male gametophyte-specific transcripts during pollen maturation (Honys and Twell 2004). Although comparison of the successful male gametophyte-specific transcriptome with the tapetum-specific transcriptome would undoubtedly be informative, it has not been possible to date because of the technical challenge of precise isolation of tapetal cells, which are attached to the middle layer of the anther wall.

Laser microdissection (LM) is a powerful tool for isolating specific cell types from sectioned specimens of heterogeneous tissues, including plant tissues (Asano et al. 2002, Kerk et al. 2003, Nakazono et al. 2003, Day et al. 2005, Nelson et al. 2006, Ohtsu et al. 2007b). A tissue section that contains the cell type of interest is placed on a microscope stage, a laser beam is targeted on the cells of interest and then the target cells are separated from the rest of the tissue. In general, there are two types of LM methodology: laser capture and laser cutting (Ohtsu et al. 2007b). Laser capture reliably targets cells for collection but sometimes also collects surrounding cells that remain attached to the target cells. On the other hand, laser cutting minimizes the collection of non-target cells, but is subject to interference by various factors such as static electricity when the target cells are very small.

However, a newly available LM system (Veritas Laser Microdissection System, Molecular Devices) can compensate for the disadvantages of laser capture and laser cutting by combining the two methods, thereby providing LM with higher precision and efficiency. Recently, many LM-microarray analyses have been conducted in plants, demonstrating wide applicability of the technology. Successfully targeted plant cell types include embryos (Casson et al. 2005, Spencer et al. 2007), coleoptile epidermis and vascular tissues (Nakazono et al. 2003), shoot apical meristems (Ohtsu et al. 2007a, Zhang et al. 2007), root pericycles (Woll et al. 2005, Dembinsky et al. 2007), silique replums (Cai and Lashbrook 2006) and stamen abscission zones (Cai and Lashbrook 2008).

In this report, and the following articles in the same issue (Hobo et al. 2008, Hirano et al. 2008), the LM-microarray approach has been used to characterize the two separated transcriptomes of the microspore/pollen and tapetum in rice. By using LM technology, the tapetal cell layers were collected with minimal contamination by other anther wall tissues or microspore/pollen. With minimum input of labeled cDNAs from the precisely separated rice microspore/pollen and tapetal cells, a 44K microarray analysis was achieved. We were able to validate the LM-microarray developed in this study by comparing our data with the results of a 4,304 cDNA array (4K array) previously published by Endo et al. (2004), which identified 156 genes specifically expressed in rice anthers, some of which were confirmed by RNA in situ hybridization.

Results and Discussion

Isolation of male gametophyte- and tapetum-specific RNAs

In order to perform the LM-microarray of microspore/pollen and tapetum in rice, we prepared cross-sections of *Oryza sativa* cv. Nipponbare anthers at various developmental stages. In rice, anther development has been precisely classified into five stages (from meiosis to tricellular pollen), according to glume length and cell numbers in the male gametophytes. These are meiosis (MEI), tetrad (TET), uninuclear microspore (UN), bicellular pollen (BC) and tricellular pollen (TC) (stages described as An4 to An8 in Itoh et al. 2005). We isolated microspore/pollen from each of the five stages. Because degradation of the tapetum begins at the BC stage, tapetum cells could only be isolated from MEI, TET and UN anthers. Using the Veritas Laser Microdissection System (Molecular Devices; Fig. 2A), the microspore/pollen or tapetal cells from each developmental stage were isolated from the anther sections. In the case of MEI, TET and UN anthers, the microspores were isolated first, followed by the tapetum; Fig. 2B.

Total RNAs were isolated from the cells dissected from 250–400 sections, corresponding to anthers of four or five

spikelets, and were subjected to the 44K rice oligoarrays with 44,000 features of 60mer oligonucleotides (Agilent Technologies), corresponding to the full-length cDNAs characterized by the Rice Annotation Project (RAP). For each microarray experiment, an average 12.6 ng (minimum 2.2 ng) of total RNAs were used for Cy3 labeling, for complementary RNA (cRNA) synthesis and amplification, according to the Agilent protocol with slight modification. Labeled cRNAs of average 660 ng (minimum 277 ng) were then used to perform the one-color microarray analysis. The microarray experiment was repeated at least three times in each stage/cell type using RNAs isolated independently. The significance of this study is the isolation of microspore/pollen- and tapetum-specific RNAs in sufficient quantity and quality for microarray analysis, making it possible to compare the male gametophyte and tapetum transcriptomes (see Hobo et al. 2008, Hirano et al. 2008 in this issue).

Gene expression profiles of reported genes in the 44K LM microarray

We focused on validating the 44K LM-microarray by re-characterizing genes previously reported as tapetum specific (see below for detail) and/or reported in a 4K microarray analysis of the developmental anther stages in rice (Endo et al. 2004). The expression profile data from the 4K microarray can be accessed at the following web address: http://www.jstage.jst.go.jp/article/ggs/79/4/79_213/_article.

Of the 156 anther-specific genes on the 4K array identified by Endo et al. (2004), the expression profiles of *Osc4*, *Osc6*, *YY1*, *OsRAFTIN1* and *TDR* have already been characterized in detail (Tsuchiya et al. 1992, Tsuchiya et al. 1994, Hihara et al. 1996, Wang et al. 2003, Li et al. 2006). These five genes are expressed mainly in the tapetum, as confirmed by RNA in situ hybridization and/or promoter analysis using a glucuronidase (GUS) gene. In addition to these, we identified six previously reported tapetum-expressed genes, *YY2*, *RA8*, *OsGAMYB*, *OsCP1*, *UDT1* and *OsGEN-L* (Hihara et al. 1996, Jeon et al. 1999, Kaneko et al. 2004, Lee et al. 2004, Jung et al. 2005, Moritoh et al. 2005). Expression profiles of these 11 known genes were re-analyzed using the 44K LM-microarray developed here (Fig. 3).

The probes for *OsGAMYB* (X98355, AK063951, AK102841 and AK119607) clearly demonstrated that this gene was highly expressed in the MEI, TET and UN tapetum, but not in the male gametes of all stages (Fig. 3), consistent with previous reports (Kaneko et al. 2004, Tsuji et al. 2006). Such distinct gene expression profiles between microspores/pollen and the tapetum indicate that the LM technology isolated each cell type accurately, and there were few contaminating tapetum RNAs detectable in the microspore cells which were isolated first from the anther sections (Fig. 2B). It is noteworthy that genes similar to β -ketoacyl-ACP synthase (AK067275), transaldolase family protein

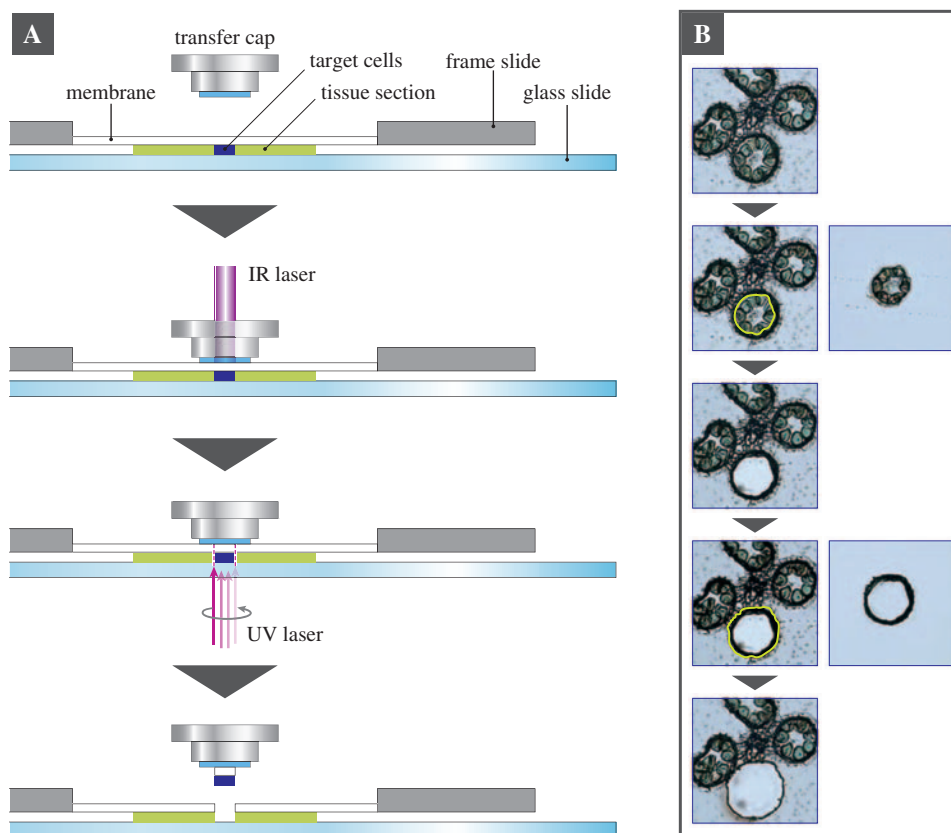


Fig. 2 Laser microdissection (LM) of pollen/microspores and tapetum cells from a cross-section of a rice anther. (A) A schematic procedure for isolating plant target cells by laser microdissection using the Veritas Laser Microdissection System (Molecular Devices), which can use both laser capture and laser cutting. In brief, a transfer cap is placed on a tissue section, which contains the target cells, mounted on a membrane of a frame slide. To anchor and capture the target cells to the transfer cap, a focused low energy infrared (IR) laser beam is targeted on the cells of interest through the transfer cap, causing the cap to fuse to the target cells. The area around the target cells is cut by a UV laser beam. The transfer cap is removed from the tissue section. The laser-microdissected target cells that fused to the transfer cap are separated from the rest of the tissue. (B) Isolation of the pollen/microspore and tapetum cells by the two-step LM. In the case of the TET anther, the microspores were isolated by the first cutting, and the tapetum cells were isolated by the second cutting.

(AK058325), acyl carrier protein III (AK058903), phytochelatin synthetase-like protein 2 (AK070472) and GCN5-related *N*-acetyltransferase domain-containing protein (AK068410) also showed similar tapetum-specific expression profiles to *OsGAMYB* in the 44K LM array (data not shown). Among these, tapetum-specific localization of transcripts for AK058903 and AK070472 has been confirmed by RNA in situ analysis (Endo et al. 2004).

Osc6, *RA8* and *OsRAFTIN1* were predominantly expressed in the UN tapetum, and lower expression was also observed in the microspore at the same stage (Fig. 3). These expression profiles are consistent with the previous reports. In promoter analyses of *Osc6* (Tsuchiya et al. 1994) and *RA8* (Jeon et al. 1999), weak or spotted GUS signals, respectively, were observed in the microspores, in addition to a predominant GUS signal in the tapetum. Wang et al. (2003) reported the possible expression of *OsRAFTIN1* in both microspores and

tapetum. In our study, hybridization patterns of two probes corresponding to *UDT1* (C1515529, Fig. 3) showed a similar expression profile to the GUS assay of the T-DNA-tagged *udt1* mutant reported by Jung et al. (2005), who suggested that *UDT1* might function in early meiosis and tapetum development. The *TDR* gene showed higher expression in the tapetum and relatively lower expression in the microspore, mainly at the MEI and TET stages (Fig. 3), consistent with RNA in situ analysis performed by Li et al. (2006), which showed that *TDR* was expressed predominantly in the tapetum at the meiosis and tetrad stage.

YY1 and *Osc4* were expressed almost identically at each stage of microspore and tapetum development, while *YY2* expression showed opposite trends at the TET and UN stages of each cell type (Fig. 3). In a previous report (Hihara et al. 1996), the existence of the *YY1* transcript in microspores before the uninuclear microspore stage was not described,

Accession No.		Description	Relative expression level (average)							
			MEI-microspore	TET-microspore	UN-microspore	BC-pollen	TC-pollen	MEI-tapetum	TET-tapetum	UN-tapetum
X98355	Os01g0812000	<i>OsGAMYB</i>	-0.541	0.076	0.165	-0.341	-1.437	0.845	0.762	1.221
AK063951	Os01g0812000	<i>OsGAMYB</i>	-0.552	0.019	0.363	-0.474	-1.425	0.965	0.791	1.288
AK102841	Os01g0812000	<i>OsGAMYB</i>	-0.476	0.099	0.251	-0.463	-1.444	0.921	0.763	1.281
AK119607	Os01g0812000	<i>OsGAMYB</i>	-0.379	0.116	0.149	-0.425	-1.558	0.983	0.837	1.111
X98355	Os01g0812000	<i>OsGAMYB</i>	-0.604	0.086	0.287	-0.418	-1.427	0.820	0.791	1.362
AK106761	Os02g0120500	<i>TDR</i>	0.659	0.709	0.110	-1.212	-1.008	1.098	0.795	0.648
AK107506	Os04g0670500	<i>OsCP1</i>	-0.288	0.330	1.864	0.157	-0.454	-1.504	-0.710	0.439
CI515529	Os07g0549600	<i>UDT1</i>	0.488	-0.224	0.909	-0.685	-1.689	0.801	-0.052	1.431
CI515529	Os07g0549600	<i>UDT1</i>	0.572	-0.064	0.869	-0.793	-1.701	0.809	0.088	1.353
AK120942	Os08g0496800	<i>OsRAFTIN1</i>	-0.441	0.155	1.233	-0.670	-1.192	0.481	0.296	1.401
AK064717	Os08g0546300	<i>Osc4</i>	0.504	0.854	0.705	-1.229	-1.398	0.655	0.882	0.907
AK109240	Os09g0480900	<i>RA8</i>	-0.106	-0.443	1.300	-0.430	-0.854	-0.238	-0.476	1.997
AK063534	Os09g0521900	<i>OsGEN-L</i>	1.061	1.645	-1.141	-0.763	-0.119	0.304	0.705	-0.774
AK107918	Os09g0525500	<i>YY1</i>	0.816	0.977	0.234	-1.284	-1.165	0.886	0.972	0.433
AB110182	Os10g0484800	<i>YY2</i>	0.002	1.193	0.663	-1.141	-1.138	0.003	1.094	1.223
AK105510	Os10g0484800	<i>YY2</i>	0.043	1.171	0.638	-1.146	-1.105	0.007	1.066	1.222
D50576	Os10g0484800	<i>YY2</i>	0.021	1.161	0.606	-1.109	-1.175	-0.007	1.066	1.278
X91811	Os10g0484800	<i>YY2</i>	-0.272	1.198	0.693	-0.913	-1.263	-0.294	1.047	1.414
AK064672	Os11g0582500	<i>Osc6</i>	-0.658	-0.570	1.646	-0.001	-0.417	-0.810	-0.881	1.912

Fig. 3 Gene expression profiles of reported genes in the 44K LM-microarray. Expression level of 19 probes corresponding to 11 selected genes, which were characterized by previous reports. The same gene accession numbers were annotated to independent probes in the case of *OsGAMYB* (X98355) and *UDT1* (CI515529). Relative values of 0.5–1.0, 1.0–1.5 and 1.5–2.0 were labeled by light orange, orange and dark orange, respectively.

although RNA in situ analysis hinted at *YY1* expression in uninuclear microspores. In the case of *Osc4* and *YY2*, Tsuchiya et al. (1994) and Hihara et al. (1996) showed tapetum-specific expression of these genes by RNA in situ hybridization. These previous data for *Osc4*, *YY1* and *YY2* differ from our results, indicating the difficulty in detecting small amounts of transcripts in the microspores (discussed below). Through LM-microarray technology, we are now able to determine the expression profiles of these genes as follows: *Osc4* is expressed in both microspores and tapetum cells at the MEI to UN stages, *YY1* is expressed at the MEI and TET stages of both cell types, and expression of *YY2* is higher at the TET stage in the microspores but higher at the UN stage in the tapetum (Fig. 3).

The expression patterns of *OsGEN-L* and *OsCP1* were distinct from those of all the other genes discussed here, namely higher expression in the microspores and relatively lower expression in the tapetum (Fig. 3). *OsGEN-L* was expressed in the MEI and TET microspores as well as in the tapetum at the same stages. Expression in microspores was observed from the MEI stage and peaked at the TET stage; the same profile was observed in the tapetum although its overall expression level was relatively lower than in microspores (Fig. 3). In previous reports, the promoter activity of *OsGEN-L* was found to be up-regulated during the post-meiotic stage in most of the diploid and haploid cells of the anthers, while knock-down *OsGEN-L*-RNAi plants displayed defects in early microspore development (Moritoh et al. 2005), all consistent with our 44K LM-microarray data. *OsCP1* was expressed predominantly in the UN microspore and weakly in UN

tapetum (Fig. 3), which might correspond to the temporal pattern of promoter activity in anther locules revealed by histochemical GUS assay (Lee et al. 2004). Although this previous report did not characterize a precise expression pattern of *OsCP1* in terms of the developmental stages of rice anther, an *oscp1* mutant displayed pollen degradation after the microspore stage, indicating that the functional role of *OsCP1* is to support development and/or maturation of pollen grains. Our data from the 44K LM-microarray are consistent with this conclusion and, moreover, determine the *OsCP1* expression profile more precisely, demonstrating the potential of LM-microarray technology for fine-scale gene expression analysis.

Cell type-specific gene expression profiles of the anther-specific genes reported in the 4K microarray

Overall, the 140 probes of the 44K LM-microarray, corresponding to the anther-specific genes identified in the 4K microarray analysis of Endo et al. (2004), were classified into 71 genes expressed specifically in male gametophytes (51%), seven genes expressed specifically in the tapetum (5%) and 62 expressed in both tissues (44%) (Fig. 4, Supplementary Tables S1, S2). The relatively small number of tapetum-specific genes identified is a reflection of the stages of the original 4K microarray analysis, which focused on the late developmental stages of anthers (from uninuclear microspore to tricellular pollen).

We further investigated the spatial expression patterns of 21 of the 140 probes by RNA in situ analysis: representative results are shown in Fig. 5. RNA in situ hybridization of

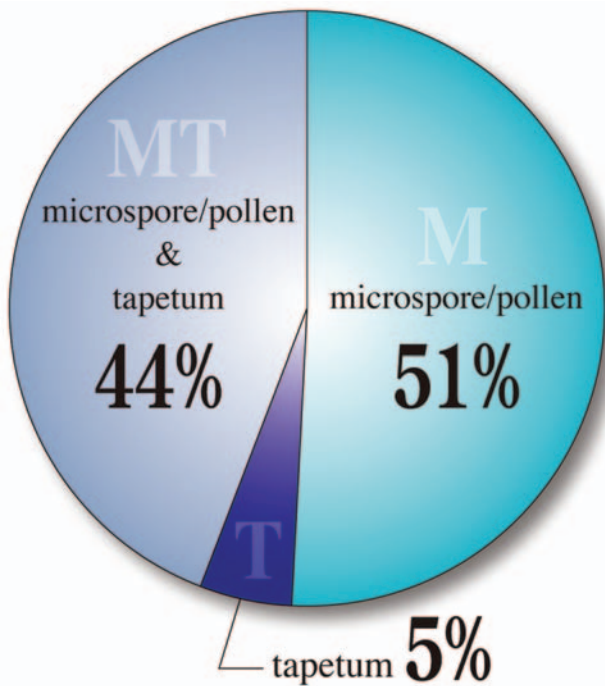


Fig. 4 Classification of 140 anther-specific genes from the previous 4K microarray in the 44K LM-microarray. Expression profiles of the 44K LM-microarray for the genes reported by Endo et al. (2004) were classified into three spatial expression patterns; microspore/pollen-specific, tapetum-specific and expressed in both tissues.

anther cross-sections with non-R1 probes tend to give faint signals in microspores, while high background signals are often obtained in the tapetum. This makes it difficult to identify small amounts of transcripts in microspores by RNA in situ analysis, presumably explaining the absence of detectable microspore expression of *Osc4*, *YY1* and *YY2* in previous reports (Tsuchiya et al. 1994, Hihara et al. 1996). This technical challenge may be common to many genes expressed in the microspore/pollen. In addition, it is a laborious task to conduct RNA in situ analyses for many genes (probes). Therefore, the LM-microarray is a useful tool to determine transcriptomes in the microspores both precisely and efficiently.

We performed a cluster analysis of the 140 probes in the 44K LM-microarray, and classified the data according to spatial and temporal expression patterns (Fig. 6). The anther transcriptome can be precisely separated into male gametophyte and tapetum transcriptomes. Since cell type-specific RNAs were isolated from the cross-sections, the developmental stage of samples was strictly determined, as was precise discrimination of the cells. Fig. 6 shows that a large number of genes in the pollen/microspore cluster were expressed in the late developmental stages (BC and TC), suggesting that maturation of pollen requires various special transcripts that accumulate in the male gametophyte.

This might be related to the large-scale gene repression that occurs during the transition from bicellular to tricellular pollen in *Arabidopsis* (Honys and Twell 2004). In the process of male gamete development, from pollen mother cell to mature pollen, the tapetum cells provide factors necessary for differentiation, development and maturation of the male gametophyte, and then begin their degradation at the bicellular pollen stage. After this, the pollen must independently produce the substances and nutrients essential for its survival, development and maturation. It has been suggested that mature pollen already contains all the transcripts necessary for the events of pollination and fertilization, e.g. pollen germination, penetration into the stigma, pollen tube elongation and double fertilization (Becker et al. 2003). An up-regulation of a large number of genes in the late developmental stage would reflect these biological features of pollen. In the pollen/microspore and tapetum cluster, synchronous expression in both cell types (e.g. high expression at the UN microspores and UN tapetum) was also a notable feature (Fig. 6). Although pollen/microspore and tapetum have independent roles, they may function by sharing some aspects of these biological events. As shown in Fig. 6, a wide variety of genes are involved in male gamete development, so it is likely that the spatial and temporal balance of their expression is critical for accurate pollen development.

Our comparative study of the 44K LM-microarray and the previous 4K-microarray suggested that the 44K gene expression profiles of male gametes and tapetum in *O. sativa* are valid, and can be used as reliable data supporting further analyses in these specialized tissues (Hobo et al. 2008, Hirano et al. 2008). Indeed, comprehensive dissection of the gene networks involved in male gametophyte and anther development, including understanding the interaction between gametophytic and sporophytic tissues, should now be possible using the 44K LM-microarray data.

Materials and Methods

Plant materials

Rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare) plants were grown in a greenhouse under normal conditions. Developing anthers of various stages were collected by confirming their developmental stage by microscopy, using one of six anthers from each flower. The stage classification of anthers is described in detail in the Results and Discussion section.

Sample preparation for LM

The anthers were fixed in Farmer's fixative (ethanol:acetate=3:1) overnight at 4°C. Dehydration and paraffin embedding were performed as described by Inada and Wildermuth (2005) by using a microwave processor. Paraffin-embedded sections were cut to a thickness of 16 µm and

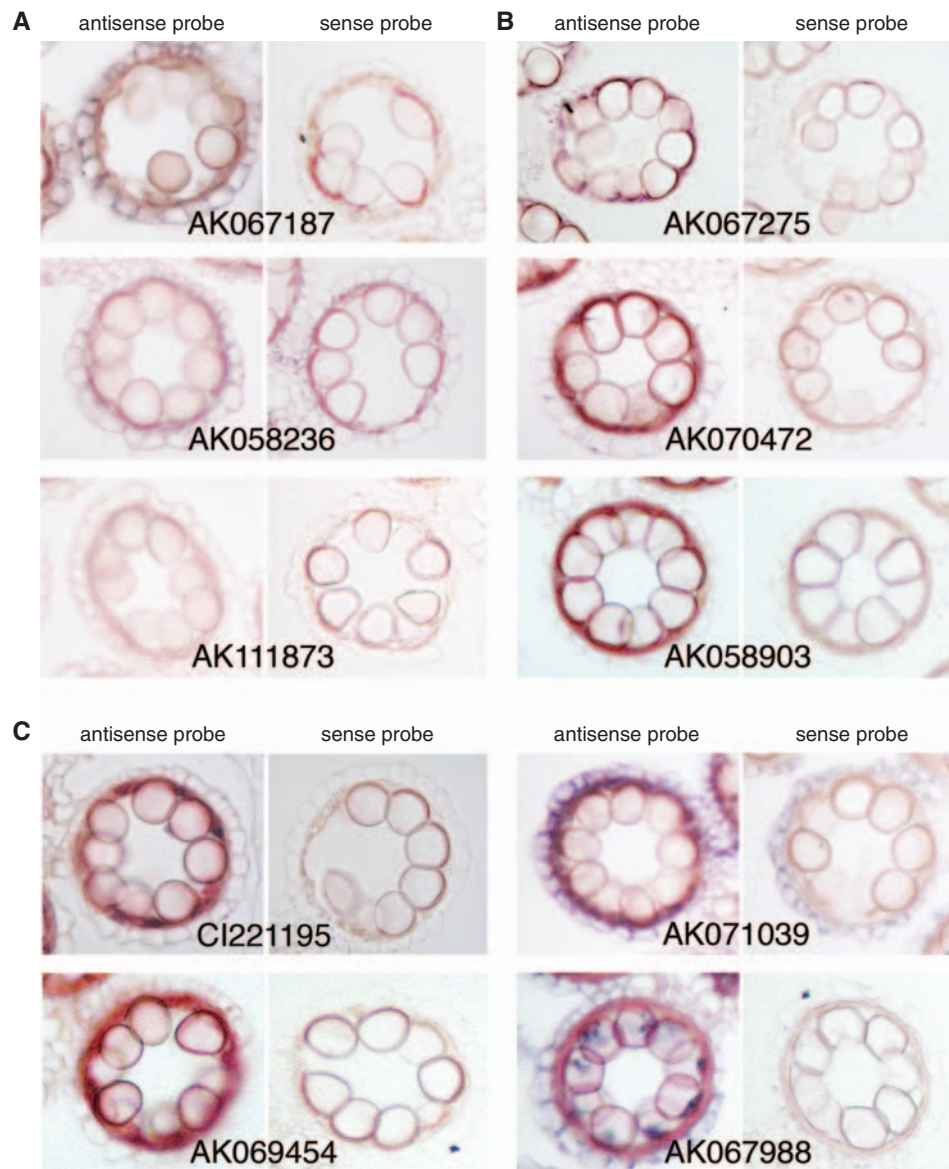


Fig. 5 Localization of the anther-specific transcripts in rice anthers. Representative results of RNA in situ hybridization of microspore/pollen-specific genes (A), tapetum-specific genes (B) and genes expressed in both microspore/pollen and tapetum (C). DIG-labeled antisense and sense (control) RNA probes were hybridized to the cross-sections of rice anthers containing immature microspores.

mounted on PEN membrane glass slides (Molecular Devices, Ontario, Canada) for LM. To remove the paraffin, slides were immersed in 100% xylene (twice), 50% xylene/50% ethanol and 100% ethanol (v/v) for 5 min each, and then air-dried completely at room temperature. Three or four individual flowers were used for each LM experiment. LM was performed using the Veritas Laser Microdissection System LCC1704 (Molecular Devices). Selected areas were captured by an infrared laser (IR laser) onto CapSure Macro LCM Caps (Molecular Devices), and were subsequently cut by a UV laser (Fig. 2). The target cells that fused to the LCM cap were collected by removing the cap from the tissue section.

Microarray analysis

Total RNAs were extracted from LM cells with a PicoPure™ RNA isolation kit (Molecular Devices). Total RNA was quantified with a Quant-iT™ RiboGreen RNA reagent and kit (Invitrogen, San Diego, CA, USA). A rice 44K oligo microarray (Agilent Technologies, Palo Alto, CA, USA) which contains ~42,000 oligonucleotides synthesized based on the nucleotide sequence and full-length cDNA data of the Rice Annotation Project (RAP) was used. Fluorescent probe labeling using the oligo-dT-T7 strand-specific amplification method, hybridization and scanning were performed according to the manufacturer's instructions with slight

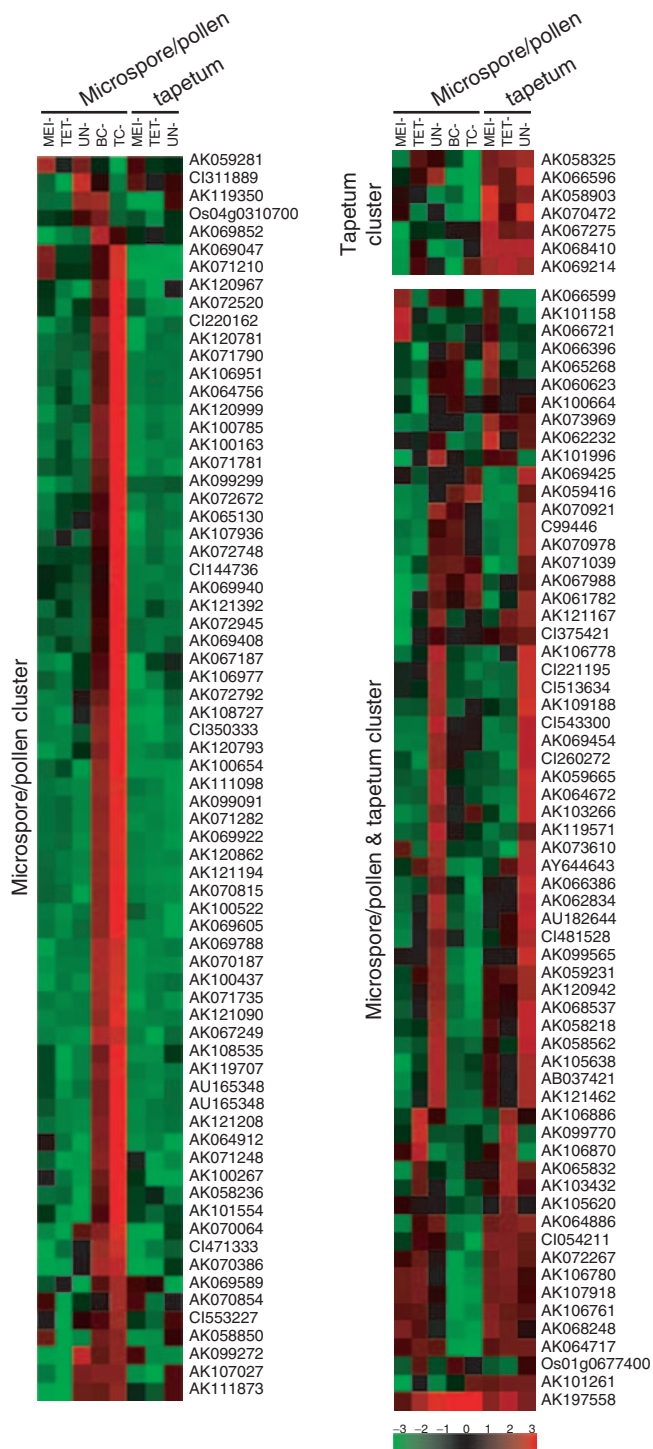


Fig. 6 Heat map view of the previously reported anther-specific genes differentially expressed in five stages of pollen/microspores and three stages of tapetum cells in the 44K LM-microarray. Cluster analysis of expression profiles of the 140 probes corresponding to the anther-specific genes characterized in the 4K array (Endo et al. 2004). Red indicates higher expression while green represents lower expression.

modification (Agilent Technologies). Microarray data were statistically analyzed for variance stabilization (VSN) and mean – SD(Z) scaling, using R software (<http://www.r-project.org/>). Cluster analysis for gene categorization was performed with Cluster and TreeView software (Eisen et al. 1998).

RNA in situ hybridization

Both antisense and sense probes were synthesized using a T3/T7 digoxigenin (DIG) RNA labeling kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Subsequent in situ hybridization was performed according to Endo et al. (2004) and Masuko et al. (2006), and tissue sections were observed under a light microscope (Eclipse E800 microscope system; Nikon, Tokyo, Japan).

Supplementary Material

Supplementary Material are available at PCP Online.

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