Plant Molecular Biology (2005) 59:151–164
DOI 10.1007/s11103-005-3958-4

Microarray analysis reveals similarities and variations in genetic programs controlling pollination/fertilization and stress responses in rice (*Oryza sativa* L.)

Lefu Lan[1,3,†], Meina Li[1,3,†], Ying Lai[1,3,†], Wenying Xu[1], Zhaosheng Kong[1], Kai Ying[2], Bin Han[2] and Yongbiao Xue[1,*]

[1]*Institute of Genetics and Development Biology and Laboratory of Molecular and Developmental Biology, Chinese Academy of Science and National Center for Plant Gene Research, 100080, Beijing, China (*author for correspondence; e-mail ybxue@genetics.ac.cn);* [2]*Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and National Center for Gene Research, 200233, Shanghai, China;* [3]*Graduate School of Chinese Academy of Sciences, 100039, Beijing, China;* [†]*These authors contributed equally to this work*

Received 29 March 2004; accepted in revised form 15 March 2005

Key words: cDNA microarray, pollination and fertilization, Real-Time PCR, rice, stress response

Abstract

Previously, we identified 253 cDNAs that are regulated by pollination/fertilization in rice by using a 10K cDNA microarray. In addition, many of them also appeared to be involved in drought and wounding responses. To investigate this relationship, we obtained their expression profiles after dehydration and wounding treatments in this study. Venn diagram analysis indicated that 53.8% (136/253) and 21% (57/253) of the pollination/fertilization-related genes are indeed regulated by dehydration and wounding, respectively, and nearly half of the genes expressed preferentially in unpollinated pistils (UP) are responsive to dehydration. These results indicated that an extensive gene set is shared among these responses, suggesting that the genetic programs regulating them are likely related. Among them, the genetic network of water stress control may be a key player in pollination and fertilization. Additionally, 39.5% (100/253) cDNAs that are related to pollination/fertilization appear not to be regulated by the stress treatments (dehydration and wounding), suggesting that the existence of additional genetic networks are involved in pollination/fertilization. Furthermore, comparative analysis of the expression profiles of the 253 cDNAs under 18 different conditions (various tissues, treatments and developmental status) revealed that the genetic networks regulating photosynthesis, starch metabolisms, GA- and defense-responses are involved in pollination and fertilization. Taken together, these results provided some clues to elucidate the molecular mechanisms of pollination and fertilization in rice.

Introduction

In flowering plants, pollination and fertilization are the key developmental stages leading to seed formation. A better understanding of the molecular mechanisms involved in these developmental processes is important for expanding our knowledge of how plant cells interact and communicate with each other as well as our ability to manipulate pollen tube growth for a practical purpose in molecular breeding. Important progresses in pollination and fertilization have been made in recent years (Sanchez *et al*., 2004; Weterings and Russell, 2004), but little is known about

the genetic regulation of pollination and fertilization in rice, which is the principal food for over half of population in the world.

Plant stress responses often mimic certain normal developmental processes (Cooper *et al*., 2003). Interaction between plant development and environmental conditions implies that some genes must be co-regulated by both environmental factors and development cues. For example, phospholipase D (PLD) and its product phosphatidic acid (PA) are involved in various stress responses (water deficits, salts, wounding, and elicitation) (Wang, 2002) and play a role in pollen germination and pollen tube growth in tobacco (Potocky *et al*., 2003; Zonia and Munnik, 2004). In *Petunia*, pollination and wounding induced nearly identical flavonol kinetics and patterns of their accumulation in the outer cell layers and exudates of the stigma (Vogt *et al*., 1994), suggesting that they share elements of a common signal transduction pathway. In recent years, microarray technique has become a useful tool for analysis of genome-wide gene expression (Schena *et al*., 1995; Eisen *et al*., 1998). A systematic regulation of gene expression has been found to drive the developmental process and stress response in *Arabidopsis* (Chen *et al*., 2002), indicating that an overlap of genes occurs between the developmental process and stress response. Moreover, a network of rice genes that are associated with stress response and seed development has been reported (Cooper *et al*., 2003).

Previously, we prepared a 10K cDNA microarray and identified 253 cDNA that appear to be responsive to pollination/fertilization in rice. Similarity analysis indicated that many of them also are potentially involved in drought and wounding responses (Lan *et al*., 2004). To investigate their expression under these stress conditions, we obtained their expression profiles after dehydration and wounding treatments, respectively. Here, we report on possible cross-talks of the genetic programs regulating the pollination/fertilization and stress responses by using a large-scale gene expression profile strategy. Genetic networks regulating water stress, photosynthesis, starch metabolisms, GA- and defense-responses have been identified to be likely involved in the pollination and fertilization in rice. These results provide some clues to elucidate the molecular mechanisms of pollination and fertilization in rice.

Materials and methods

Plant materials, stress treatments and RNA isolation

Rice (*Oryza sativa* L. ssp. *indica*) seed was germinated and grown in water on a piece of tissue paper in Petri dish. Rice seedlings were harvested from 14-day-old-plants grown in a growth chamber at 26 °C under 16 h light/8 h dark. For dehydration treatment, seedlings were removed from the water and desiccated in plastic dishes at 26 °C. For wounding treatments, shoots were cut into 1 cm length using a razor blade and then incubated in water at 26 °C. Unpollinated pistils (UP) were collected at the stage of 1–2 days before floret flowering and 5hP (pistils of 0–5 h after pollination) dissected out 0–5 h after pollination, respectively. RNA was isolated using RNeasy kit (Qiagen, Valencia, CA, USA). Isolation of poly (A) (RNA from the total RNA was preformed using Qiagen mRNA Extraction Kit.

Labeling, hybridization, washing, scanning and data acquisition

Labeling was performed using a CyScribe^{TM} Post-Labeling Kit (Amersham Biosciences, Piscataway, NJ, USA). Hybridization and washing were performed as described in CyScribe^{TM} Post-Labeling Kit and CMTTM Hybridization Chamber (CORNING) user manuals similar to our previous report (Lan *et al*., 2004). Scanning and data acquisition was performed on a GenePix 4000B scanner using GENEPIX 4.0 software (Axon Instruments, Union City, CA, USA). More detailed microarray information is freely available in our microarray database: http://plantbiol.genetics.ac.cn/.

Data analysis

The GenePix Pro 4.0 output files were converted to TIGR Multi Experiment Viewer file (.mev) by ExpressConverter V1.4 (http://www.tigr.org/software/tm4/utilities.html) and normalized (local lowess) by MAIDS (MicroArray Informatics Discovery System) (http://www.tigr.org/software/tm4). Then, the spots flagged Bad or Not Found by Genepix software were removed from further data analysis and only those spots that showed

fluorescent intensity levels in at least one channel above the 'background (local) +2SD' were used for further analysis. Moreover, only array elements that had a summatory intensity (in both channels) values ≥50 after background subtraction were used for further analysis. Furthermore, those spots that exhibited a large difference between the duplicate experiments (dye-swap) were regarded as outliers and removed manually. Hierarchical clustering was performed as described by Eisen *et al.* (1998). Similarity analyses were performed using the BLAST program (Altschul *et al.*, 1990).

Real-Time PCR

Reverse transcription was performed using TaqMan Reverse Transcription Regents Kit (Applied Biosystems, Foster City, CA, USA). 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 using the SYBR Green Master mix with an ABI 7900 sequence detection system according to the manufacture's protocol (Applied Biosystems). Gene-specific primers were designed by using PRIMEREXPRESS 1.0 software (Applied Biosystems) and listed in Table (S1). The relative quantization method ($\Delta\Delta C_T$) was used to evaluate quantitative variation between the replicates examined. The amplicon of 18S rRNA was used as an internal control to normalize all data.

Results

Identification of genes regulated by dehydration and wounding

Previously, we monitored gene expression profiles in rice and identified 253 cDNAs that are likely regulated by pollination and (or fertilization using a 10K cDNA microarray and found that the majority of them also are possibly related to genes involved in stress responses (in particular, drought and defense responses) (Lan *et al.*, 2004). To investigate this further and identify genes whose transcripts are regulated by dehydration and (or wounding, we harvested tissues (shoots) at four time points: 0, 2, 6 and 12 h after the dehydration treatment and two time points: at 2 and 6 h time points after wounding, respectively. The dehydration reduced the fresh weight of the seedlings to 47.7, 34.9 and 30.2% of that of the untreated plants (0 h), respectively. The 0-h time point was used as a reference sample to which the other time points of treatments were compared. To control the biological variation that could interfere with data interpretation, all the samples included in the study were pooled from at least 96 individual seedlings receiving the same treatment or receiving the same treatment in replicated experiments. In each case, the hybridization experiment was replicated by reversing the fluorescent dyes. Microarray hybridization, washing, scanning and data analysis were carried out as described in Materials and methods. The data from the dye-swapped experiment showed a strong correlation (Figure 1A), indicating a good reproducibility between slides.

To find out a useful cut-off for the differentially expressed genes, the same sample was hybridized to two 10K microarray slides, respectively. The distribution of the average $\log_2$-ratios is shown in Figure 1B. 99.8% of cDNA showed the ($\log_2$-ratios) less than 0.5 and only 0.01% of cDNA showed the ($\log_2$-ratio) larger than 1, indicating the consistence of the microarray data. The 2-fold cut-off was chosen in this study and the false positive rate in each dye-swap experiment was estimated separately and listed in Table (S2). The range of false positive rates is 0–0.1%. The detail information about how to calculate the false positive rate is presented in Table S2. Using this 2-fold change, 580, 2038 and 2241 cDNAs showed a difference of expression (>2-fold change) at 2, 6 and 12 h time points during dehydration process, respectively (Table S3). We regarded 3758 cDNAs, with one expression ratio greater than or equal to 2-folds at least at one time-course point, as dehydration-regulated genes. In addition, 8957 cDNAs, with an expression ratio lesser than 2-fold change during the dehydration process, were regarded as dehydration-invariable genes. Moreover, 546 and 731 cDNAs showed a differential expression at 2- and 6-h time points during the wounding process, respectively (Table S3), and 969 cDNAs were regarded as the wounding-regulated genes and 10617 cDNAs were invariable under the wounding condition used in this study.

Table 1. Verification of microarray results by Real-Time RT-PCR.[a]

Acc#	Putative function	5hP vs. UP		dr-12h vs. ck		w-12h vs. ck		w-6h vs. ck	
		Microarray	Q-RT	Microarray	Q-RT	Microarray	Q-RT	Microarray	Q-RT
BX898165	Receptor-like protein kinase	2.287	1.895	−1.647	−1.787	MV	−3.54	−2.513	−3.607
BX899544	LIP5	−2.127	−1.86	3.69	2.303	2.184	NA	−0.945	NA
BX899347	Receptor protein kinase	2.013	1.035	−0.211	−2.808	−0.369	NA	0.202	NA
BX901668	MADS20	−1.247	−2.255	0.843	0.015	MV	NA	MV	NA
BX900023	No significant homology	2.461	1.926	−0.48	−1.706	−0.486	NA	−0.291	NA
BX900201	Chitinase	−4.05	−4.45	1.92	NA	MV	NA	MV	NA
BX900828	Putative LHY protein	−1.003	−5.792	−3.421	−5.106	−0.602	−0.41	−4.376	−3.793
BX927912	Protein LEA type 1	−1.29	−1.1	4.235	3.682	−0.185	NA	−1.495	NA
CR278485	LIP9	−1.857	−0.267	4.011	4.966	1.303	NA	−1.376	NA
CR278984	Abcisic acid-inducible protein kinase	−1.037	0.454	1.855	1.515	0.47	NA	−0.031	NA
CR282197	OSJNBa0006B20.1	2.679	3.395	−1.703	−3.743	MV	NA	MV	NA
CR286861	LTI6B	−1.756	−1.21	2.091	1.539	−0.748	NA	−1.36	NA
CR285538	AP2 domain transcription factor EREBP	−1.244	−1.267	2.167	4.506	MV	4.007	MV	3.202
CR285756	Floral organ regulator 1	−2.356	−2.89	1.851	1.913	MV	NA	0.465	NA
CR287245	Non-specific lipid-transfer protein	2.444	3.45	−2.193	−2.4	−0.176	NA	0.197	NA
CR287685	Isoflavone reductase-like protein	2.258	3.232	−1.343	−2.152	0.145	NA	−0.274	NA
CR288152	Vacuolar acid invertase	−2.658	−4.47	−0.247	−0.53	0.12	NA	0.698	NA
CR288425	Beta-expansin EXPB4	−1.379	−1.932	−1.815	−5.293	−0.678	NA	−0.234	NA
CR287151	Cellulose synthase-like protein OsCs1A6	1.427	1.098	−1.6	−1.964	MV	NA	0.728	NA
CR288956	Putative GABA-A transaminase subunit precursor isozyme 3	1.592	1.129	1.216	−1.743	MV	NA	−0.127	NA
CR290553	Neurofilament triplet M protein-like protein	−1.685	−0.354	2.402	1.53	0.824	NA	−0.391	NA
CR290344	NAC6	−1.047	−2.785	3.386	2.804	1.029	NA	0.567	NA
CR291219	No significant homology	1.14	2.789	−3.43	−4.966	−2.39	−1.962	−3.155	−4.589
CR292239	Longevity factor-related protein	−1.688	−0.272	2.704	0.994	1.992	NA	−0.785	NA
CR291522	CBL-interacting protein kinase 23	1.621	2.624	2.056	0.772	2.032	2.308	2.245	2.786
CR293108	Putative zinc finger transcription factor	−1.114	−1.414	−0.215	0.329	−0.841	NA	−0.788	NA
CR292670	Probable dormancy-associated protein	−1.256	−1.15	0.613	−0.228	1.009	NA	1.205	NA
CR289346	Boron transporter	0.341	−1.623	−2.713	−1.961	MV	NA	MV	NA
CR292244	Abscisic acid- and stress-induced protein	1.255	1.163	1.188	5.373	−0.433	NA	−0.447	NA
CR284320	GABA-A receptor epsilon-like subunit	−1.049	−1.347	3.123	1.683	−0.165	NA	−0.675	NA

[a] In this table, the ratios were log (base 2) transformed. MV: missing value, the ratio was not determined due to: (1) weak expression of gene; (2) high background; (3) dusts on the cDNA spots. NA: no application. dr: dehydration; w: wounding; h: hours. Acc: accession number. Q-RT: Real Time RT-PCR. The primers used for Real-Time RT-PCR are listed in Table S1.

Concordance between cDNA microarray and Real-Time PCR analysis

We performed Real-Time PCR analysis on 30 genes to confirm the validity of the cDNA microarray data. The expression ratios obtained by cDNA microarray analysis are in good correlation with those obtained by Real-Time PCR analysis (Figure 1C). This is consistent with our previous report (Lan *et al.*, 2004). Nevertheless, regardless of the high correlation, gene expressions of six genes showed variations generated by the two methodologies for a particular experiment (Figure 1C and Table 1). For example, a putative GABA-A transaminase subunit precursor gene (CR288956) is both up-regulated by pollination/fertilization and dehydration (at 12-h time point) in our microarray analysis. However, Real-Time

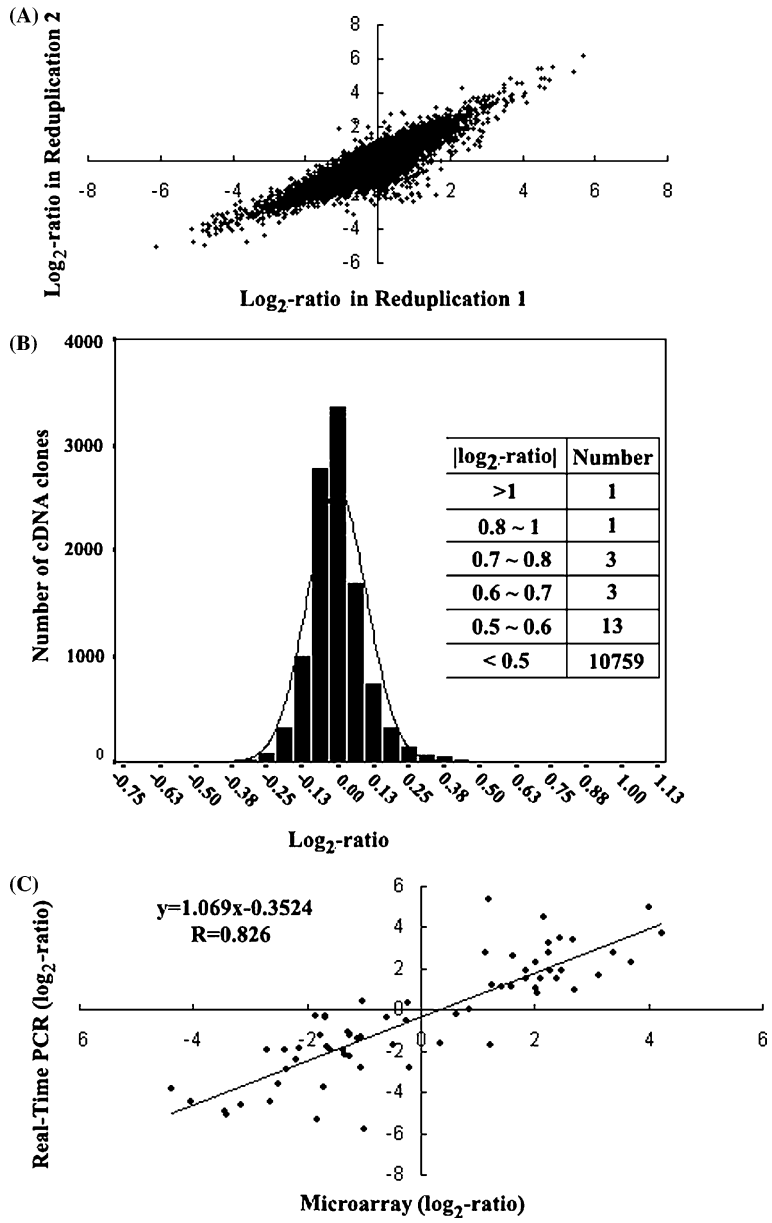

Figure 1. Reliability of the microarray data. (A) Correlation of the $\log_2$-ratios for a dye-swap experiment. Transcriptions ratios (Cy5/Cy3) determined from a microarray hybridization (Reduplication 1, in 12-h time point) were $\log_2$ transformed ($\log_2$-cy5/cy3) and plotted against those ($\log_2$-cy3/cy5) obtained from another microarray hybridization (Reduplication 2, in 12-h time point). (B) The distribution of the $\log_2$-ratios from the 'self-to-self' experiments. (C) Verification of microarray results with Real-Time RT-PCR. Transcriptions ratios determined from the microarray hybridization were $\log_2$ transformed and plotted against those obtained from Real-Time RT-PCR analysis. R: Pearson coefficient.

PCR analysis showed that its transcripts are up-regulated by pollination(fertilization, but down-regulated by dehydration (at 12-h time point) (see Table 1). By Blastx analysis, CR288956's top hit is BAD05337 (on chr8). In rice, BAD05337 showed 84, 76 and 76% amino acid identities to CAE04333 (on chr4), CAE04332 (on chr4) and BAD07632 (on chr2), respectively. Considering that the primers for Real-Time PCR are gene-specific, it was likely that the cross-

hybridization between some members of a gene family had occurred in our microarray experiments.

An extensive set of genes is shared between pollination/fertilization and stress responses

In total, we identified 3758 dehydration-regulated cDNAs and 969 wounding-regulated cDNAs using the 10K cDNA microarray, respectively. Based on Venn diagram analysis, we examined possible cross-talks of gene expression between the responses to pollination(fertilization and that to the stresses (dehydration and wounding) (Figure 2). 53.8% (136(253) and 21% (57(253) of the cDNAs which are responsive to pollination/fertilization also are regulated by dehydration and wounding, respectively (Figure 2A).

Among the 253 cDNAs, 166 cDNAs were down-regulated and 87 cDNAs were up-regulated by pollination(fertilization (Lan *et al.*, 2004). As shown in Figure 2B, 47% (68 + 10) of the cDNAs that are down-regulated by pollination(fertilization responded to dehydration (at 12-h time point) and, among them, 87%(68) are dehydration-inducible. Moreover, 43.7% (30 + 8) of the up-regulated cDNAs by pollination/fertilization also are regulated by dehydration and of those 79% (30) are dehydration-repressible. However, there are relatively small numbers of cDNAs, only 37 (14.6%), that responded to the wounding treatment (at 2-h time point) (Figure 2B).

Interestingly, 153 (96 + 40 + 17) cDNAs, amounting to 60.5% cDNAs regulated by pollination(fertilization, are *likely* involved in the stress responses (see Figure 2A). The expression profiles of the 153 cDNAs during the stress processes are shown in Figure 3. Ninety-nine cDNAs were down-regulated (Figure 3A) and 54 cDNAs were up-regulated (Figure 3B) by pollination/fertilization, respectively. Furthermore, most pollination-/fertilization repressed genes tended to be induced by dehydration, including the regulatory proteins involved in signal transduction and gene expression (transcription factors, protein kinases, protein phosphatases and other signaling molecules, such as calmodulin,

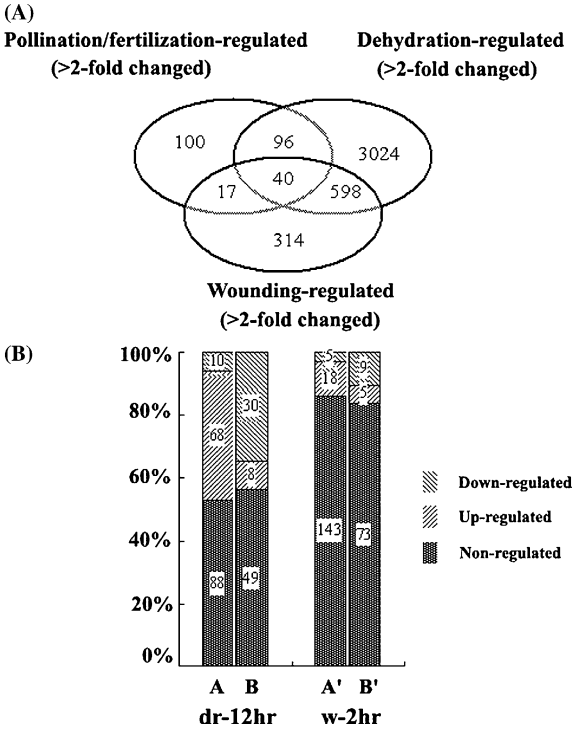

Figure 2. Relationships of genes involved in pollination(fertilization and stress responses. (A) Venn diagram of cDNAs regulated by pollination(fertilization, dehydration and wounding. Numbers are based on cDNA probes with 2-fold changes of signal intensity at least at one time point of the respective treatment(s). (B) Relationships of cDNAs regulated by pollination(fertilization and dehydration (at 12-h time point, dr-12 h), wounding (at 2-h time point, w-2 h). A and A′, cDNAs down-regulated by pollination/fertilization; B and B′, cDNAs up-regulated by pollination/fertilization.

and EF-hand Ca^{2+}-binding protein) and functional proteins (enzymes involved in osmo-protectant synthesis/degradation, protein degradation, protease inhibitor, LEA, protein, LTP, heat-shock protein, lipid transfer protein, and plant defense-related genes) (see Figure 3). In addition, two phospholipase d1 (PLD) precursor genes (CR283145 and CR290260), involved in a number of signaling pathways, were up-regulated by dehydration and down-regulated by pollination/fertilization in our microarray analyses. Recent studies have shown that PLD plays a role in the process of pollen tube growth (Potocky *et al.*, 2003) and is responsive to the perturbation of the extracellular osmotic potential (Zonia and Munnik, 2004).

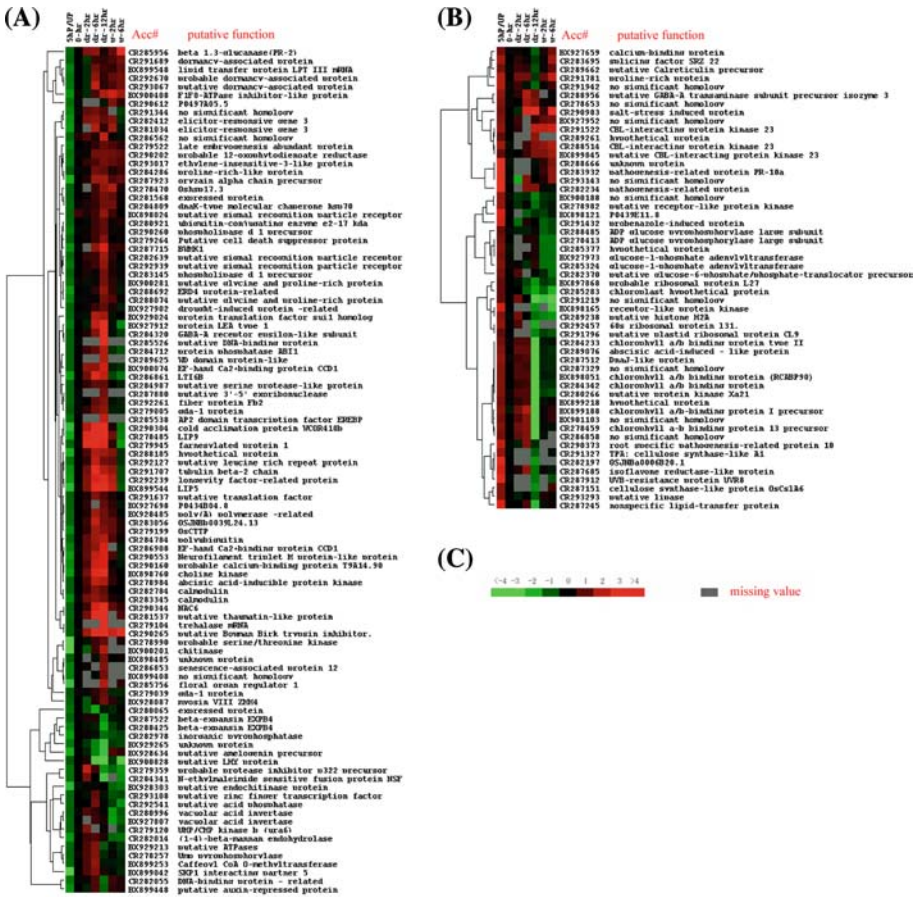

Figure 3. Clustering analysis of genes involved in pollination/fertilization and stress responses. (A) Expression profiles of the 99 cDNAs that are down-regulated by pollination/fertilization; (B) Expression profiles of 54 cDNAs that are up-regulated by pollination/fertilization. dr: dehydration; w: wounding; hr: hours.

Functional classification of cDNAs regulated by pollination/fertilization but not by the stress treatments

Although an extensive set of genes is shared between pollination/fertilization and stress responses, 39.5% (100/253) cDNAs that are related to pollination/fertilization appear not to be regulated by the stress treatments (dehydration and wounding), suggesting that the existence of additional genetic networks are involved in pollination/fertilization. To examine their possible functions in pollination/fertilization, we classified the 100 cDNAs into 16 groups based on their putative functions (Figure 4). The largest category consists of genes (22 cDNAs) of unknown functions. The second largest category consists of genes (10 cDNAs) that are potentially involved in carbohydrate metabolism and defense response, respectively. Moreover, 16 cDNAs have been annotated as regulatory proteins involved in signal transduction and transcription, indicating that there is a complex gene expression network operating during pollination and fertilization.

However, the stringent threshold used to select the significantly differential expressed genes in this study (see Figure 1B) could overestimate the number of the cDNAs regulated by pollination/fertilization but not to be regulated by the stress treatments (dehydration and wounding). Moreover, the expression levels of some of these genes were not high enough to be detected (large than, local background (2SD') in shoots and were excluded for further analysis (with missing value)

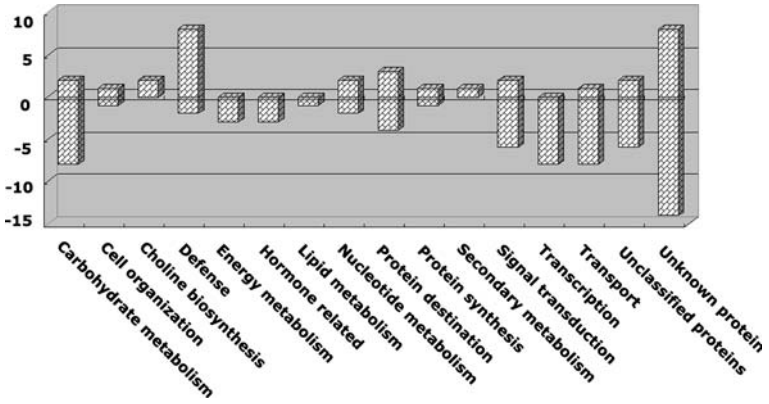

Figure 4. Functional classification of cDNAs regulated by pollination/fertilization but not by the stress treatments. The similarity analysis for each sequence was done using the Blastx and Blastn database search program, and cDNA sequences with a Blastx E-value lesser than 10^{-10} or a Blastn scores equal to or greater than 100 were classified according to their putative function and MIPS (Munich Information Center for Protein Sequences) functional categories.

although they were expressed at a high level in the pistils.

Nearly half of the genes expressed preferentially in unpollinated pistils also are responsive to dehydration and most of them are dehydration-inducible

The specialized reproductive functions of the angiosperm pistil must depend in part upon a regulated activation of numerous genes expressed predominantly in this organ system. As shown in Figure 2B, nearly half (47%) of the cDNAs, down-regulated by pollination/fertilization, also are responsive to dehydration, indicating that some genes that function in unpollinated pistil (UP) also are regulated by dehydration. To further examine the expression of these UP-predominant genes in a dehydration environment, their expression profiles were classified into three subgroups: dehydration-invariable, dehydration-repressible and dehydration-inducible during the dehydration process (at 12-h time point) (Figure 5). Furthermore, 133 cDNAs were found to be expressed preferentially in unpollinated pistils (greater than or equal to 4-fold changed at least in one tissue comparisons: in UP vs. Shoot experiments, UP vs. 5hP experiments or in UP vs. 5DAP experiments) and their expression profiles during the dehydration process are clustered in Figure 6. Together, these results showed that nearly half of the genes that expressed preferentially in UP are also regulated by dehydration in the shoots and most of them are dehydration-inducible.

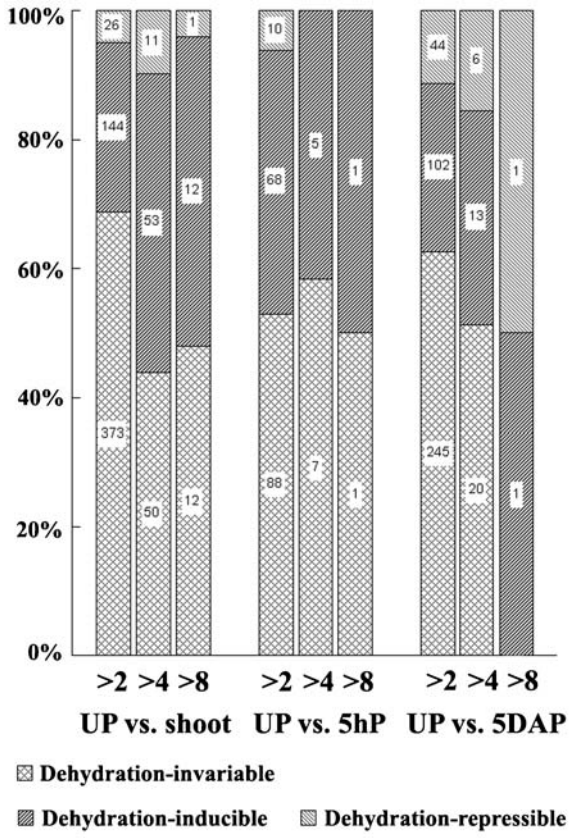

Figure 5. Relationships of cDNAs expressed predominantly in unpollinated pistil (UP) and responded to dehydration (at 12-h time point). UP: pistil of unpollinated; 5hP: pistil of 0–5 h after pollination; 5DAP: pistil of 5 days after pollination. The gene expression profiles of the UP vs. shoot, UP vs. 5hP and UP vs. 5DAP were derived from our previous study (Lan *et al*., 2004).

Figure 6. Expression profiles of the UP-predominantly expressed gene under dehydration treatment. UP/S: UP vs. shoot; UP/5hP: UP vs. 5hP; UP/5DAP: UP vs. 5DAP. dr:dehydration; w: wounding. hr: hours.

Clustering analysis of the pollination/fertilization-related genes under different conditions

Cluster analysis mathematically arranges genes according to the similarity of gene expression and shows expression patterns of different genes over a number of different experiments. Thus, data of the 253 cDNAs regulated by pollination/fertilization were compared with that from seven microarray experiments available in our previous study (Lan *et al*., 2004), five microarray experiments in this study and other five microarray experiments (our unpublished data, but see Figure 7). These 18 experiments, conducted with the same clone set, included comparisons among different tissue type, hormone treatments and a floral mutant vs. wild type. A selection of four major clusters is shown in Figure 7, and the whole cluster is shown in Figure (S1).

There are a total of 20 genes in cluster I, expressed predominantly in the shoots and 18 of them are down-regulated by the dehydration (at 12-h time point). Moreover, 18 of them were up-regulated by pollination/fertilization except two genes, a putative LHY (late elongated hypocotyl) protein and an unknown protein, which are down-regulated by pollination/fertilization. Similarity analysis revealed that most of them are potentially involved in photosynthesis. Interestingly, a putative LHY protein gene encodes a Myb-related DNA binding protein and its transcripts were strongly down-regulated by the pollination/fertilization, dehydration and wounding, respectively (Figure 7 and Table 1). The *LHY* gene has been shown to play a role in circadian clock function (Schaffer *et al*., 1998). Cluster II contained 12 genes. Five of them were of unknown function, and three (CR283932, CR290373 and CR291432) are potentially involved in defense responses and two are annotated to be receptor-like kinases, respectively. They were all up-regulated by pollination/fertilization and seven of them were induced by GA treatment. A recent study has shown that gibberellins are required for both seed development and pollen tube growth in *Arabidopsis* (Singh *et al*., 2002). Twenty-six genes in cluster III preferentially expressed in UP, were induced by the dehydration and down-regulated by the pollination/fertilization. In cluster IV, 35 genes were up-regulated by pollination/fertilization and preferentially expressed in 5hP. Similar analysis revealed that many genes listed in this cluster are

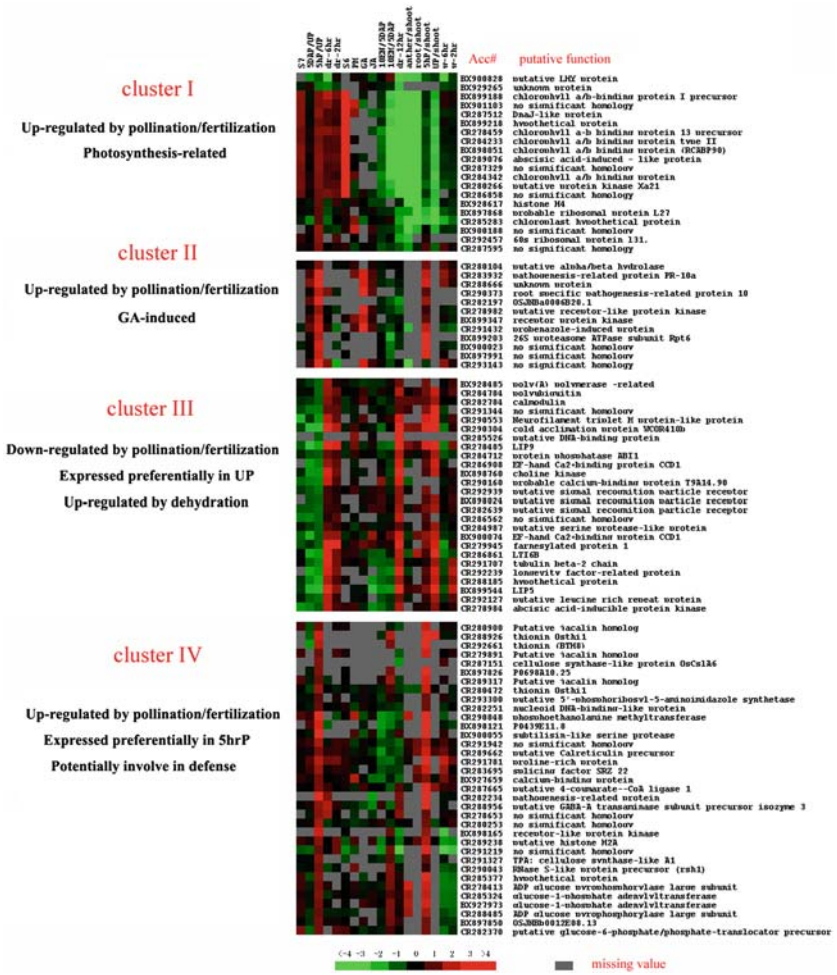

Figure 7. Clusters analysis of the pollination- and fertilization-related genes under different conditions. UP: pistil of unpollinated; 5hP: pistil of 0–5 h after pollination; 5DAP: pistil of 5 days after pollination. 10EM: embryos derived from 10-day-old seed; 10EN: endosperms derived from 10-day-old seed; S6: rice spikes derived from development stage 6 (meiotic division of pollen mother cells) in a floral mutant-1[a] and compared with that (spikes in stage 6) derived from wild type; S7: rice spikes derived from development stage 7 (filling stage of pollen) in the mutant-1[a] and compared with that derived from wild type; PM: spikes derived from development stage 5 (formation of the pollen mother cells) in a floral mutant-2[b] and compared with that derived from wild type; GA: rice callus cell suspension culture (GA treatment) vs. rice cell suspension culture (no treatment); JA: rice callus cell suspension culture (JA treatment) vs. rice cell suspension culture (no treatment); [a]Phenotypes of the floral mutant-1: spikes with decreased anthers, fleshy stigma, unclosed and multiiple lemma and palea. [b]Phenotypes of the floral mutant-2: completely degenerated pistil and anthers, mulriple lemma and palea.

potentially involved in the defense response, such as thionin, jacalin homolog, pathogenesis-related protein, *S*-like RNase protein precursor and cell wall-related proteins. ADP glucose pyrophosphorylase (large subunit), a key enzyme of starch biosynthesis, was preferentially expressed in another and 5hP (the shoot as a control), up-regulated by pollination/fertilization but was down-regulated by the wounding. However, its function in the pollination/fertilization remains unknown.

Discussion

An overview of cDNA microarray

Microarray has been shown to be a powerful tool for generating large amounts of data for parallel gene expression analyses. However, confirmation of the data by other means (such as RNA gel analysis and Real-Time PCR) is recommended to overcome the technical limitations of the microarrays (such as cross-hybridization between closely

related genes, cross-contamination of the PCR product and mislabeled for the cDNA clones) as well as biological variance. In this study, we have used gene-specific primers for Real-Time PCR to validate the results from cDNA microarray and confirmed the key microarray results of 30 genes. Real-Time PCR analysis indicated that the differentially expressed genes are highly predictive and the microarray data have a high correlation with the results from Real-Time PCR analysis. Although microarray data provide a measure of steady-state transcripts levels or relative steady-state transcripts levels only (Gygi *et al*., 1999), the gene expression profiles provide useful starting points for more in depth functional analyses.

Water stress may be an important factor in response to pollination and fertilization

Transport of sperm to the egg in flowering plants is a complex process involving many opportunities for signaling, including pollen tube guidance. Although specific mechanisms of the guidance for pollen tubes through the pistil are not clear, the female tissues play a critical role in the event (Rotman *et al*., 2003). For example, an elegant study using the laser cell ablation on ovules of *Torenia* has definitively shown that the cue for the pollen attraction is a diffusible signal emitted by the synergid cells surrounding the egg cells (Higashiyama *et al*., 2001). In our study, 53.8% pollination/fertilization related genes were responsive to the dehydration, indicating that water stress plays an important role in pollination and fertilization, and these dehydration-related genes are likely associated with the necessary changes in osmotic tensions to facilitate the rapid hydration/germination of pollen gains or establishing the gradient of the diffusible signal, which is required for guiding the pollen tube to the ovule. In the case of tobacco, a lipidic ECM (extracellular matrix) provides a gradient of water that is thought to give a directional cue of a physical nature of the pollen tube penetration into the stigma tissue (Wolters-Arts *et al*., 1998). A recent study has revealed that a GABA (gamma-aminobutyric acid) gradient along the tube path controls the regulation of pollen tube growth and guidance, and the insights into the role of GABA in pollination were gained from the studies of *pop*2 encoding a GABA-degrading transaminase (Palanivelu *et al*., 2003). Experimental evidence supports the involvement of GABA synthesis in pH regulation, nitrogen storage, plant development and defense, as well as a compatible osmolyte and an alternative pathway for glutamate utilization (Shelp *et al*., 1999; Bouché and Fromm, 2004). Whether GABA plays a specific role (i.e. osmolyte or osmoprotectant) under dehydration or is metabolized (e.g. to support the production of known osmolytes, such as proline) remain to be established. In our study, a putative GABA-A transaminase (CR288956) is up-regulated by pollination/fertilization and down-regulated (Real-Time PCR analysis) by the dehydration treatment. Moreover, a GABA-A receptor epsilon-like subunit gene (CR284320), involved in the signaling pathway of GABA, is down-regulated by pollination/fertilization and up-regulated by the dehydration, respectively (see Figure 3 and Table 1). Furthermore, nearly half of the genes expressed preferentially in the unpollinated pistils also are responsive to the dehydration (Figures 5 and 6), suggesting *a likely* evolutionary adaptation of pistil in response to various stresses, and the dehydration-regulated genes are probably involved in maintaining water homeostasis under the stress conditions, thus providing an optimal environment to support the pollen tube growth.

Photosynthesis- and carbohydrate metabolism-related genes are involved in pollination/fertilization

Cluster analysis indicated that many photosynthesis-related genes are up-regulated by pollination/fertilization and down-regulated by the dehydration, respectively. The down-regulation of the photosynthesis-related genes is consistent with a previous report that water stress inhibits photosynthesis (Tezara *et al*., 1999). Although the style is not thought to be a photosynthetic organ, it possesses well-developed chloroplasts, and indeed, enhanced photosynthetic flux was detected following pollination (Jansen *et al*., 1992). The up-regulation of the photosynthetic genes in the pistil is likely to attribute directly to sugars metabolic interactions between the style and the elongating pollen tube. Moreover, ADP glucose pyrophosphorylase (large subunit), a key enzyme in starch biosynthesis up-regulated by pollination/fertilization (Figure 7, cluster IV), is probably involved in the availability/allocation of carbohydrates during pollination/fertilization. Starch,

present all along the stylar tract at anthesis, vanishes as the pollen tubes pass by, indicating that the pollen tube growth is heterotrophic (Herrero and Arbeloa, 1989). In tobacco, the carbohydrate moiety associated the TTS (Transmitting Tissue-Specific) protein is arranged to form a gradient of glycosylation, increasing toward the bottom end of the style, and this gradient may has a chemotropic effect on the growing pollen tubes (Wu *et al.*, 1995). The vacuolar acid invertase (CR288152) transcripts are strongly down-regulated (about 22-fold change) by pollination/fertilization (Table 1). Invertase is a hydrolase, cleaving sucrose into glactose and fructose. In connection with the various roles that sucrose plays in plants (nutrient, osmoticum, and signal molecule), invertases likely function in pollination. Possibly, invertases cleave sucrose into hexoses to provide with carbon and energy for the synthesis of numerous different compounds in the growing pollen tube. One study in favor of this possibility has been published (Ylstra *et al.*, 1998). These authors showed the presence of a wall-bound invertase activity and uptake of sugars in the form of monosaccharides by the growing pollen tube. Alternatively, sucrose may not or only be used for metabolism but rather to modulate the availability of water and could thus represent a signal in pollen tube guidance. Lush *et al.* (1998) showed that the directional supply of water, i.e. the availability of water to the pollen tube, could establish a cue for the guidance of pollen tubes in tobacco.

Correlation of the gene expression between pollination/fertilization and defense responses

Similarity analysis revealed that many genes up-regulated by pollination/fertilization are potentially involved in defense responses, such as LTP (lipid transfer protein), thionin, jacalin homolog, pathogenesis-related protein, probenazole-inducible protein, *S*-like RNase protein precursor, isoflavone reductase-like protein, salt-induced protein and cell wall-related proteins (Table 1, Figures 3B and 7). In addition, proteomic approaches have recovered that the rice pathogen-related protein class 10 (OsPR-10), isoflavone reductase-like protein, salt-induced protein and probenazole-inducible protein were induced by rice blast fungus (Kim *et al.*, 2003). This co-ordinated gene expression indicates that the pollen tube may be acting like as a fungal hypha by eliciting a response from the style. In the pistils of *Solanum tuberosum*, the expression of an isoflavone reductase-like gene was enhanced by pollen tube growth (van Eldik *et al.*, 1997). Pollination, as well as wounding of the style, induced an increase in *SPP2* dioxygenase mRNA (*Solanum pollinated pistil2*) steady-state levels at a distance, in the ovary (Lantin *et al.*, 1999). Further, it has been reported that pollination and wounding induced nearly identical flavonol kinetics and patterns of accumulation in the outer cell layers and exudates of the stigma (Vogt *et al.*, 1994). In our study, 12 cDNAs are both up-regulated by pollination/fertilization and the wounding (Figure 3B), including five Ca^{2+}-related protein genes (BX927659, CR289662, CR291522, CR288514 and CR288514) (see Figure 3B). These results indicate that pollination and wounding likely share elements of a common signal transduction pathway. The need for such proteins (defense-related and signal transduction) may result from the physical penetration and, thereby, the wounding of the stigma by pollen tubes and/or resistance against pollen tube-mediated pathogen ingress.

GA response pathway in pollination/fertilization in rice

Seven cDNA*s including* three defense-related genes (CR283932, CR290373 and CR291432) are all up-regulated by pollination/fertilization and GA treatment in our study (Figure 7, cluster II), suggesting that the GA response pathway may cross-talk with pollination/fertilization and/or the pathogen-related pathway in rice. However, GA has not been reported to be associated with pathogen infection. In calli of rice, genes such as those for pathogen-related thaumatin-like protein, class III chitinase, phenylalanine ammonia-lyase, and thionin were responsive to GA, further analysis revealed that thionin functions under various conditions and is not just related to the pathogen response (Yazaki *et al.*, 2003). Thus, the biological functions of some defense-related genes, which are regulated by pollination/fertilization, require further work. In addition, a recent report has shown that the cellular distribution of bioactive GAs might be altered under different light and temperature conditions in *Arabidopsis* (Yamauchi *et al.*, 2004). Considering GA is required for the pollen

tube growth (Singh *et al*., 2002), it is tempting to suppose that the environmental factors, such as light and temperature, play an important role in pollination/fertilization. In fact, the light intensity and temperature have a profound effect on the pollen tube growth and embryo survival in some species (Campbell *et al*., 2001;Young *et al*., 2004). In this study, 24 cDNAs were both responsive to GA and pollination/fertilization (Figure S1) and their further functional analysis will help shed light on the GA response pathway during this important biological process.

Additional genetic networks in pollination/fertilization

A large number of genes described here have no similarity to proteins in the database and 100 cDNAs were not responsive (<2-fold change) to the stress treatments (dehydration and wounding). Among them, 44 cDNAs showed no significantly differential expression profiles (>2-fold change) in 14 microarray experiments (except four microarray experiments, viz., UP vs. shoot, 5hP vs. shoot, 5hP vs. UP and UP vs. 5DAP) (Figure S1), indicating that some of them were mainly involved in pollination/fertilization. For example, CR 278661, derived from OSAP1 (aspartic protease-like protein) gene, up-regulated by pollination/fertilization, was expressed preferentially in 5hP (shoot as a control) and with missing value in many microarray experiments (Figure S1). OSAPI shows high similarity (Blastp, *E*-value = 1e-83) to the nucellin in barley, which was expressed specifically in nucellar cells during their degeneration after pollination (Chen and Foolad, 1997).

In this study, a number of candidate genes have been identified to be likely involved in both pollination/fertilization and the dehydration and wounding responses, but their direct relationships need further experimental validation. Nevertheless, their detailed functional characterization is expected to provide a better understanding of the genetic programs controlling pollination/fertilization in rice.

Acknowledgements

We thank Kang Chong and Zhen Wang for generously sharing their microarray data related to GA and JA treatments for the 253 cDNAs, Ms. Lingfeng Chen for her technical assistance in microarray scanning, and Dr Zhen Su for the development of China cDNA Microarray Database (RIFGP-CDMD). This work was supported in part by grants from the Ministry of Science and Technology (MOST) of China (G199 9011602), the Transgenic Plant Initiative of the MOST, National Natural Science Foundation of China (30221002) and Chinese Academy of Sciences.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool J. Mol. Biol. 215: 403–410.

Bouché, N. and Fromm, H. 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9: 110–115.

Campbell, A.W., Griffin, W.B., Burritt, D.J. and Conner, A.J. 2001. The importance of light intensity for pollen tube growth and embryo survival in wheat × maize crosses Ann. Bot. 87: 517–522.

Chen, F. and Foolad, MR. 1997. Molecular organization of a gene in barley which encodes a protein similar to aspartic protease and its specific expression in nucellar cells during degeneration Plant Mol. Biol. 35: 821–831.

Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, JL., Wang, X. and Zhu, T. 2002. Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses Plant Cell 14: 559–574.

Cooper, B., Clarke, J.D., Budworth, P., Kreps, J., Hutchison, D., Park, S., Guimil, S., Dunn, M., Luginbuhl, P., Ellero, C., Goff, S.A. and Glazebrook, J. 2003. A network of rice genes associated with stress response and seed development Proc. Natl. Acad. Sci. USA 100: 4945–4950.

Dixit, R., Rizzo, C., Nasrallah, M. and Nasrallah, J. 2001. The *brassica* MIP-MOD gene encodes a functional water channel that is expressed in the stigma epidermis Plant Mol. Biol. 45: 51–62.

Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. 1998. Cluster analysis and display of genome-wide expression patterns Proc. Natl. Acad. Sci. USA 95: 14863–14868.

Franklin-Tong, V.E. 1999. Signaling and the modulation of pollen tube growth Plant Cell 11: 727–738.

Gygi, S.P., Rochon, Y., Franza, B.R. and Aebersold, R. 1999. Correlation between protein and mRNA abundance in yeast Mol. Cell Biol. 19: 1720–1730.

Herrero, M. and Arbeloa, A. 1989. Influence of the pistil on pollen tube kinetics in peach ({IPrunus persica}) Am. J. Bot. 76: 1441–1447.

Higashiyama, T., Yabe, S., Sasaki, N., Nishimura, Y. Miyagishima, S., Kuroiwa, H. and Kuroiwa, T. 2001. Pollen tube attraction by the synergid cell Science 293: 1480–1483.

Jansen, M.A.K., Sessa, G., Malkin, S. and Fluhr, R. 1992. PEPC-mediated carbon fixation in transmitting tract cells reflects style-pollen tube interactions Plant J. 2: 507–515.

Kim, S.T., Cho, K.S., Yu, S., Kim, S.G., Hong, J.C., Han, C.D., Bae, D.W., Nam, M.H. and Kang, K.Y. 2003. Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells Proteomics 3: 2368–2378.

Lan, L.F., Chen, W., Lai, Y., Suo, J.F., Kong, Z.S., Li, C., Lu, Y., Zhang, Y.J., Zhao, X.Y., Zhang, X.S., Zhang, Y.S., Han, B., Cheng, J. and Xue, Y.B. 2004. Monitoring of gene expression profiles and isolation of candidate genes involved in pollination and fertilization in rice (*Oryza sativa* L.) using a 10K cDNA microarray Plant Mol. Biol. 54: 471–487.

Lantin, S., O'Brien, M. and Matton, D.P. 1999. Pollination, wounding and jasmonate treatments induce the expression of a developmentally regulated pistil dioxygenase at a distance, in the ovary, in the wild potato *Solanum chacoense Bitt*. Plant Mol. Biol. 41: 371–386.

Lush, W.M., Grieser, F. and Wolters-Arts, M. 1998. Directional guidance of *Nicotiana alata* pollen tubes *in vitro* and on the stigma Plant Physiol. 118: 733–741.

Palanivelu, R., Brass, L., Edlund, A.F. and Preuss, D. 2003. Pollen tube growth and guidance is regulated by *POP2*, an *Arabidopsis* gene that controls GABA levels Cell 114: 47–59.

Potocky, M., Elias, M., Profotova, B., Novotna, Z., Valentova, O. and Zarsky, V. 2003. Phosphatidic acid produced by phospholipase D is required for tobacco pollen tube growth Planta 217: 122–130.

Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F. and Faure, J.E. 2003. Female control of male gamete delivery during fertilization in *Arabidopsis thaliana* Curr. Biol. 13: 432–436.

Sanchez, A.M., Bosch, M., Bots, M., Nieuwland, J., Feron, R. and Mariani, C. 2004. Pistil factors controlling pollination Plant Cell 16(Suppl) S98–S106.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A. and Coupland, G. 1998. The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering Cell 93: 1219–1229.

Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray Science 270: 467–470.

Shelp, B.J., Bown, A.W. and McLean, M.D. 1999. Metabolism and functions of gamma-aminobutyric acid Trends Plant Sci. 4: 446–452.

Singh, D.P., Jermakow, A.M. and Swain, S.M. 2002. Gibberellins are required for seed development and pollen tube growth in *Arabidopsis* Plant Cell 14: 3133–3147.

Tezara, W., Mitchell, V.J., Driscoll, S.P. and Lawlor, D.W. 1999. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP Nature 401: 914–917.

van, Eldik G.J., Ruiter, R.K., Colla, P.H., Herpen, M.M.van, Schrauwen, J.A. and Wullems, G.J. 1997. Expression of an isoflavone reductase-like gene enhanced by pollen tube growth in pistils of *Solanum tuberosum* Plant Mol. Biol. 33: 923–929.

Vogt, T., Pollak, P., Tarlyn, N. and Taylor, L.P. 1994. Pollination- or wound-induced kaempferol accumulation in *petunia* stigmas enhances seed production Plant Cell 6: 11–23.

Wang, X. 2002. Phospholipase D in hormonal and stress signaling Curr. Opin. Plant Biol. 5: 408–414.

Weterings Russell, K.S.D. 2004. Experimental analysis of the fertilization process Plant Cell 16(Suppl) S107–S118.

Wolters-Arts, M., Lush, W.M. and Mariani, C. 1998. Lipids are required for directional pollen-tube growth Nature 392: 818–821.

Wu, H.M., Wang, H. and Cheung, A.Y. 1995. A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower Cell 82: 395–403.

Ylstra, B., Garrido, D., Busscher, J. and van Tunen, A.J. 1998. Hexose transport in growing petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide transporter Plant Physiol. 118: 297–304.

Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y. and Yamaguchi, S. 2004. Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds Plant Cell 16: 367–378.

Yazaki, J., Kishimoto, N., Nagata, Y., Ishikawa, M., Fujii, F., Hashimoto, A., Shimbo, K., Shimatani, Z., Kojima, K., Suzuki, K., Yamamoto, M., Honda, S., Endo, A., Yoshida, Y., Sato, Y., Takeuchi, K., Toyoshima, K., Miyamoto, C., Wu, J., Sasaki, T., Sakata, K., Yamamoto, K., Iba, K., Oda, T., Otomo, Y., Murakami, K., Matsubara, K., Kawai, J., Carninci, P., Hayashizaki, Y. and Kikuchi, S. 2003. Genomics approach to abscisic acid- and gibberellin-responsive genes in rice DNA Res. 31: 249–261.

Young., L.W., Wilen., R.W. and Bonham-Smith, P.C. 2004. High temperature stress of *Brassica napus* during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production J. Exp. Bot. 55: 485–495.

Zonia, L. and Munnik, T. 2004. Osmotically induced cell swelling versus cell shrinking elicits specific changes in phospholipid signals in tobacco pollen tubes Plant Physiol. 134: 813–823.