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Monitoring of gene expression profiles and isolation of candidate genes involved in pollination and fertilization in rice (*Oryza sativa* L.) with a 10K cDNA microarray

Lefu Lan[1,+], Wei Chen[1,+], Ying Lai[1], Jinfeng Suo[1], Zhaosheng Kong[1], Can Li[2], Ying Lu[2], Yujun Zhang[2], Xiangyu Zhao[3], Xiansheng Zhang[3], Yansheng Zhang[1], Bin Han[2], Jing Cheng[4] and Yongbiao Xue[1,*]

[1]*Institute of Genetics and Development Biology, Chinese Academy of Science and National Center for Plant Gene Research, Beijing 100080, China (*author for correspondence; e-mail ybxue@genetics.ac.cn);* [2]*National Center for Gene Research, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200233, China;* [3]*College of Life Sciences, Shandong Agricultural University, Taian 271018, China;* [4]*Department of Biological Science and Biotechnology, Tsinghua University and Beijing National Biochip Research and Engineering Center, Beijing 100084, China;* [+] *these authors contributed equally to this work*

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Abstract

To monitor gene expression profiles during pollination and fertilization in rice at a genome scale, we generated 73 424 high-quality expressed sequence tags (ESTs) derived from the green/etiolated shoot and pistil (0–5 h after pollination, 5hP) of rice, which were subsequently used to construct a cDNA microarray containing ca. 10 000 unique rice genes. This microarray was used to analyze gene expression in pistil unpollinated (UP), 5hP and 5DAP(5 days after pollination), anther, shoot, root, 10-day-old embryo (10EM) and 10-day-old endosperm (10EN). Clustering analysis revealed that the anther has a gene-expression profile more similar to root than to pistil and most pistil-preferentially expressed genes respond to pollination and/or fertilization. There are 253 ESTs exhibiting differential expression ($e \pm 2$-fold changes) during pollination and fertilization, and about 70% of them can be assigned a putative function. We also recovered 20 genes similar to pollination-related and/or fertility-related genes previously identified as well as genes that were not implicated previously. Microarray and real-time PCR analyses showed that the array sensitivity was estimated at 1–5 copies of mRNA per cell, and the differentially expressed genes showed a high correlation between the two methods. Our results indicated that this cDNA microarray constructed here is reliable and can be used for monitoring gene expression profiles in rice. In addition, the genes that differentially expressed during pollination represent candidate genes for dissecting molecular mechanism of this important biological process in rice.

Introduction

Pollination and fertilization are the key steps leading to seed formation. Almost 80% of the world's foods are derived from seeds, including such staple crops as maize, wheat and rice. Pollination in flowering plants begins when pollen land on a stigma. After hydration and germination, the pollen tube carrying sperms goes a long journey to reach the ovary where double fertilization occurs. Thus, pollination represents an attractive model system for investigation of polarized tip growth, cell-to-cell interactions, and signal transduction (Franklin-Tong, 1999).

The pollen coat contains many molecules involved in the initial interaction with the stigma

(Dickinson and Elleman, 2000). In *Arabidopsis*, lipophilic molecules in the pollen exine mediate pollen–stigma adhesion (Zinkl *et al*., 1999). After adhesion, the pollen grain hydrates. This step is somewhat aided by the presence of pollen coat, which contains predominantly lipases and oleosins in *Arabidopsis* (Mayfield *et al*., 2001). Wolters-Arts *et al*. (1998) showed that specific lipids play a role in providing a directional supply of water which is important for the pollen tube to penetrate through the stigma. Although the aquaporin described by Ikeda *et al*. (1997) has been specifically implicated in the control of self-incompatibility, aquaporin-like proteins in the stigma may act as water channels in controlling water flow into the grain from the stigma (Dixit *et al*., 2001). Once pollen is correctly hydrated it must germinate. Phytosulfokine, a 5 amino acid sulfated peptide, is capable of inducing pollen germination (Chen *et al*., 2000). Importantly, Rop GTPase has been shown to play an important role in regulating Ca^{2+}-dependent pollen tube growth (Li *et al*., 1999). In the transmitting tract, arabinogalactans such as *Nicotiana* TTS1 and TTS2 proteins are secreted into the extracellular matrix and provide nutritional and guiding cues that support tube migration (Wu *et al*., 2001). Adhesion of pollen tubes to the transmitting tract is regulated in lily by a 9 kDa lipid transfer protein (Park *et al*., 2000). Parallels between mechanisms involved in pollen tube and axon guidance have recently been reviewed by Palanivelu and Preuss (2000). A recent study demonstrated that *POP2*, encoding a class III transaminase, regulates the pollen tube growth and guidance by controlling GABA (γ-aminobutyric acid) levels (Palanivelu *et al*., 2003).

Although an impressive progress has been made in this area, relatively little is known about the overall processes that regulate pollination and fertilization, the essential first step in seed formation. Recently, microarray technology has emerged as a powerful tool that allows simultaneous monitoring the expression levels of numerous genes. Many reports of the use of microarray analysis in plants have been published and provide important information on the genome-wide regulatory networks of a particular response (Schena *et al*., 1995; Ruan *et al*., 1998; Ma *et al*., 2001; Kawasaki *et al*., 2001; Oono *et al*., 2003). Previously, we performed a preliminary study on genes involved in pollination in rice with a cDNA-AFLP technique and several candidate genes were subsequently identified (Chen *et al*., 2001). To extend this investigation, in this study, we generated 73 424 high-quality expressed sequence tags (ESTs) from rice, which is the most important food crop for more than half of the world's population and also the major model species for cereal genome research. Subsequently, a cDNA microarray containing about 10 000 unique rice genes was constructed. Here, we report the initial analysis of a genome-wide gene expression in response to pollination and fertilization in rice.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. ssp. *indica*) roots, green shoots and etiolated shoots were harvested from 7-day-old plants grown in a greenhouse. The other plant materials used in this study described below were harvested from rice grown in field. Unpollinated pistils (UP) and anthers were collected at a stage 1–2 days before floret flowering; 5hP (pistils 0–5 h after pollination) and 5DAP (5 days after pollination) were dissected out 0–5 h and five days after anthesis, respectively; 10-day-old embryos and endosperms were dissected from grains 10 days after flowering.

Immunofluorescence microscopy

Rice pistils were collected 0–5 h after anthesis for each time point, stained with aniline blue, and observed under a fluorescent microscope.

RNA isolation and cDNA library construction

RNA was isolated with the RNeasy kit (Promega). Isolation of poly(A)$^+$ RNA from the total RNA was performed with the Qiagen mRNA Extraction Kit. The cDNA libraries were constructed from 5hP (pistil 0–5 h after pollination, Figure 1) and 7-day-old green/etiolated shoots with a SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA) and the cDNA clones were converted to plasmid according to manufacturer's instructions. The titer was estimated at 1.2×10^6 pfu for the 5hP library and 1×10^6 pfu for the shoot library. An average insert size of 800 bp in the 5hP library

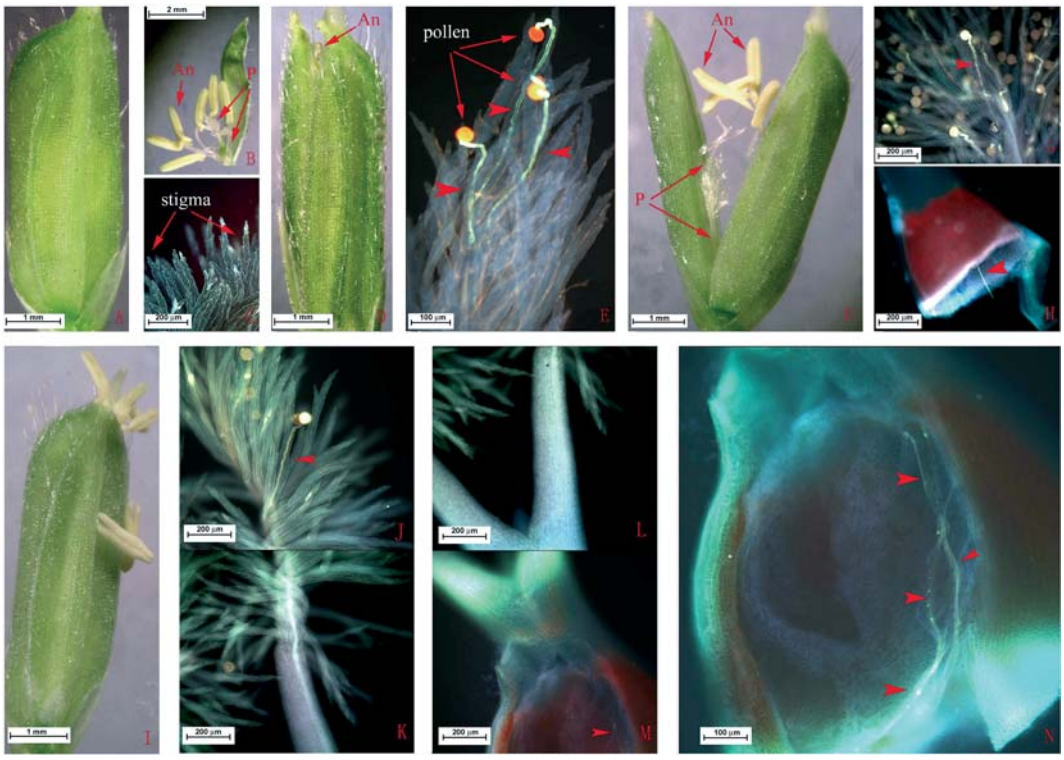

Figure 1. Rice florets before and after pollination. Except for A, D, F and I, the pistil was stained with anilin-blue solution and observed under fluorescence microscope. (A) A floret before anthesis. (B) Anatomy of the floret in stage A; An, anther; Pi, pistil. (C) Part of the stigma at stage A. (D) Prior to anthesis, the tips of the lemma and palea begin to open. (E) Part of the stigma in stage D. (F) Maximum opening of the floret. (G) Part of the stigma at stage F. (H) Part of the ovary at stage F.(I) An closed floret. (J) Part of the stigma at stage I. (K and L) Part of the style at stage I. (M and N) Part of the ovary at stage I. Large arrowheads indicate the pollen tube; Bars = 100 μm (E and N), 200 μm (C, G, H, J, K, L and M), 1 mm (A, D, F and I), 2 mm (B).

and 900 bp in the shoot library was calculated based on analysis of 96 randomly picked cDNA clones.

EST sequencing, assembly and sequence analysis

A total of 23 040 and 22 080 cDNA clones were randomly selected from 5hP and shoot cDNA libraries respectively and sequenced from both 5′ and 3′ ends. The sequence primers were 5′-TCCGAGATCTGGACGAGC (5′ primer) and 5′-TAATACGACTCACTATAGGGC (3′ primer). DNA templates (plasmid) for sequencing were isolated by using a 96-well alkaline lysis miniprep and purified through a 96-well MultiScreen filter (Millipore). Sequencing reactions were carried out with the DYEnamic ET dye terminator kit (MegaBACE, Amersham Pharmacia Biotech) and analyzed on a MegaBACE 1000 sequencer. A total of 88 387 individual ESTs were obtained (http://ncgr.ac.cn/EST.html). In order to identify ESTs derived from the same genes, we used CAP3 (Huang and Madan, 1999) to organize redundant ESTs into overlapping contigs, resulting in a total of 7258 contigs. After removing poor-quality sequences (i.e. EST sequence of 100 nucleotides or less and with a Phred score <20), a total of 4613 individual singletons was retained. In summary, 11 871 unique sequences (4613 singletons and 7258 contigs) were obtained. Homology searches were performed with the BLAST program (Altschul *et al*., 1990).

Preparation of cDNA microarrays

The cDNA clones corresponding to 11 871 putative unique sequences were selected and their inserts amplified by PCR. The primers were 5′-CAAGCTCCGAGATCTGGACGAGC (forward primer) and 5′-TAATACGACTCACTATAGGGCGAA (reverse primer). Plasmid templates

(1–2 ng) were added to 100 μl of a PCR mixture containing 0.25 mM of each nucleotide, 0.2 μM of each primer, 1×Ex *Taq* buffer (Takara Shuzo, Kyoto, Japan), and 1.5 units of Ex *Taq* polymerase (Takara Shuzo). PCR was performed as follows: at 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 3 min; and 72 °C for 10 min. To clean up PCR products and prepare the DNA for printing, we precipitated PCR products in isopropanol and re-suspended the DNA in 20 μl 50% DMSO. Of each finished reaction 1 μl was electrophoresed on a 1% agarose gel to confirm amplification and quantity. PCR products <100 ng/μl or showing several DNA fragments were not considered for further use (ca. 15% of 11 871 clones). If possible, we re-amplified them or replaced them with alternative clones. As a result, about 96.5% of 11 871 clones were finally utilized in the experiment and the average length of the PCR product was ca. 850 bp based on analysis of 384 randomly picked clones. Further BLAST analysis showed that ca. 10 000 sequences were indeed unique and the rest (ca. 1000) redundant. Therefore, the unique sequences most likely derived from about 10 000 individual unique rice genes, and the microarray was thus referred to as 10K array. The identity of 109 randomly picked plasmid samples was confirmed by re-sequencing, and 104 sequences matched their original EST sequences, but five were misidentified, which gave rise to a duplication error rate of about 5%.

PCR products and Lucidea Microarray ScoreCard reagents were arrayed from a 384-well microtiter onto a poly-lysine-coated micro slide glass (Amersham Pharmacia Biotech) with an Array Spotter Generation III (Amersham Pharmacia Biotech). The printed slide were dried and subjected to UV cross-linking. For more details about Lucidea Microarray ScoreCard reagents, we refer to the Lucidea Microarray ScoreCard user manuals.

Labeling, hybridization, washing, scanning and data analysis

Labeling was performed with a CyScribe Post-Labeling Kit (Amersham Biosciences). Hybridization and washing were performed as described for the CyScribe Post-Labeling Kit and the CMTTM Hybridization Chamber (Corning) user manuals. Data acquisition and analysis were performed on a GenePix 4000B scanner with GENEPIX 3.0 software (Axon Instruments). We used GProcesser 1.0 (http://keck.med.yale.edu/biostats/software.htm) to normalize (loess methods) and merge the replicated GenePix Pro 3.0 output data. With this program, we pooled replicated data sets of each experiment. First, spots flagged Bad or Not Found by GenePix software were removed from the final data analysis. Second, those spots that exhibited a large difference between the ratio mean and ratio median were considered as outlier and eliminated from the final data analysis. Third, only those spots that showed fluorescent intensity levels in at least one channel above three times the local background were used for further analysis. Fourth, only the results derived from at least two independent hybridizations were accepted in our study and the threshold for significantly differentially expressed gene set as $|\log_2 \text{ratio}| \geq 1$, combining a P value < 0.05 (t-test). Hierarchical clustering was performed as described by Eisen *et al*. (1998).

Real-time PCR

Reverse transcription was performed with the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). The cDNA samples were diluted to 1, 0.5, and 0.1 ng/μl. Triplicate quantitative assays were performed on 1 μl of each cDNA dilution with the SYBR Green Master mix with an ABI 7900 sequence detection system according to the manufacturer's protocol (Applied Biosystems); the gene-specific primers were designed by using PRIMEREXPRESS software (Applied Biosystems). The relative quantification method ($\Delta\Delta C_T$) was used to evaluate quantitative variation between replicates examined. The amplicon of 18S rRNA was used as an internal control to normalize all data.

Results and discussion

Microarray hybridization and reproducibility

We constructed a rice 10K cDNA microarray which allowed us to examine the expression of about 10 000 genes simultaneously. To assess the reproducibility of the microarray analysis and the use of 10K array to analyze a well-defined bio-

logical system, poly(A)$^+$ RNA from 7-day-old root and 7-day-old green shoot was labeled and hybridized with the 10K array for materials that are relatively easy to obtain. We performed the tissue comparisons five times as described in Table A1. Six dynamic range controls were used to evaluate the detection limits and dynamic range, the control mRNA (33, 10, 1, 0.33, 0.1, 0.033 ng) was added to 500 ng sample mRNA, thereby resulting in control mRNA to sample mRNA ratios of 6.6%, 2%, 0.2%, 0.066%, 0.02% and 0.0066%. Microarray hybridization, washing, scanning and data analysis were performed as described in Materials and methods. The data from different microarrays showed a good correlation (Table S1 and Figure A1), indicating an excellent reproducibility between slides. In spite of the high correlation, the results shown in Figure A1 also suggested that replicate experiments are needed to obtain the reliable microarray data since some cDNA clones showed different hybridization patterns in the replicate experiments. Moreover, DR (dynamic range control) and RC (ratio control), which were spotted at different positions on the same slide (Figure A2), showed similar hybridization patterns indicating the reliability of the data within the same slide.

There were 1098 ESTs preferentially expressed in shoot and 318 ESTs preferentially expressed in root, respectively (Table A2). As expected, a number of genes involved in photosynthesis and CO_2 fixation such as chlorophyll *a*/*b*-binding protein and small subunit of Rubisco were found to be expressed highly in the shoot (Table A3, also see Figure 2), whereas genes associated with defense/stress activity were expressed at a high level in root (Table A3). In fact, several of them have been verified experimentally in previous studies (Sakamoto *et al*., 1989; Hsieh *et al*., 1995; Huang *et al*., 1998; Higuchi *et al*., 2001; Ogihara *et al*., 2003). Thus we conclude that the 10K array developed in this study produces reliable gene expression profiles consistent with those achieved by other analytic methods.

Expression profiling of genes involved in pollination and fertilization

To monitor gene expression profiles of pollination and fertilization at a genome-wide scale in rice and to isolate pollination- and fertilization-associated genes, mRNA samples from UP (pistil unpollinated) and 5hP(pistil 0–5 h after pollination) were labeled and hybridized to the 10K array. To prepare mRNA sample from pollinated pistils, the time course of the pollen-tube growth in a rice pistil was examined (Figure 1). Pollination takes place immediately after the anthesis (Figure 1A–D), within 2–3 min, the pollen landing on stigma starts to germinate (Figure 1E). About 60 min

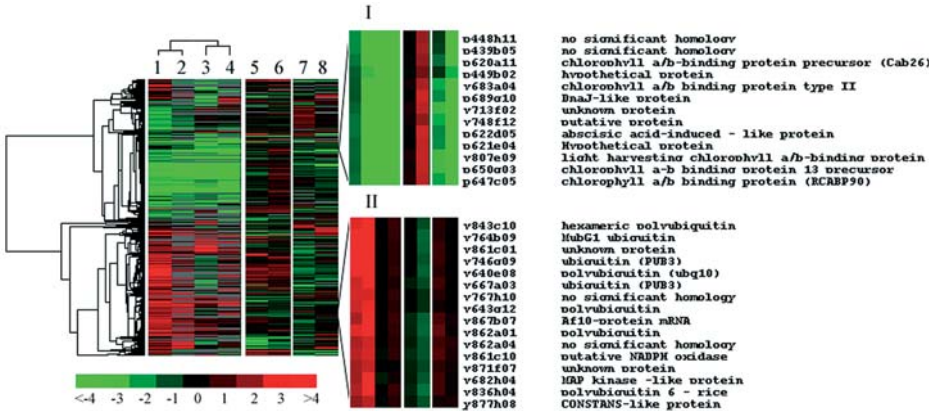

Figure 2. Hierarchical clustering analysis of 5133 differentially expressed transcripts. To ensure reproducible regulation profile, we selected the gene for cluster analysis if it shows significantly differential expression (set as two-fold) in at least one tissue comparison. Lanes: 1, 5hP/shoot; 2, UP/shoot; 3, anther/shoot; 4, root/shoot; 5, 5DAP/UP; 6, 5hP/UP; 7, 10EN/5DAP; 8, 10EM/5DAP. The fold changes were $\log_2$-transformed and subjected to average hierarchical clustering; up-regulated and down-regulated genes are shown in red and green, respectively: the higher the absolute value of the fold change, the brighter the color. The color scale is shown at bottom; grey represents missing values. I sub-cluster shows that some photosynthesis-related genes were induced in the 5hP tissue, II sub-cluster containing a group of ubiquitin genes which are highly expressed in pistil (UP and 5hP, shoot as a control).

after floret opening (Figure 1H), the pollen tubes reach the bottom of the ovary (Figure 1G–N). Fertilization usually occurs within 2–4 h after anthesis. A rice floret remains open for 1–2 h. In our study, 5hP pistil tissues were harvested 0–5 h after anthesis, thus they corresponded to the stages ranging from the release of pollen from the anthers to the penetration of the micropyle by the pollen tube tip. We performed a dye-swapped experiment and the sample duplicate experiment using the same techniques of the first dye-swapped experiment except that the RNA sample were independently isolated from the materials harvested at the same time. There were 370 differentially expressed ESTs detected in our study as described in Table A2. These include 170 down- and 200 up-regulated ESTs. Figure A3 shows the average hybridization signal intensity of 6DR, 5DR, 4DR, 3DR and 370 differentially expressed ESTs. More detailed information about the transcripts abundance and distribution of these genes are given in Table A4. There are 43.5% (74/170) ESTs in the down-regulated group and 55.5% (111/200) ESTs in the up-regulated group with their mRNA relative abundance less than 0.0066%, respectively. This suggests that despite their low levels of expression, they may play important roles in pollination and fertilization in rice and could be identified by our microarray analysis.

To further obtain the expression profiles of the 370 ESTs in a variety of tissues in rice, cy3- and cy5-labeled cDNA pairs of anther plus shoot, un-pollinated pistil (UP) plus shoot, 5hP pistil plus shoot, UP plus 5DAP pistil, 5DAP pistil plus 10-day-old embryo (EM) and 5DAP plus 10-day-old endosperm (EN) were prepared and hybridized to the 10K array as described for the UP vs. 5hP experiment. A good correlation between the direct and the indirect results demonstrated an internal consistency of the array data (Figure A4).

The numbers of cDNA clones with preferential expression ratios in various developmental stages or tissues are listed in Table A2. The set of genes highly expressed with a putative function is presented in Table A3. Our data indicated that tissue types could be classified on the basis of their specific patterns of gene expression and transcription accumulation. Figure 2 shows the relationship of the expression data from several tissue/organ comparisons, pistil gene expression profiles (shoot as a control) at two developmental states (UP and 5hP) grouped together as expected since there were a small number of genes differentially expressed in the 5hP vs. UP experiment (Table A2). Interestingly, anthers have a gene expression profile more similar to roots than to pistils, although anthers and roots are very different in developmental origin, functions and location during the rice life cycle. Maybe, at least in part, this is due to the fact that they are both non-photosynthetic organs. Moreover, some photosynthesis-related genes are up-regulated by pollination (Figure 2I). Although the style is not thought to be a photosynthetic organ, it contains well-developed chloroplasts, and indeed, enhanced photosynthetic flux was detected following pollination (Jansen *et al.*, 1992). The up-regulated expression of the photosynthetic genes in the pistil may coincide with floret opening and respond to an increase in available light, in addition to, or instead of, being attributed directly to sugars metabolic interactions between the style and the elongating pollen tube.

Cluster analysis of cDNA highly expressed in pistil tissue

As listed in Table S3, the putative functions of the tissue-preferentially expressed genes are closely related to the biological character of the tissues, respectively. It is tempting to suppose that some genes that are highly expressed in pistil may also be likely involved in pollination; thus, we performed cluster analysis of 46 genes which were highly expressed in the pistil [pistil (5hP or UP)/shoot>8 or UP/5DAP>8] (Figure 3). Of these 46 genes, 30 were derived from the 5hP library. Moreover, 10 cDNA clones corresponding to cluster I (Figure 3) were more highly expressed in 5hP tissue than in other stages (UP) and organ/tissue, and were all derived from the 5hP library. This indicates that it is important to construct a specialized cDNA library for identification of tissue- or organ-preferentially expressed genes. Among 46 pistil-abundant clones, 30 (65.2%) showed differential expression in the UP vs. 5hP experiment, indicating that most genes which were specifically/preferentially expressed in pistil tissue are responsive to pollination and fertilization in rice.

Twelve (26%) cDNA clones showed no significant homology to known sequences (blastx

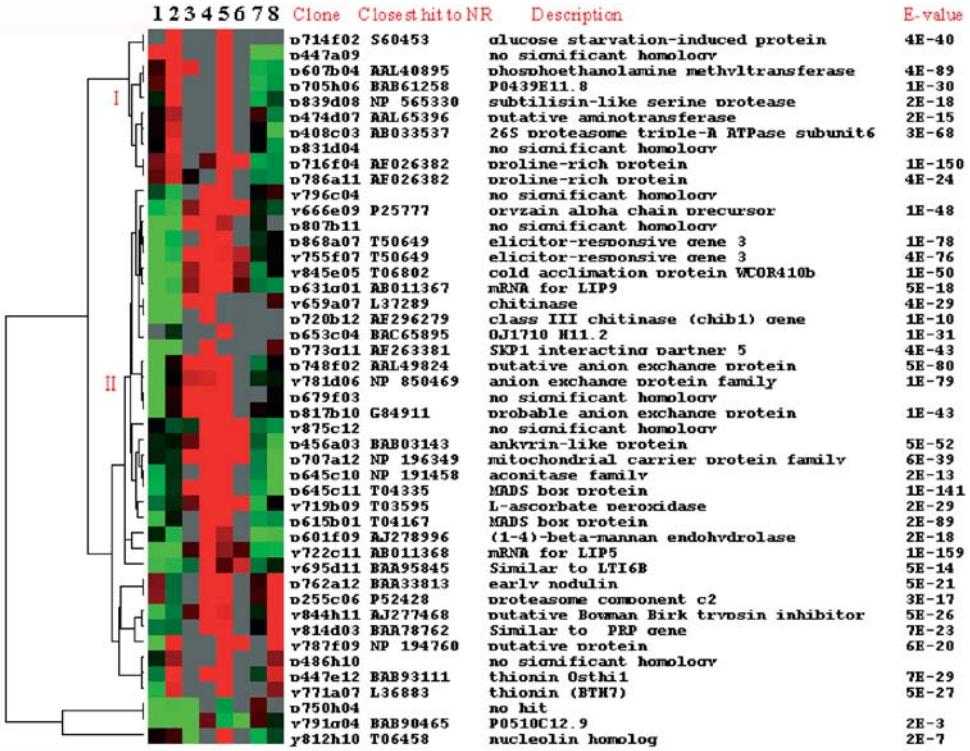

Figure 3. Clustering analysis of 46 genes that are predominantly expressed in pistil. Lanes: (1) 5DAP vs. UP; (2) 5hP vs. UP; (3), anther vs. shoot; (4), UP vs. shoot; (5), 5hP vs. shoot; (6), root vs. shoot; (7), 10EN vs. 5DAP; (8), 10EM vs. 5DAP. The clone and the protein which top hit in NR (non-redundant database) are shown. For other descriptions, see legend to Figure 2.

E-value > 1E−5) or were similar to proteins with unidentified functions and 16 (34.8%) showed database matches with defense/stress-related genes. Together, these genes may function in pathogenic defense, since exudates secreted from stigma tissues of the pistil provides a favorable environment for the growth of pathogens. Four low-temperature-induced proteins, which are predominantly expressed in UP, are all down-regulated by pollination. These include LIP9, LIP5, the low-temperature- and salt-responsive protein LTI6B, and the cold acclimation protein WCOR410b. It has been reported that some *cer* mutants are temperature sensitive and can be successfully pollinated at low temperature, indicating that temperature is critical during the time that hydration takes place (Hülskamp *et al*., 1995). In *Arabidopsis*, several *cer* mutants (*cer1*, *cer2*, *cer3*, and *cer6*) exhibit defective pollen recognition and failed pollen hydration and, consequently, are male-sterile (Preuss *et al*., 1993; Hulskamp *et al*., 1995). Maybe, the low-temperature-induced proteins are associated with necessary changes in osmotic tensions to facilitate rapid hydration/germination of pollen gains in rice.

Three clones matched to the genes (SKP1 interacting partner 5, proteasome component c2 and 26S proteasome regulatory particle triple-A ATPase) which function in the ubiquitin/proteasome protein degradation pathway preferentially expressed in pistil. Moreover, a group of ubiquitin genes are more highly expressed in pistil than in root and anther (Figure 2). Two members of MADS-box protein gene family were also present in this group, and several MADS-box proteins have been found to act as important regulators of inflorescence development, floral organ identity, ovule development, flowering timing and cell-type specification in floral organs (Jack, 2001; Favaro *et al*., 2003). Furthermore, four ESTs highly expressed in anther and pistil were grouped together (Figure 3, cluster II) with a high correlation ($r = 0.9747$). Three of them are highly similar to a putative/probable anion exchange protein that contains a highly conserved domain, pfam00955, $HCO3^-$ cotransp, belonging to the

HCO_3^- transporter family. This family contains Band 3 anion exchange proteins that exchange Cl^-/HCO_3^-. It has been reported that Cl^- ion dynamics is an important component in the network of events that regulate the pollen tube homeostasis and growth (Zonia *et al*., 2002). Clone p474d07, top hit to rice *POP*2 (AF251073) in NR (non-redundant database), highly expressed in 5hP. In *Arabidopsis*, *POP*2 regulates pollen tube growth and guidance by controlling GABA levels (Palanivelu *et al*., 2003).

Assessing the microarray data by real-time PCR

To confirm the differentially expressed genes identified by the microarray analysis, real-time (RT) PCR was used to examine the values selected based on the microarray data. Fifty-three genes representing a range of fluorescence signal intensity (Table A4) and expression profiles (Figure 4A) in the 5hP vs. UP experiment were subjected to analysis. There was a high degree of concordance ($r = 0.823$) between data generated by the two methods (Figure 4A). In addition, a high correlation ($r = 0.875$) between the results from the microarray and RT-PCR was observed (Figure 4B) for four genes, a non-specific lipid-transfer protein precursor gene (p596c09), an aquaporin gene (p811e06), a low-temperature- and salt-responsive protein LTI6B gene (y695d11) and an abscisic acid- and stress-induced protein gene (y831f12) (Tables A5 and A6), which were selected to evaluate the microarray data across the eight sets of tissue comparisons. Moreover, some randomly selected tissue- or organ-preferentially expressed genes also have been confirmed by RT-PCR analysis (Table A3). In summary, the validation tests indicate that differential expression by microarray analysis is highly predictive (>75% confirmation) of differential expression as determined by RT-PCR. The RT-PCR results of some genes were listed in Table A5.

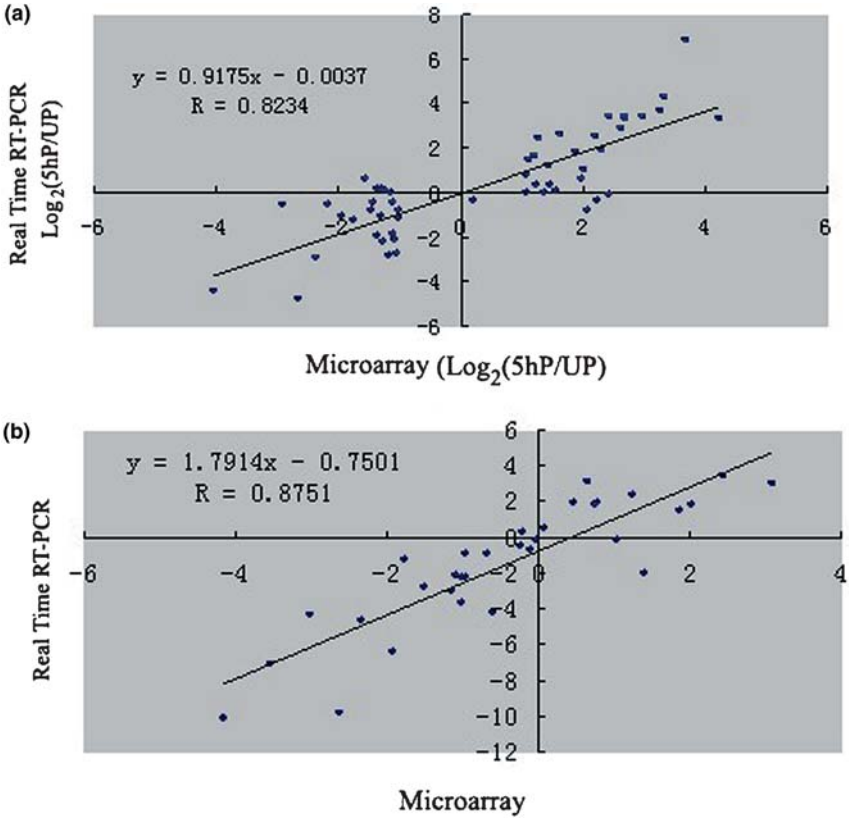

Figure 4. Correlation between the microarray and RT-PCR data. A. Log-transformed fold changes (base 2) for 52 differentially expressed genes and one constitutively expressed gene in the 5hP vs. UP experiment. B. Log-transformed fold changes (bases 2) for four genes in eight experiment sets.

Twenty-four genes (Table A4), whose relative abundances were less than 0.0066%, were selected to validate their expression based on the microarray experiment. The result showed a high correlation with RT-PCR data ($r = 0.8135$, similar to 0.823, a Pearson correlation factor between the microarray data and RT-PCR data in the 5hP vs. UP experiment, see Figure 4A). Moreover, a MADS box (MADS20) gene and a receptor-like protein kinase gene, transcripts with the lowest abundance (ca. 5 × less than 0.0066%) of the down-regulated gene and the up-regulated gene, respectively, were confirmed by RT-PCR (Table A5). Thus, the detection limit of our microarray system is around 0.0013% mRNA or 1–5 copies per cell according to the estimate that the total number of average-sized transcripts ranges from 100 000 to 500 000 per cell in higher plants (Kamalay and Goldberg, 1980).

Since the size of the pistil in rice is very small (Figure 1) and the pollination and fertilization takes place in a short time, the collection of the tissue is very laborious. Our data indicated that cDNA microarray is an effective tool for monitoring gene expression during pollination and fertilization at a genome scale. However, to obtain reliable data, especially those 2–3-fold change genes, a cDNA microarray and RT-PCR should be combined.

Functional classification of differentially expressed genes during pollination and fertilization

To further annotate the genes differentially expressed during pollination and fertilization, the similarity analysis for each sequence was done using the Blastx and Blastn database search program, and cDNA sequences with a Blastx E-value less than 10^{-10} or Blastn scores ≥100 were classified according to their putative function. Although functional assignment based only on sequence homology needs further experimental verification, it nonetheless provides a measure of diversity of the genes in the regulated gene collection and help shed light on the biological process. We cannot exclude the possibility that the sequence obtained from some cDNA clones may not include their ORF sequence if they have a long 5′- or 3′-untranslated regions. Moreover, some cDNA annotated to the same functional genes were probably derived from the same gene or from redundant homologous genes. The functional groups of these genes were given in Figure A5. As expected, the largest category consists of genes with unknown or no significant homology/no hit consistent with the genome annotation in rice (Goff *et al.*, 2002; Yu *et al.*, 2002). Overall, genes associated with amino acid metabolism, carbohydrate metabolism and protein synthesis tended to be up-regulated in 5DAP, consistent with their proposed roles in embryo and endosperm development.

To examine the expression pattern of each individual pollination- or fertilization-related gene in detail, we analyzed normalized data from four microarray experiments by hierarchical clustering (Figure 5). The expression of most ribosomal genes and starch synthesis-related genes up-regulated in 5hP, and with a more higher expression level in 5DAP (sub-clusters I and II), indicating the initiation of active protein biosynthesis and starch synthesis at the 5hP stage. So we considered that the genes, which have a 5hP/UP ≥ 2 combined with 5hP/5DAP ≥ 2 [calculated by log 2 (5hP/5DAP) = log 2 (5hP/UP)−log 2 (5DAP/UP)] expressed patterns are most likely to represent the pollination-induced/enhanced or sexual reproduction-associated genes. These candidate genes are listed in Table 1 and grouped into 16 functional classes. Moreover, in this gene list, we also recovered 20 genes similar to pollination-related or fertility-related genes previously identified in other plants (Table 2), demonstrating the reliability of the work.

A total of 253 ESTs, including 76 ESTs of hitherto unidentified function, were responsive to pollination and fertilization, and have a wide range of potential functions based on the sequence homologies. The microarray contains 16–20% of the total rice genes, based on the estimate of around 50 000–60 000 genes in rice (Goff *et al.*, 2002; Yu *et al.*, 2002). Our data indicate that a relatively small number of genes, about 1000–1400, are regulated during pollination and fertilization in rice. However, this number may be an underestimate to some extent because only those values that showed fluorescent intensity levels in at least one channels above three times the local background were used to calculate the ratios.

Sub-cluster III (Figure 5) contains 12 cDNA clones, half of them with unknown function. They are all up-regulated in 5hP but down-regulated in 5DAP (UP as a control) and with a more higher

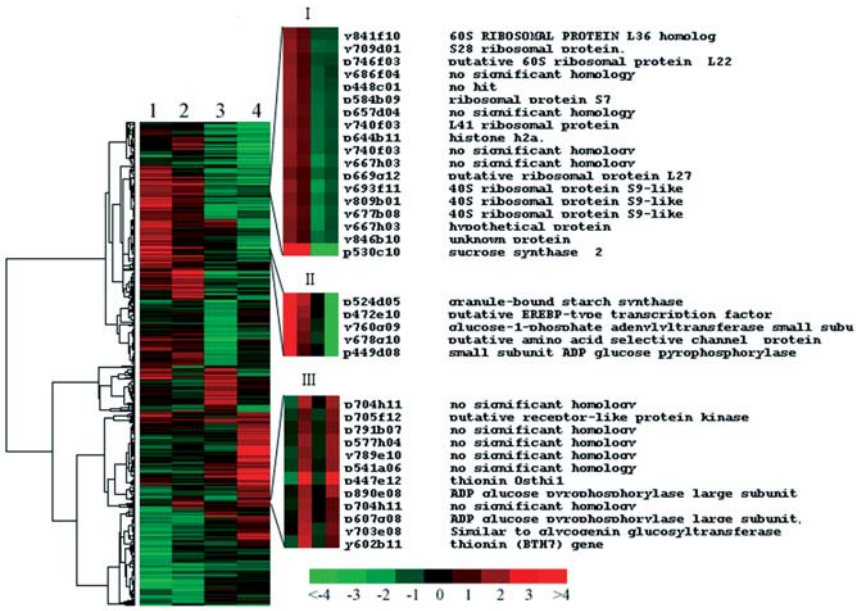

Figure 5. Clustering analysis of gene expression in pistil and/or seed development. To ensure a reproducible regulation profile, we selected the genes for cluster analysis if they showed significantly differential expression (set as two-fold) in at least one tissue comparison, moreover, genes with missing value(s) are not included for analysis. Lanes: (1), 5DAP vs. UP; (2), 5hP vs. UP; (3), 10EN vs. 5DAP; (4), 10EM vs. 5DAP. For other descriptions, see Figure 2.

expression level in 10EM (10-day-old embryo) than in 10EN (10-day-old endosperm), indicating that they are potentially involved in pollination and fertilization as well as embryo development in rice. Taken together, the gene expression profiles contributed to generating a hypothesis on the involvement of uncharacterized genes in pollination and fertilization.

Down-regulation of gene expression also might play important roles in pollination. Transcript levels for 166 ESTs were found to be decreased and only 87 ESTs were considered as pollination-induced/enhanced in our study. In addition, nine transcription factors or putative transcription factor genes were all down-regulated by pollination. Considering that the down-regulation response was extensive, they might be responsible for the suppression of these genes.

Large proportions of the differentially expressed genes during pollination and fertilization are involved or potentially involved in defense and/or stress response

Among the 177 ESTs that can be assigned a putative function, 75 (42%) are involved or potentially involved in defense/stress response and regulation (signal transduction and transcription factor). It is tempting to speculate that an extensive overlap occurs between pollination/fertilization and defense- or stress-response pathways, at least at the mRNA level. Maybe, the penetration of the pistil by the growing pollen tube causes stress/defense response during pollination.

Four genes encoding thionin proteins and a lipid transfer protein (LTP) gene (p596c09) were up-regulated by pollination. LTPs and thionins are thought to be a group of plant antimicrobial peptides, including defensins (Broekaert *et al.*, 1997). Park *et al.* (2000) suggest that an antimicrobial LTP in lily was recruited evolutionarily as pollen tube adhesion and guidance molecule. In addition, studies on defensins have demonstrated that these molecules could also act as chemotactic molecules with a role in signaling (Yang *et al.*, 1999). Perhaps antimicrobial LTP and thionin in rice play a role in plant–pathogen interactions and/or pollen tube growth regulation. In addition, four up-regulated genes, a pathogenesis-related protein PR-10a gene (p898a08), two probenazole-induced proteins (y756b11 and y841e12) and an RNase S-like protein precursor gene (y747d05),

Table 1. Candidate genes involved in pollination and fertilization classified by putative function.[a]

Clone ID	Hit to NR	Description	E-value	Log 2 (5hP/UP)[b]
Carbohydrate metabolism (12)				
y703e08	BAA82375	Similar to glycogenin glucosyltransferase	4.00E−81	1.504
p607g08	AAD39597	Identical to gb|D50317 ADP glucose pyrophosphorylase large subunit	3.00E−60	1.415
p577h04	D50317	mRNA for ADP glucose pyrophosphorylase large subunit	1.00E−176	1.288
p890e08	T02965	Glucose-1-phosphate adenylyltransferase large chain	5.00E−14	1.266
p801e10	AY042874	Similar to glucose-6-phosphate/phosphate translocator	2.00E−32	1.244
y789e10	D50317	MRNA for ADP glucose pyrophosphorylase large subunit	1.00E−173	1.125
y790c04	AF276704	Vacuolar acid invertase (INV3) gene	8.00E−12	−1.062
p780f04	AF276704	Vacuolar acid invertase (INV3) gene	4.00E−45	−1.177
y741h03	AF093629	Inorganic pyrophosphatase	1.00E−120	−1.466
y747f01	AF093629	Inorganic pyrophosphatase	1.00E−120	−1.718
y836b05	AF093629	Inorganic pyrophosphatase	1.00E−120	−1.954
y718g04	AF276703	Vacuolar acid invertase (INV2) gene	8.00E−98	−2.658
Cell fate (2)				
y697h10	BAC24888	Putative ATP-dependent DNA helicase II, 70 kDa subunit	5.00E−70	−1.373
y696e11	AAK98735	Putative cell death suppressor protein	1.00E−103	−1.069
Cell organization (5)				
p713h11	BAB44136	Putative histone H2A	4.00E−26	1.464
y750f09	HSWT41	Histone H4	2.00E−41	1.108
y609c12	A59311	Myosin VIII ZMM4	4.00E−89	−1.08
y838b03	BAB93319	Putative Tub family protein	3.00E−41	−1.255
y719e05	Q9ZPN9	Tubulin β-2 chain	1.00E−132	−1.547
Cell-wall-related (10)				
p716f04	AF026382	Proline-rich protein	1E−150	2.046
p786a11	AF026382	Proline-rich protein.	7.00E−24	1.967
p497f04	AAL25127	Cellulose synthase-like protein OsCslA6	1.00E−64	1.427
y683d01	CAA57810	Proline-rich-like protein	1.00E−10	−1.008
p723f12	AF261272	β-Expansin	1.00E−176	−1.052
y775f07	AF261272	β-Expansin	4.00E−86	−1.379
p525a02	CAB45149	Caffeoyl CoA O-methyltransferase	1.00E−126	−1.182
y657d10	AJ242804	Putative glycine- and proline-rich protein	1.00E−24	−1.253
y681f10	AJ242804	Putative glycine- and proline-rich protein	1.00E−37	−1.372
p601f09	AJ278996	(1-4)-β-mannan endohydrolase	2.00E−18	−1.7
Hormone-related (6)				
p622d05	NP_567713	Abscisic-acid-induced-like protein	4.00E−24	1.488
y658g08	AB028602	D1 Gene for α-subunit of GTP-binding protein	2.00E−32	−1.289
y668d03	T06822	Gda-1 protein	4.00E−65	−1.008
y656d05	P27057	Gast1 protein precursor	3.00E−27	−1.025
y709e09	NP_180639	Putative gibberellin-regulated protein	1.00E−29	−1.066
y666e09	P25777	Oryzain alpha chain precursor	1.00E−48	−1.915
Lipid metabolism (8)				
p607b04	AAL40895	Phosphoethanolamine methyltransferase	4.00E−89	2.979
y861d06	AAL40895	Phosphoethanolamine methyltransferase	2.00E−40	1.958
p835c01	AAL61542	Isoflavone reductase-like protein	1E−43	2.258
y607b11	BAB39417	Putative lipase	7.00E−68	1.356
y843e01	Q43007	Phospholipase d 1 precursor	2.00E−89	−1.098
y825g01	Q43007	Phospholipase d 1 precursor	7.00E−36	−1.355
y938b11	CAD30692	Fatty acyl coA reductase	2.00E−26	−1.386
y745c05	T08817	CCholine kinase	2.00E−42	−1.646

Table 1. (Continued).

Clone ID	Hit to NR	Description	E-value	Log 2 (5hP/UP)[b]
Other metabolism activities (7)				
p474d07	AAL65396	Putative aminotransferase	2.00E−15	1.592
y835b06	P52424	Phosphoribosylformylglycinamidine cyclo-ligase precursor	6.00E−76	1.009
y783f04	T11580	Probable 12-oxophytodienoate reductase	2.00E−36	−1.225
y685e01	AF187063	UMP/CMP kinase b (ura6)	7.00E−17	−1.002
p433h09	P93394	UUracil phosphoribosyltransferase	1.00E−20	−1.098
y685g03	AF187063	UMP/CMP kinase b (ura6)	2.00E−78	−1.117
y721a04	AAD30579	Similar to dTDP-D-glucose dehydratase	3.00E−20	−1.166
Photosynthesis (5)				
y713f02	AAC15992	Chlorophyll *a*/*b*-binding protein	1.00E−152	1.367
y683a04	AAL29886	Chlorophyll *a*/*b*- binding protein type II	1.00E−78	1.256
p650g03	P27489	Chlorophyll *a*-*b*- binding protein 13 precursor	3.00E−97	1.436
p426b01	A44956	Chlorophyll *a*/*b*-binding protein I precursor	2.00E−18	1.392
p647c05	AF061577	Chlorophyll *a*/*b*-binding protein (RCABP90)	1.00E−142	1.401
Protein destination/chaperone (12)				
p408c03	AB033537	26S proteasome regulatory particle triple-A ATPase	3.00E−68	1.983
p689g10	CAC39071	DnaJ-like protein	1.00E−19	1.511
y689b08	AAK16647	F-box containing protein TIR1	5.00E−45	−1.022
y862d08	AAM13204	Putative serine protease-like protein	2.00E−42	−1.08
p659a09	U83669	Low-molecular-mass heat-shock protein Oshsp17.3	3.00E−30	−1.089
y746g03	P35135	Ubiquitin-conjugating enzyme e2-17 kDa	2.00E−48	−1.12
y814g03	S20925	Polyubiquitin	0	−1.135
y826d09	S53126	DnaK-type molecular chaperone hsp70	1.00E−171	−1.147
y746g03	P35135	Ubiquitin-conjugating enzyme e2-17 kda	2.00E−48	−1.165
y802f02	BAB63884	Putative cysteine protease	1.00E−150	−1.182
p680b07	T50779	Copper chaperone homologue CCH	2.00E−15	−1.427
p773g11	AF263381	SKP1-interacting partner 5	4.00E−43	−2.898
Protein synthesis (7)				
y684b05	dJ553F4.5	60S ribosomal protein l31	1.00E−42	1.516
p861c03	NP_187110	Putative tryptophanyl-tRNA synthetase	3.00E−35	1.122
p644b05	P25864	50S ribosomal protein l9 chloroplast precursor	6.00E−41	1.094
p551d12	T02788	Probable ribosomal protein L27	4.00E−70	1.026
y746f09	P35614	Eukaryotic peptide chain release factor subunit 1	1.00E−18	−1.009
y802f09	P33278	Protein translation factor sui1 homologue	2.00E−54	−1.062
y856f09	P33278	Protein translation factor sui1 homologue	2.00E−52	−1.101
Signal transduction (24)				
p751d05	NP_191470	Receptor-like protein kinase	7E−8	2.287
p638g09	NP_196564	Receptor protein kinase	9.00E−37	2.013
y657h05	BAB91716	Putative receptor-like protein kinase	5.00E−35	1.573
p672f02	BAB03621	Putative protein kinase Xa21	2.00E−58	1.334
p447d03	AF314176	Protein serine/threonine kinase PBS1 (PBS1)	4.00E−31	1.008
y801b03	AY035226	CBL-interacting protein kinase 23	1.00E−83	1.689
y758h05	AY035226	CBL-interacting protein kinase 23	6.00E−63	1.621
p817e01	AY035226	CBL-interacting protein kinase 23	8.00E−36	1.37
p795f11	AY035226	CBL-interacting protein kinase 23	2.00E−93	1.115
p732f09	AF093604	Apyrase (Atapy1)	7.00E−16	1.866
p583h05	AB021259	mRNA for calcium-binding protein	1.00E−114	1.26
y813b05	NP_564003	Receptor-like serine/threonine kinase	6.00E−14	−1.301
y730g07	NP_199829	Protein kinase ATN1-like protein	2.00E−36	−1.089
y659a10	T14736	Probable serine/threonine kinase	4.00E−40	−2.972

Table 1. (Continued).

Clone ID	Hit to NR	Description	E-value	Log 2 (5hP/UP)[b]
y668g06	BAB02016	MAP kinase	9.00E−66	−1.067
p834d07	AF177392	BWMK1 mRNA	0	−1.55
y641a10	Q02066	Abcisic-acid-inducible protein kinase	2.00E−37	−1.037
y687a02	AF181661	EF-hand Ca^{2+}-binding protein CCD1 (ccd1)	3.00E−15	−1.438
p835f04	AF181661	EF-hand Ca^{2+}-binding protein CCD1 (ccd1)	7.00E−38	−1.033
y792d10	A54588	Protein phosphatase ABI1	2.00E−24	−1.071
p808d10	AF042840	Calmodulin	1.00E−121	−1.456
y723a12	NP_850097	Calmodulin	1.00E−11	−1.042
y786e06	T06080	Probable calcium-binding protein T9A14.90	3.00E−17	−1.51
p579a06	CAD48128	Farnesylated protein 1	5.00E−37	−1.105
Stress/defense (42)				
p714f02	AAL11444	Pathogenesis-related protein (PR4)	4.00E−40	4.234
p839d08	NP_565330	Subtilisin-like serine protease, putative	2.00E−18	3.252
p751f10	T06213	Probable aspartic proteinase	4E−7	2.699
y661f05	T50662	UVB-resistance protein UVR8	6E−8	2.626
p447e12	BAB93111	Thionin Osthi1	7.00E−29	2.309
y625d11	BAB93111	Thionin Osthi1	9.00E−69	2.216
y602b11	BAB93111	Thionin Osthi1	5.00E−27	1.67
y771a07	L36883	Thionin (BTH7)	5.00E−27	2.02
p596c09	P19656	Non-specific lipid-transfer protein	7E−6	2.444
y610a11	P24120	Salt-stress-induced protein	1.00E−31	1.161
y756b11	T02973	Probenazole-induced protein	1.00E−85	2.245
y841e12	T02973	Probenazole-induced protein	3.00E−46	2.089
p898a08	AF274850	Pathogenesis-related protein PR-10a	5.00E−58	3.321
y747d05	AF182197	RNase S-like protein precursor (rsh1)	1.00E−79	1.249
y717f08	AF017360	Lipid transfer protein LPT III mRNA	3.00E−91	−1.378
y748d03	AY029319	Seven-transmembrane protein Mlo8 mRNA	3.00E−16	−1.119
y688h04	AF126425	Trehalase mRNA	1.00E−105	−1.04
y622d04	AF082032	Senescence-associated protein 12	8.00E−16	−1.408
y844h11	AJ277468	Putative Bowman Birk trypsin inhibitor.	5.00E−26	−1.081
y747g10	P20346	Probable protease inhibitor p322 precursor	2.00E−18	−1.195
y725g06	T02212	NOI protein	8.00E−42	−1.653
p631g01	AB011367	mRNA for LIP9	5.00E−18	−1.857
y722c11	AB011368	mRNA for LIP5	1.00E−159	−2.127
y695d11	AAC97511	Low-temperature- and salt-responsive protein LTI6B	2.00E−11	−1.756
y845e05	T06802	Cold acclimation protein WCOR410b	1.00E−50	−2.164
p684b11	T03779	Protein LEA type 1	6.00E−15	−1.29
y770a01	BAB19059	Putative late-embryogenesis-abundant protein	3.00E−62	−1.381
y770b05	T52190	Probable dormancy-associated protein	5.00E−26	−1.256
y781h09	AAL78369	Putative dormancy-associated protein	5.00E−10	−1.297
y813c04	T06255	Dormancy-associated protein	1.00E−10	−1.043
y874e09	AJ238697	Glutathione peroxidase-like protein	3.00E−79	−1.075
y793c07	T03405	Probable chitinase (EC 3.2.1.14) IIb	3.00E−60	−1.368
y666f03	AF332458	Putative endochitinase protein	2.00E−10	−2.118
p720b12	AAM08776	Putative class III chitinase	1.00E−12	−3.904
y703b01	AF296279	Class III chitinase (chib1)	7.00E−42	−1.443
y874c04	AF296279	Class III chitinase (chib1)	1.00E−76	−1.77
y931e01	T03614	Chitinase	2.00E−72	−2.235
y659a07	L37289	Chitinase	4.00E−29	−4.05
y616h12	P22503	Endoglucanase precursor	1.00E−30	−1.393
y674c04	AB027429	β-1,3-Glucanase (PR-2)	1.00E−105	−1.172
y755f07	T50649	Elicitor-responsive gene 3	4.00E−76	−1.712
p868a07	T50649	Elicitor-responsive gene 3	1.00E−78	−1.554
Transcription (9)				
y712d12	BAB12694	Putative zinc-finger transcription factor	2.00E−14	−1.114

Table 1. (Continued).

Clone ID	Hit to NR	Description	E-value	Log 2 (5hP/UP)[b]
	BAB18280	Putative transcription factor	5.00E−30	−1.028
p552b04				
p465c09	AAF21887	Putative transcription factor X1	6.00E−63	−1.015
p604f11	AAF21887	Putative transcription factor X1	5.00E−26	−1.057
y794c10	CAD12767	LHY protein	2.00E−10	−1.003
y824d02	AF254558	NAC6 (NAC6) gene	0	−1.047
y709g08	BAB78462	Ethylene-insensitive-3-like protein	1.00E−114	−1.299
y937b05	AF049930	PGPD14 mRNA	2.00E−18	−1.207
p732c02	AAO92341	MADS20	3.00E−23	−1.247
Transport (12)				
p411f10	AAD25756	Contains phosphatidyl-inositol-transfer protein domain	9.00E−62	1.256
y675c04	AF326494	Plasma membrane integral protein ZmPIP2-4	1.00E−132	−1.06
p811e06	AJ224327	Aquaporin	1.00E−125	−1.088
y844h01	AJ224327	Aquaporin	1.00E−159	−1.188
p753e04	AY035003	Putative permease 1	1.00E−10	−1.188
y826c04	BAB90366	Putative amino acid transport protein	1.00E−15	−1.312
y668f05	AAD39650	Similar to gb|Z70524 PDR5-like ABC transporter	4.00E−32	−1.392
y820b08	BAB39957	GABA-A receptor å-like subunit	2E−8	−1.049
p656c12	NP_194789	Signal recognition particle receptor-like protein	1.00E−17	−1.056
y676g05	NP_194789	Signal recognition particle receptor-like protein	2.00E−40	−1.176
y843b04	NP_194789	Signal recognition particle receptor-like protein	3.00E−53	−1.31
y666d01	BAB01839	Phosphate permease-like protein	1.00E−38	−1.486
Unclassified (16)				
p602f03	S26981	Pvs protein 1	3.00E−15	1.017
p585a02	AAG13432	Putative α/β hydrolase	1.00E−53	1.377
y746g05	AJ309302	Putative membrane protein exons1-3 allele 2	5.00E−91	1.155
p465g10	AAN11203	Putative jacalin homologue	4.00E−24	1.214
y732b09	AAN11203	Putative jacalin homologue	2.00E−31	1.214
p762b06	AAN11203	Putative jacalin homologue	1.00E−78	1.143
p709b07	NP_191008	Nucleoid DNA-binding-like protein	5.00E−15	1.082
y726f09	P35681	Translationally controlled tumor protein homologue	1.00E−85	−1.007
y677f11	NP_177891	Putative 3́-5́-exoribonuclease	1.00E−36	−1.067
y933c01	AAM54146	Putative reverse transcriptase	2.00E−17	−1.081
y673f05	BAB02803	WD domain protein-like	4E−6	−1.086
y850c12	NP_199049	Arm-repeat-containing protein	8.00E−41	−1.103
p726e10	AAM08898	Putative ATPases	7.00E−29	−1.181
y685c07	T48471	F1F0-ATPase inhibitor-like protein	1E−8	−1.563
y722f08	AAF66103	LAG1 homologue 2	2E−7	−1.688
y742d10	AAL58252	Putative amelogenin precursor	3.00E−24	−1.969

Unknown protein/hypothetical protein/no significant homology/no hit (up-regulated: 25 cDNA clones; down-regulated: 51 cDNA clones).

[a]The genes expressed profiling with $\log_2(5hP/UP) \leq -1$ or $\log_2(5hP/UP) \geq 1$ and $\log_2(5hP/5DAP) \geq 1$ were shown; Log_2 (5hP/5DAP) is calculated by $\log_2$ (5hP/UP)−$\log_2$ (5DAP/UP).

[b]Average $\log_2$ (5hP/UP) of at least two replicate values and combining with a P value less than 0.05 (t-test).

NR: non-redundant database.

are structurally related to ribonucleases, and it is tempting to suppose that they might be active against pollen-tube-mediated pathogen ingress.

A directional supply of water is essential for pollen–pistil interaction (Ikeda *et al.*, 1997). In our microarray analysis, four low-temperature-induced proteins are down-regulated by pollination. These include LIP9, LIP5, the low-temperature- and salt-responsive protein LTI6B, and the cold acclimation protein WCOR410b. In addition, three genes

Table 2. Some cDNA clones show similarity to well-known fertility-related and pollination-related genes in the literature.[a]

Clone description	Biological function	Reference
y938b11 Male sterility 2-like protein	Male sterility	Aarts et al. (1997)
p602f03 sterility protein 1	Male sterility	Johns et al. (1992)
p607b04 phosphoethanolamine methyltransferase	Male sterility	Mou et al. (2002)
y850c12 arm-repeat-containing protein	Regulating cell adhesion during pollination	Gu et al. (1998)
p751d05 receptor-like protein kinase	Pollen–pistil interactions	Muschietti et al. (1998)
p638g09 receptor protein kinase	Pollen–pistil interactions	Muschietti et al. (1998)
p732f09 apyrase	Pollen germination	Steinebrunner et al. (2003)
p811e06 aquaporin	Pollen germination	Ikeda et al. (1997)
y675c04 aquaporin	Pollen germination	Ikeda et al. (1997)
y937b05 PGPD14	Pollen germination	Guyon et al. (2000)
p497f04 cellulose synthase-like protein	Pollen tube growth	Goubet et al. (2003)
p835c01 isoflavone reductase-like protein	Pollen tube growth	van Eldik et al. (1997)
y741h03 inorganic pyrophosphatase	Pollen tube growth	Rudd et al. (2003)[b]
y843e01 phospholipase D	Pollen tube growth	Potocky et al. (2003)[b]
p474d07 aminotransferase	Pollen tube growth and guidance	Palanivelu et al. (2003)
y659a07 chitinase	Protecting the ovary against pollen-tube-mediated pathogen ingress	Wemmer et al. (1994)
y793c07 probable chitinase	Protecting the ovary against pollen-tube-mediated pathogen ingress	Wemmer et al. (1994)
y874c04 class III chitinase	Protecting the ovary against pollen-tube-mediated pathogen ingress	Wemmer et al. (1994)
y931e01 chitinase	Protecting the ovary against pollen-tube-mediated pathogen ingress	Wemmer et al. (1994)
p751f10 aspartic proteinase	Nucellar cell death after pollination	Chen and Foolad (1997)

[a]In this table, the similarity analysis for each sequence was done with Blastx (http://www.ncbi.nlm.nih.gov/). Subsequently, the top hit in NR was extracted and compared with the gene that has been reported in the literature by using blastp (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html), the E-value less than 1E−30, respectively.
[b]No available gene/protein sequence from the literature.

encoding aquaporin proteins, which enable fast and controlled translocation of large volumes of water across biological membranes, two hydrophilic LEA proteins that are preferentially expressed in response to dehydration or hyperosmotic stress and three putative dormancy-associated proteins are all down-regulated by pollination. Dormancy is an important developmental program allowing plants to withstand extended periods of extreme environmental conditions, such as low temperature or drought. Moreover, the LTI6B (y695d11) gene has been demonstrated as drought-induced expression in shoot of rice by our RT-PCR analysis (data not shown).

It is important to note that microarray data provide a measure of steady-state transcripts levels or relative steady-state transcripts levels only (Gygi *et al.*, 1999). Nevertheless, expression profiles will provide useful starting points for more in-depth analyses. Identification and determination of the biological relevance of these differential expression gene during pollination and fertilization will help shed light on the molecular mechanism of this important biological process in rice.

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