Various Spatiotemporal Expression Profiles of Anther-Expressed Genes in Rice

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The male gametophyte and tapetum play different roles during anther development although they are differentiated from the same cell lineage, the L2 layer. Until now, it has not been possible to delineate their transcriptomes due to technical difficulties in separating the two cell types. In the present study, we characterized the separated transcriptomes of the rice microspore/pollen and tapetum using laser microdissection (LM)-mediated microarray. Spatiotemporal expression patterns of 28,141 anther-expressed genes were classified into 20 clusters, which contained 3,468 (12.3\%) anther-enriched genes. In some clusters, synchronous gene expression in the microspore and tapetum at the same developmental stage was observed as a novel characteristic of the anther transcriptome. Noteworthy expression patterns are discussed in connection with gene ontology (GO) categories and gene annotations, which are related to important biological events in anther development, such as pollen maturation, pollen germination, pollen tube elongation and pollen wall formation.

Keywords: Anther • Gene ontology • Laser microdissection • Microarray • \textit{Oryza sativa} L. • Synchronous gene expression.

Abbreviations: BC, bicellular; GO, gene ontology; LM, laser microdissection; MEI, meiosis; miRNA, microRNA; TC, tricellular; TET, tetrad; UN, uninuclear; VSN, variance stabilization normalization.

Introduction

Anther tissues in flowering plants are important for microsporogenesis and dispersing the male gamete, pollen. Anther tissues consist of three layers, L1, L2 and L3. The epidermis and stamium are differentiated at the final developmental stage in the L1 layer. The microspore/pollen, endothecium and outer tapetum are formed in the L2 layer, while the L3 layer differentiates into vascular bundle and inner tapetum (Goldberg et al. 1993). Although the sporogenous cells and parietal cells originate from the same L2 layer, their final fates are different. In the case of sporogenous cells, following meiosis, microspores are released into the anther locule, and each microspore divides into vegetative and generative cells. In rice, the generative cell further divides into two sperm cells. Desiccated mature pollen grains are dispersed from anthers onto the stigma surface ready for fertilization. In contrast, parietal cells differentiate into...
endothecium and outer tapetum. Tapetum cells function as nurse cells for pollen development; the tapetum supports pollen development by secreting nutrients and other secondary metabolites. The tapetum constitutes the pollen outer surface, but degrades before anther dehiscence (Goldberg et al. 1993). Therefore, while microspore/pollen and outer tapetum originate from the same cell lineage, the L2 layer, their fetal functions are completely different.

In order to understand the molecular mechanisms of anther development, particularly microspore/pollen differentiation and tapetum development, transcriptome analyses have been conducted in anthers of rice and *Lotus japonicus* (Endo et al. 2002, Endo et al. 2004). Some anther-specific genes were further characterized by RNA in situ hybridization analysis, which is extremely laborious and not comprehensive. These studies demonstrated the existence of microspore/pollen-specific and tapetum-specific genes (Endo et al. 2004, Masuko et al. 2006). Briefly, in the mature pollen grain, coordinated expression of pollen-specific transcripts was detected. These transcripts are related to the cell cytoskeleton, cell wall re-organization, methionine metabolism, proton pumps and sugar transporters, and are presumably important for pollen germination and pollen tube elongation (Endo et al. 2002, Endo et al. 2004, Masuko et al. 2006). In contrast, tapetum-specific transcripts were detected relating to secondary metabolism, fatty acid biosynthesis, protein secretion and the gibberellin signaling cascade (Endo et al. 2004, Masuko et al. 2006). The different characteristics of the two transcriptomes suggested that gene functions in pollen and tapetum are distinct. However, further comprehensive analysis of microspore/pollen-specific and tapetum-specific genes is necessary for a complete functional understanding of these cell types.

Because laser microdissection (LM) is a useful technique to separate cell types within complex 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. 2007), we performed a 44K microarray with RNAs derived from LM-separated microspore/pollen and tapetum cells in rice. The reliability of the data was confirmed by comparison with the published gene expression profile of 156 previously identified anther-specific genes in rice (refer to Suwabe et al. 2008 in this special issue). Having validated the technique, the genome-wide expression profiles of microspore/pollen and tapetum in rice were determined in this study according to the developmental stages. From the cluster analysis, 28,141 genes were categorized into 20 clusters according to their characteristic expression patterns, and gene ontology (GO) was applied to each cluster. We discuss noteworthy characteristics of the microspore/pollen and tapetum transcriptomes in rice.

## Results and Discussion

### Various spatiotemporal expression patterns of genes in rice anthers

We conducted a 44K LM-microarray analysis of microspore/pollen and tapetum cells of *Oryza sativa* cv. Nipponbare, at each of the five stages of anther development: meiosis (MEI), tetrad (TET), uninuclear (UN) microspore, bicellular (BC) pollen and tricellular (TC) pollen (Suwabe et al. 2008). Because tapetum degradation occurs at the BC stage, the tapetum transcriptome was only characterized at the MEI, TET and UN stages. After normalization of Z-scored 44K array data, a cluster analysis was performed with TIGR MeV software (Fig. 1, Supplementary Figs. S1–S20, Tables S1–S20). It should be noted that the microspore/pollen and tapetum transcriptomes were successfully separated without contamination in the present LM-microarray, as described by Suwabe et al. (2008) in this issue. This conclusion was further supported by the existence of the anther-specific and microspore-specific clades in the heat map (Fig. 2). In total, 28,141 anther-expressed genes were categorized into 20 clusters, and the average number of genes in each cluster was 1,407. Expression levels of genes in six clusters (Nos. 4, 5, 6, 8, 10 and 19) were higher in the microspore/pollen than in the tapetum, indicating that these clusters were microspore/pollen dominant. On the other hand, no obvious tapetum-dominant profiles were observed in the 20 clusters, mainly because only a small proportion of genes showed the strictly tapetum-dominant expression pattern, and these were distributed among several clusters in this analysis.

To discriminate genes preferentially expressed in anthers, we compared the LM-microarray data of microspore/pollen and tapetum with microarray data sets obtained for rice roots and leaves (Y. Nagamura, unpublished results). In the present study, anther-expressed genes with a vegetative expression signal score <50 were defined as ‘anther-specific’ genes. According to this definition, 3,468 anther-specific genes were identified, accounting for 12.3% of the total 28,141 anther-expressed genes. The percentage of anther-specific genes was then calculated in each cluster (Fig. 1), and relatively lower ratios of anther-specific genes (~2–4%) were observed for the clusters of constitutively tapetum-expressed genes (Nos. 13, 14, 15 and 18). In contrast, in the clusters of genes expressed in MEI microspore (clusters 12 and 17) and in late developmental stages of pollen (clusters 5 and 10), >20% of genes were anther specific. The increased proportion of anther-specific transcripts in clusters 5 and 10 is consistent with analysis of the *Arabidopsis* male gametophyte-specific transcriptome (Honys and Twell 2004), which showed that the transition from BC to TC pollen was accompanied by an increase in the proportion of male gamete-specific transcripts.
Fig. 1 Gene expression patterns of 20 clusters in microspore/pollen and tapetum. In each cluster, Z-scored data with a standard error bar were plotted according to developmental stages. The number of genes in the cluster is indicated at the upper left side. The calculated percentage of anther-enriched genes in each cluster is shown at the lower right side. The high synchronous gene expression pattern between microspore/pollen and tapetum is shown by red circles in clusters 2 and 20. The low synchronous pattern is shown by blue circles in clusters 3, 7, 12, 13, 16 and 17.
When we focused on the tapetum transcriptome, genes in clusters 12, 20 and 2 were preferentially expressed only at one stage (MEI, TET and UN stages, respectively), suggesting that the biological functions of tapetum genes differed in the three stages. In other clusters, genes in cluster 7 were predominantly expressed in both MEI and TET stages, while those in clusters 13, 14, 15 and 18 were expressed continuously in all the three stages.

In contrast, analysis of the microspore/pollen transcriptome revealed complex profiles. Expression patterns in the BC pollen were variable in some clusters, e.g. in cluster 1, expression was high in the UN microspore, gradually down-regulated in the late BC pollen, and low in the TC pollen. Such gradual down-regulation within the BC stage was also observed in clusters 15 and 16, whereas gradual up-regulation was seen in clusters 5, 6, 10, 11 and 19. In view of these patterns, it would be possible to divide the BC stage in the pollen transcriptome into early BC and late BC stages.

Throughout the developmental stages from MEI to TC, there are many expression patterns among the microspore/pollen genes. For example, gradual up-regulation of genes from MEI to TC was observed in clusters 5 and 6, while gradual down-regulation occurred in cluster 16. In the case of clusters 9 and 19, genes were gradually down-regulated from MEI to UN, but then gradually up-regulated again from BC to TC pollen. Another pattern was shown in cluster 3, with constant expression in the microspore/pollen at the UN to TC pollen stages. The variety of expression profiles in the

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**Fig. 2** Heat map view of the microspore/pollen-specific clade (A) and tapetum-specific clade (B). Microspore/pollen- and tapetum-specific clades were extracted from clusters 3 and 2, respectively. Red indicates higher, while green represents lower expression.

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microspore/pollen indicated that biological events in the male gametophyte are developmentally regulated by many anther-expressed genes with various expression patterns.

Interestingly, synchronous gene expression in both microspore and tapetum was observed in eight clusters: Nos. 2 and 20 with a high degree of synchrony (indicated by the red color in Fig. 1), and Nos. 3, 7, 12, 13, 16 and 17 with low synchronization (indicated by the blue color). These synchronous profiles cannot be attributed to contamination artifacts during the LM procedure, because microspore-specific and tapetum-specific clades could be identified (Fig. 2, Suwabe et al. 2008), and the expression patterns of microspore and tapetum were fundamentally independent in the other 12 clusters. The number of genes classified into the eight synchronous clusters was 10,810 (38.4%). Synchronous expression in the microspore/pollen and tapetum is one of the major features of the expression profiles in anthers, and argues against the conventional idea that tapetum cells function solely as nurse cells for feeding microspores in anther locules, with genes in different categories being expressed in the microspore and tapetum. The finding that synchronous transcription of some groups of genes occurs in both the microspore and tapetum suggests that these cells do share many developmental pathways as described in Scott et al. (2004), and is also consistent with the fact that both the microspore and tapetum are derived from the same cell lineage, L2.

Characterization of specific gene clusters based on GO

Genes with functional annotation were classified in each cluster based on GO by using Gene Ontology Slim terms (http://www.tigr.org/tdb/e2k1/osa1/batch_down load.shtml). We focused on five categories with likely functional significance in anther development: transcription, translation, lipid metabolism, secondary metabolism and the cell cycle (Fig. 4).

Genes related to transcription occupied 1.8–7.2% of genes in each cluster, and were frequently observed in clusters 7, 13, 14 and 20 (6.0–7.2% in each cluster, Fig. 4). These clusters were all characterized as tapetum expression, indicating that transcriptional regulation might be significantly active in the tapetum. In particular, clusters 13 and 14, showing similar expression profiles, included 312 genes (28.0%) out of the 1,114 transcription-related genes in all 20 clusters. Clusters 13 and 14 contained various transcription factor genes, with annotations including bHLH, MYB, GRAS, MADS, HD-Zip and Zn-finger-type transcription factors.

Translation-related genes were frequently observed in clusters 1 and 4 (6.3 and 7.3% in each cluster, respectively); the two clusters contained 149 genes (34.6%) out of the 431 translation-related genes in all the clusters. Common characteristics of the expression profiles in clusters 1 and 4 were higher gene expression in early BC pollen. The profiles in the clusters with a high percentage of transcription-related genes (Nos. 7, 13, 14 and 20) and translation-related genes (Nos. 1 and 4) were considerably different; in particular, the expression profiles of clusters 4 and 14 were almost opposite (Fig. 1). Thus, translation in the BC pollen might be achieved using transcripts produced in earlier stages of pollen (TET and UN microspores).

Lipid metabolism is important in pollen development, and failure of lipid synthesis frequently causes male sterility (Mariani and Wolters-Arts 2000, Ariizumi et al. 2003). In our GO analysis, lipid metabolism genes often appeared in cluster 2, whose profiles were characterized by high expression in the UN microspores and tapetum. This indicates that regulation of lipid metabolism-related genes might function at the UN stage. Several fatty acid synthesis genes, such as genes for β-ketoacyl-CoA synthase and β-keto acyl reductase, were also observed in clusters 13, 14 and 15 (described further in the following section), although the percentage coverage of lipid metabolism genes in these clusters was not so high.

Genes related to secondary metabolism were the most frequently observed in clusters 2 and 20 (3.9 and 3.2% in each cluster), including chalcone synthase, chalcone reductase and phytoene synthase. These two clusters were categorized as showing synchronous expression in the TET or UN microspore and tapetum.

A relatively small portion of cell cycle-related genes (0.0–1.4%) was categorized in the GO analysis. However, most of these (19.8%; 17 genes out of all the 86 cell cycle genes) were included in cluster 17. This might be due to the existence of genes related to meiosis, because notable gene expression was observed at the MEI stage in cluster 17. Genes annotated as cyclin, cyclin–F-box and rad51/recA were identified in cluster 17.

Functional relationships between other characteristic genes in each cluster and biological events in the microspore/pollen and tapetum

During the maturation of anther tissues, many characteristic biological events can be related to the microspore/pollen and tapetum transcriptomes. For example, important biological events for the male gamete are pollen maturation, pollen germination and pollen tube elongation. To date, many pollen-specific genes, related to carbohydrate metabolism (glycosyltransferase, glycoside hydrolase, sugar transporter, etc.), cytoskeleton re-organization (actin), Ca2+ signaling (calmodulin) and tip growth (small GTPase), have been identified and characterized in terms of these biological events (Franklin-Tong 1999, Endo et al. 2000, Endo et al. 2002, Golovkin and Reddy 2003, Hony and Twell 2003, Endo et al. 2004, Jiang et al. 2005, Huang and Yang 2006, Malhó et al. 2006, Shimamura et al. 2007, Cheung and Wu 2008). These genes, whose transcripts are present in mature pollen grains and are translated during pollen germination (Hoekstra and Bruinsma 1979, Mascarenhas et al. 1984, Schrauwen et al. 1990), were confirmed in our study to be predominantly
expressed in the late BC and TC pollen (clusters 5, 6, 10, 11 and 19).

During pollen tube elongation, the secretion of products to the tube tip is important, but only a few genes involved in the transport of secretory vesicles during tube growth have been identified to date (Sanderfoot et al. 2001). In our array data, genes encoding secretory vesicle molecules (SNARE, SNAP, synaptobrevin, clathrin, etc.) were identified in the late BC and TC pollen (clusters 5, 6, 10, 11 and 19). This new finding should be important in understanding the molecular machinery for vesicle trafficking in the pollen tube.

Clusters 13, 14 and 15 showed similar predominant expression in the MEI, TET and UN tapetum, and contained known genes related to pollen wall formation and fatty acid synthesis. Rice WDA1 (Jung et al. 2006), which is involved in the biosynthesis of very long chain fatty acids in the anther and is necessary for pollen exine formation, was categorized in cluster 14. As potential orthologs of known fatty acid biosynthesis genes, genes similar to Arabidopsis CER6/CUT1 (Fiebig et al. 2000) and FIDDLEHEAD (Pruitt et al. 2000) were categorized in cluster 13, while genes homologous to maize glossy8 (Xu et al. 1997) and Arabidopsis ECR (Zheng et al. 2005)
were in cluster 15. The tapetum cells have a central role in pollen wall formation, and transfer lipids onto the exine surface. Transcriptional regulation of the lipid-related genes observed in these clusters is undoubtedly important in male gametophyte development.

Clusters 13, 14 and 15 also contained ethylene signaling-related genes (ethylene receptor genes, EIN2, EIN3, EIL3, ETO1, ERF genes, etc.) and ubiquitin–proteasome system-related genes (genes for 26S proteasome, ubiquitin, E2 (E3)-ligase, F-box protein, SUMO, etc.). Ethylene is an important phytohormone in senescence, fruit ripening and stress responses, and it has been reported that ethylene signaling is regulated by the proteolytic degradation of transcription factor EIN3 (Guo and Ecker 2004, Schwechheimer and Villalobos 2004). Taken together, involvement of these sets of genes in clusters 13, 14 and 15 suggests that the phenomenon of gradual tapetum shrinking from the MEI to UN stages may be regulated by ethylene-mediated senescence-like events in the tapetum.

The argonaute gene and micro RNAs (miRNAs) were observed in 13 clusters (clusters 3, 5, 7–13, 15, 16, 18 and 19). The expression profiles of these clusters were diverse, with active transcription patterns distributed through all the developmental stages of the microspore/pollen and tapetum. The argonaute proteins are integral players in all known small RNA-directed regulatory pathways (Vaucheret 2008), and miRNAs have emerged as key guide molecules in the regulation of various biological processes (Ramachandran and Chen 2008). Thus, our LM-microarray analysis indicated that transcriptional regulation by small

![Table 1](https://example.com/table1.png)

Table 1: Representative common motifs in the 140 male gamete- and tapetum-expressed genes.

| Motif | Factor or site name | Description |
|-------|---------------------|-------------|
| CANNTG | EBOXBNNAPA | E-box |
| YACT | -- | -- |
| AAAG | DOFCORE2M | Core site required for binding of Dof proteins |
| CAAT | CAATBOX1 | CAAT promoter consensus sequence in the legA gene |
| GRWAAW | GT1CONSENSUS | Consensus GT-1-binding site in many light-regulated genes |
| GATA | GATABOX | GATA motif in the CaMV 35S promoter |
| TGAC | WRKY71OS | A core of the TGAC-containing W-box |
| GTGA | GTGANTG10 | GTGA motif in the promoter of the tobacco late pollen gene g10 |
| ATATT | ROOTMOTIFTAPOX1 | Motif in promoters of rolD |
| AGAAA | POLLEN1ELELAT52 | One of two co-dependent regulatory elements responsible for pollen-specific activation of tomato LAT52 gene |
| ACGT | ACGBTATRED1 | Required for etiolation-induced expression of erd1 in Arabidopsis |

Fig. 4 Representative results of the GO classification in each cluster. In each category, clusters with a high percentage coverage, shown in Fig. 3, are highlighted. For the lipid metabolism process category, we selected three additional clusters (Nos. 13, 14 and 15). They are indicated by asterisks in the three cells.
Characterization of cis-elements in the male gamete- and tapetum-specific genes

Using LM-microarray technology, we found that 28,141 genes were ‘anther-expressed’. Of these, 3,468 ‘anther-specific’ genes were identified by comparison with gene expression in vegetative tissues, as described above. We were interested in identifying specific cis-elements regulating spatiotemporal gene expression in the promoter regions of anther-expressed genes. A representative sample of 140 genes, characterized as male gamete-specific, tapetum-specific, and genes expressed in both tissues in our LM-microarray (Suwabe et al. 2008), were analyzed for cis-elements. A total of 164,188 cis-elements, classified into 256 cis-motifs, were found using the PLACE database. Among the 19 clusters, the frequency of the 11 common elements, as a percentage of the total cis-elements in that clusters, ranged from 51.2 to 86.3% (Table 1). Among these common elements, two were pollen-specific elements, GTGANTG10 and POLLEN1LELAT52. The GTGA motif in the promoter of the tobacco late pollen gene g10 was found to be an element responsible for gene expression in the late pollen stage (Rogers et al. 2001). The AGAAA motif is one of two co-dependent regulatory elements responsible for pollen-specific expression of the tomato LAT52 gene (Bate and Twell 1998). Because these two elements were originally characterized in tobacco and tomato, Solanaceae, and are also found as common cis-elements in pollen small cysteine-rich proteins in rice (Park et al. 2006), they can be regarded as common elements responsible for gene expression in pollen and widely conserved among plant species. By comparing cis-elements between the microspore/pollen-expressed clusters (Nos. 4, 5, 6, 8, 10 and 19) and the tapetum-expressed clusters (Nos 2, 7, 12, 13, 14, 15, 18 and 20), five male gamete- and 35 tapetum-specific cis-elements were found (Table 2). They included various kinds of motif elements from a wide variety of plant species, but we are far from deducing how male gamete- or tapetum-specific gene expression is controlled by these cis-elements. Interestingly, >130,000 elements were found in cluster 15, and the ATATT motif accounted for approximately 70% of elements in this cluster. In contrast, the ATATT motif was not found in any of the cis-elements identified by the PLACE database in clusters 7 and 9, even though synchronous and predominant gene expression at MEI and TC pollen stages, was observed in microspore and tapetum in clusters 7 and in 9, respectively. It is difficult to rule out experimental bias in these clusters, but the difference in cis-element frequencies might truly reflect biological characteristics. As discussed above, there were many kinds of cis-elements identified, indicating considerable complexity in the regulation of gene expression during male gamete and tapetum development.

Characterization of Tos17 insertion mutant lines in male gamete- and tapetum-specific genes

As a systematic rice mutant resource, Tos17 insertion lines are convenient for field cultivation, although there is preference for a Tos17 insertion sites within the rice genome (Miyao et al. 2003). By using the Tos17 mutant panel database (http://tos.niasaffrc.go.jp/), Tos17 mutant lines were surveyed among the 140 representative genes (Suwabe et al. 2008). A total of 114 independent lines were identified among 17 of these genes (12.1%). From the mutant phenotype descriptions available on the database, several phenotypes were observed (e.g. sterile, low fertility, semi-dwarf, dense panicle and pale green leaf). In particular, nine lines (52.9%) showed sterile or low fertility phenotypes, indicating that these genes are likely to be important in pollen development (data not shown).

Conclusion

In this study, using cluster analysis, we found various spatiotemporal expression patterns in anther-expressed genes. Interestingly, many genes were synchronously expressed in microspore/pollen and tapetum. This synchronous expression in different cell types should shed light on the mechanisms of anther development.

These global gene expression data from the LM-array in microspore/pollen and tapetum will form an important basis for future research in plant reproduction, as well as other fields in plant science. In order to utilize this large body of data effectively, it will be necessary to combine several methodologies (e.g. in silico, morphological, genetic and biochemical analyses).

Materials and Methods

Plant materials

Rice (O. sativa L. ssp. japonica cv. Nipponbare) plants were grown in a greenhouse under normal conditions. Developing anthers at each of five stages, MEI, TET, UN microspore, BC...
Table 2

| Motif          | Factor or site name | Description                                                                 |
|----------------|---------------------|-----------------------------------------------------------------------------|
| Tapetum        |                     |                                                                             |
| AATATTTTATT    | AT1BOX              | AT-1 box (AT-rich element)                                                   |
| AATGGAAATG     | mRNA3ENDTAH3        | Cis element in the 3′ end region of wheat histone H3 mRNA; 3′ end formation |
| ACGTCATAGA     | LS7ATPR1            | A positive salicylic acid-inducible element in the Arabidopsis PR-1 gene promoter |
| AGACCGTTG      | MSAACRCYM           | Motif in the Catharanthus roseus B-type cyclin (CYM) promoter               |
| ATAGAAATCAG    | BOX1PGS2            | Box 1 element in the pea glutamine synthetase (GS2) gene                    |
| ATCATTTTACT    | RBCSBOX3PS          | 5′ upstream region (−114) of the pea rbcS5 gene                             |
| ATGTACGAAGC    | ABAREG2             | Motif related to ABA regulation                                              |
| CAACCTCTAT     | RSPEVGRP1           | RSE (root-specific element) of the bean GRP1.8 gene                         |
| CAATWATTG      | ATHB1ATCONSSENSUS   | Recognition sequence of Arabidopsis Athb-1 protein                         |
| CAGAAAGATA     | RGATAOS             | R-GATA (GATA motif-binding factor)-binding site                             |
| CATGGGCCGGG    | RE1ASphyA3          | RE1 (putative repressor element) responsible for Prf-directed repression of the oat phyA3 phytochrome gene |
| CATTAATTAG     | GMHDLMVSPB          | Binding site of the soybean homeodomain leucine zipper proteins (GmHdIL5, GmHdIL57) |
| CCACACCCCCC    | ACIIPVPA2           | ACI element found at −131 to −120 of the bean PAL2 promoter                 |
| CCTACACNNNNNCT | HBOXCONSUSPVCHS     | Consensus sequence of H-boxes in the bean chs15 gene promoter              |
| CCTACCNNNNNCTCPNNNA | HBOXPVCHS15     | Binding site of two bean protein factors, KAP-1 and KAP-2                 |
| CTTTCCGTAC     | GLUTECOREOS         | Core site required for binding of the trans-acting factor in the promoter region of rice glutelin (type 2) |
| GAAWTTGTGA1    | S659BOXLELAT5659    | A sequence motif shared between the tomato LAT56 and LAT59 promoters        |
| GATCATCGATC    | RNFG1OS             | RNFG1-binding site                                                          |
| GCCACCTCAT     | O2F2BE2S1           | opaque-2 recognition site F2 in Bertholletia excelsa 2S storage protein gene |
| GCCACGTACA     | ABRE3HVA22          | ABA-responsive element, ABRE3                                               |
| GCCACGTGGG     | ABREAZMRA28         | ABA-responsive element, ABRE A                                               |
| GCCACTTGTG     | ABREDISTBBNNAPA     | dist B (distal portion of the B-box) in the napA gene of Brassica napus      |
| GCCGGAAA       | E2F1OSPCNA          | re2f-1 in the promoter of the rice PCNA gene                                |
| GCCGTAATT      | GT2OSPHYA           | GT-2- (a rice nuclear protein) binding site in the rice phyA promoter       |
| GCTAAACAAT     | BOXSITE1STPAT       | 10 bp motif (site 1) within the B-box in the potato patatin gene promoter    |
| GGCCCGGGG      | AMMoresVDCRNIA1     | Motif (VD) in the Chlamydomonas Nia1 gene promoter                          |
| GGTCAANNAGT    | ELENTCHN50          | Elicitor-responsive element (EBE) in the tobacco basic class I chitinase gene |
| GTACGGTGGGG    | ACGTSEED            | ACGT motif related to seed expression                                        |
| NNWNCWAVWVWTRGWWAN | BOXIIIPCCHS-like | Core of box II/G box in the parsley chs genes                               |
| RTACGGTGCCR    | OCTYPEiINTHISTONE  | Oct-containing composite element type II in the tobacco histone gene promoter |
| TACGGCGGTAC    | OCETYPEiiNTHISTONE |                                                                              |
| TGCCACGCG      | ABRECIIHVA22        | CE1 (coupling element 1) of the barley HVA22 gene                           |
| TGGGCCTC      | SITEIIAOSPCNA       | Site Ia of rice PCNA (proliferating cell nuclear antigen) gene              |
| TGGTCCACCAC   | SITEIIbosPCNA       | Site Iib of rice PCNA (proliferating cell nuclear antigen) gene             |
| Male gamete    |                     |                                                                             |
| ACCTATTTAAA    | CONSERVED11NTZMATP1 | Conserved 11 nt sequence in maize                                           |
| GCGGGCGGGG    | ABREMTIIOSRAB168    | Motif III in the promoter of the rice rab168 gene                           |
| GCTCCTTGG     | VSF1PVPGRP1         | VSF-1-binding site in the French bean gpl1.8 gene promoter                  |
| TAGTGCTGT     | D4GMAUX28           | DNase I-protected sequence in the soybean auxin-responsive gene, Aux28, promoter |
| TCTACGTCAC    | LSSATPR1            | A negative regulatory element in the Arabidopsis PR-1 gene promoter         |
pollen and TC pollen, 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 Suwabe et al. (2008) in this issue.

**Laser microdissection**
The fixation, dehydration and paraffin embedding of anthers were described in Suwabe et al. (2008) in this issue. Briefly, paraffin-embedded sections were cut to a thickness of 16 μm and mounted on PEN membrane glass slides (Molecular Devices, Ontario, Canada) for LM. To remove paraffin, slides were immersed in 100% xylene (twice), 50% xylene/50% ethanol (v/v) and 100% ethanol for 5 min for each step, 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). The target cells that fused to the CapSure Macro LCM cap (Molecular Devices) were collected by removing the cap from the tissue section.

**Microarray and cluster analysis**
Microarray analysis was performed as described by Suwabe et al. (2008) in this issue. Briefly, total RNAs were extracted from LM cells with a PicoPure™ RNA isolation kit (Molecular Devices), and were 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) with ∼42,000 oligonucleotides based on the Rice Annotation Project (RAP) was used. Fluorescent probe labeling using the oligo-dT-T7 strand-specific amplification method and hybridization were performed according to the manufacturer’s instructions (Agilent Technologies) with slight modifications. The slide images were scanned with a DNA microarray scanner (Agilent Technologies, http://www.home.agilent.com/) using the manufacturer’s Feature Extraction software. Microarray experiments using each stage sample were replicated at least three times.

Microarray data were statistically analyzed by the variance stabilization normalization (VSN) method using R software (http://www.r-project.org/). Normalization by VSN, Z-scoring and one-way ANOVA analysis of variance were performed by Bioconductor (http://www.bioconductor.org/). Advanced data analyses including cluster analysis (K-means) were performed using Microsoft Excel and TIGR MeV (Saeed et al. 2003; http://www.jcvi.org/cms/research/software/#c622).

**Gene ontology analysis**
GO Slim terms for genes (probes) were obtained from the TIGR database site (http://www.tigr.org/tdb/e2k1/osa1/batch_download.shtml). To obtain GO terms, locus identifiers (IDs) of TIGR were assigned for probes by the RAP-DB and our perl scripts. For each cluster, GOSlim terms for locus IDs of TIGR were searched by the TIGR web site and counted.

**Cis-element analysis**
For identifying the cis-elements of anther-specific genes, the promoter region of each gene was compared with the PLACE database (http://www.dna.afrc.go.jp/PLACE/). Identified cis-elements were classified by sequence similarity.

**Identification of Tos17 insertion mutant lines**
The Tos17 mutant panel database (http://tos.nias.afrc.go.jp/) was used for identification of Tos17 insertion mutant lines. The number of mutant lines was counted, and their mutant phenotypes were surveyed.

**Supplementary Material**
Supplementary Material are available at PCP Online.

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