Plant Molecular Biology (2005) 58:721–737
DOI 10.1007/s11103-005-8267-4
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Microarray analysis of gene expression involved in anther development in rice (*Oryza sativa* L.)

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Received 13 August 2004; accepted in revised form 1 June 2005

Key words: anther development, DNA microarray, expression profile, GA_3, JA, rice

Abstract

In flowering plants, anthers bear male gametophytes whose development is regulated by the elaborate coordination of many genes. In addition, both gibberellic acid (GA_3) and jasmonic acid (JA) play important roles in anther development and pollen fertility. To facilitate the analysis of anther development genes and how GA_3 and JA regulate anther development, we performed microarray experiments using a 10-K cDNA microarray with probes derived from seedlings, meiotic anthers, mature anthers and GA_3- or JA-treated suspension cells of rice. The expression level change of 2155 genes was significantly (by 2-fold or greater) detected in anthers compared with seedlings. Forty-seven genes, representing genes with potential function in cell cycle and cell structure regulation, hormone response, photosynthesis, stress resistance and metabolism, were differentially expressed in meiotic and mature anthers. Moreover, 314 genes responded to either GA_3 or JA treatment, and 24 GA_3- and 82 JA-responsive genes showed significant changes in expression between meiosis and the mature anther stages. RT-PCR demonstrated that gene y656d05 was not only highly expressed in meiotic anthers but also induced by GA_3. Strong RNA signals of y656d05 were detected in pollen mother cells and tapetum in *in situ* hybridization. Further characterization of these candidate genes can contribute to the understanding of the molecular mechanism of anther development and the involvement of JA and GA_3 signals in the control of anther development in rice.

Abbreviation list: EST, expressed sequence tag; GA_3, gibberellic acid; JA, jasmonic acid; RT-PCR, reverse transcription-polymerase chain reaction

Introduction

The life cycle of flowering plants alternates between the diploid sporophyte and haploid gametophyte generations. The stamen is the male reproductive organ, consisting of an anther where the male gametophyte develops and a filament that provides water and nutrients to the anther. In general,

anther development is divided into two phases (Goldberg *et al.*, 1993; Zhao *et al.*, 2002). During phase I, anther structure is established including the differentiation of different cells types. One key event during phase I is meiosis of pollen mother cells. During phase II, microspores develop into pollen grains, and anthers dehisce to release pollen grains. Anther development involves cell division, cell differentiation and cell death. Many genes contribute to anther development, although only a small number are known to be specifically involved in this complex developmental process (Klimyuk and Jones, 1997; Kapoor *et al.*, 2002).

Many studies have identified and characterized genes regulating anther development. In *Brassica napus*, the expression of *I3*, designated an 'early' stage gene, reaches its highest level during meiosis and decreases as anthers matured (Roberts *et al.*, 1991). Some 'late' stage genes such as *NTP303* and *Bcpl* were detected at their highest expression level in mature anthers (Theerakulpist *et al.*, 1991; Weterings *et al.*, 1992). Silencing studies revealed that *NTP303* plays roles in pollen tube growth (de Groot *et al.*, 2004). Moreover, anther and/or tapetum-specific genes were also isolated from *Arabidopsis* (Rotman *et al.*, 2005), maize (Lauga *et al.*, 2000) and tobacco (Cecchetti *et al.*, 2004). Recently, molecular approaches have facilitated the definition of genes with function in anther cell patterning (Walbot and Evans, 2003). In *Arabidopsis*, the *SPL/NZZ* gene is required for tapetum and microsporocyte formation (Yang *et al.*, 1999). Both *EMS1/EXS1* and *TPD1* regulate tapetal cell fate determination (Zhao *et al.*, 2002; Yang *et al.*, 2003). In rice, *MSP1* gene plays a role similar to *EMS1/EXS1* in anther development (Nonomura *et al.*, 2003).

Meiosis occurs in anther development. Many genes are involved in a wide range of meiotic processes such as chromosome cohesion and condensation, recombination, synapsis, segregation and cell cycle regulation (Higgins *et al.*, 2004). Considering the complexity of anther development, it is necessary to discover additional genes that are essential for anther development. Studies have also been performed to identify genes regulating anther development on a larger scale, particularly with the application of microarray techniques. Early in the 1990s, genes involved in microspore development were isolated through the screening of specific cDNA libraries that were generated from sorted cells by flow cytometry during microsporogenesis (Mascarenhas, 1990). Using subtractive hybridization, 13 anther-specific genes were isolated from *Arabidopsis* (Rubinelli *et al.*, 1998). Many more genes were found to be associated with flower development from expression studies involving microarray techniques (Hennig *et al.*, 2004; Wellmer *et al.*, 2004). However, the functions of most of these genes remain unknown. Furthermore, it is not clear how these genes are related to anther development.

Both gibberellic acid (GA_3) and jasmonic acid (JA) play important roles in anther development and pollen fertility. Studies showed that GA_3 promotes the formation and release of mature pollen grains (Goto and Pharis, 1999; Swain *et al.*, 2004). Further studies revealed that GA_3 controls cell elongation of stamen filaments and the formation of mature pollen grains (Cheng *et al.*, 2004). JA and JA signaling are required for pollen development and anther dehiscence. JA-insensitive mutant *coi1* is male sterile and JA synthesis-deficient mutants are defective in anther dehiscence (Zhao and Ma, 2000). Twenty-five JA-regulated anther-development genes were detected in *Arabidopsis* by differential display (Mandaokar *et al.*, 2003). However, our understanding of the molecular mechanism of GA and JA signaling in anther development is scant. With the completion of the whole-genome sequencing, rice, a major crop of the world, has become a model plant organism for addressing both fundamental and applied questions in plant sciences.

To better understand how genes control anther development at the genome level, we compared gene expression patterns in rice anthers at different developmental stages using a 10-K cDNA microarray (Lan *et al.*, 2004). We also profiled gene expression pattern with GA_3 or JA treatment of suspension-cultured cells from rice. A comparison of mature anthers and seedlings revealed 2155 genes with 2-fold or more change in expression. Forty-seven genes were differentially expressed in meiotic anthers and mature anthers. Moreover, among the 314 genes responding to GA_3 or JA treatment, 24 GA_3- and 82 JA-responsive genes showed more than 2-fold expression changes in meiosis compared with at mature anther stages. Our results could contribute to further characterization the function of genes and dissection the roles of JA and GA_3 signaling in rice anther development.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. ssp. *japonica* cv Zhonghua 10) seeds were germinated once a week for 5 weeks to continuously provide enough plants for anther isolation. Before being transplanted to the field, some of the 2-week-old seedlings were harvested for RNA extraction. In general, anthers at the meiotic stage (meiotic anthers) were dissected from rice flowers when the distance between the last two leaf collars was within 2 cm (−1 cm to +1 cm) (Figure 1A, Chen *et al.*, 2005). When the collar of the flag leaf reached the collar of the penultimate leaf, the upper-middle, middle and lower florets of the spike were dissected from plants to determine the developmental stage of the anthers. In one spike, meiotic anthers were from florets growing adjacent to and with a similar length as florets containing meiotic anthers determined according 4′-6-diamidino-2-phenylindole (DAPI) staining. Meiotic anthers were collected for RNA preparation (Figure 1B–G), while anthers with obvious pollen were harvested right before anthesis and for RNA extraction of mature anthers (Figure 1H).

Chromosome observation

To determine the developmental stages of rice anthers, anthers were dissected from fresh rice florets and then stained with DAPI (1 μg/ml) for 3–5 min on a microscope slide. Coverslips were tapped to squash the anthers and release their content. Chromosomes were observed under a fluorescence microscope (Zeiss, Germany) (Park *et al.*, 1998).

Suspension cell culture

Rice calli were cultured in N_6 liquid medium (Chu *et al.*, 1975) containing 2,4-D (1 mg/l) in a rotary shaker at 150 rpm at 25 °C for 4 weeks with the medium refreshed every week. Then the suspension cells (0.15 g/ml) were treated with 10 μM JA (Sigma, St. Louis, MO, USA) or GA_3 (Sigma) for 5 and 4 h, respectively. The JA- or GA_3-treated cells and untreated cells were filtered through a 2×2 mm^2 sieve. The cells passing through the sieve were collected and used for RNA extraction.

RNA preparation

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from the meiotic anthers, mature anthers and rice suspension cells, including untreated controls and those treated with GA_3 or JA. The quality of total RNA was examined with use of a DU 640 Nucleic Acid & Protein Analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Total RNA at a ratio of OD_{260} to OD_{280} >1.8 was further used to prepare mRNA with use of an mRNA Extraction Kit (Qiagen, Valencia, CA, USA).

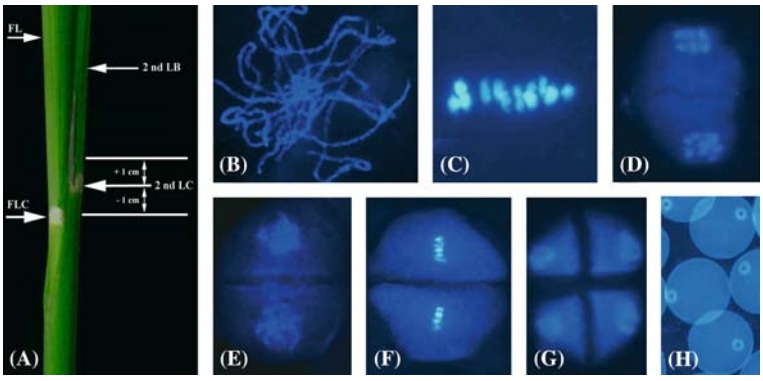

Figure 1. The developmental stages of rice anther (Chen *et al.*, 2005). (A) The location of rice flag leaf when meiosis occurs in anther (FL: flag leaf. FLC: flag leaf collar. 2nd LB: the second leaf blade. 2nd LC: the second leaf collar). (B)–(G) Meiosis stages in rice anther. Anthers in the selected stages were stained with DAPI (1 μg/ml) and images were acquired under a fluorescence microscope (Zeiss, Germany). (B) Pachytene. (C) Metaphase I. (D) Telephase I. (E) Interkinesis. (F) Metaphase II. (G) Telophase II. (H) Mature pollen grains just before anthesis.

Probe labeling, microarray hybridization, scanning and data acquisition

One microgram of Poly (A^+) RNA was labeled with the fluorescence dye Cy3 or Cy5 according to instructions for the CyScribe^{TM} Post-Labeling Kit (Amersham Biosciences, San Francisco, CA, USA). Purification, hybridization and washing were performed according to the manufacturer's instructions. Hybridized slides were scanned with use of a GenePix 4000B scanner (Axon Instruments Inc., Union City, CA, USA) at 532 and 635 nm to capture the emission of Cy3 and Cy5, respectively. The intensity of each spot at the two wavelengths was transformed into the ratio value by use of GenePix 4.0 software. The overall intensity of the hybridized slide was then normalized by GenePix 4.0 software. Spots automatically flagged 'Bad' or 'Not Found' by the software and whose [media of signal (S)/media of background (B)] <4 were discarded (Lan *et al.*, 2004). Thus, only the spots whose signal intensity was at least 4-fold higher than the background were further analyzed. In addition, we ruled out those spots whose regulation pattern was contradictory in two no-dye-exchange replicates. Only data with $|\text{Log}_2 \text{ ratio}| \geq 1$ in all four replicates were subjected to further clustering analysis with us of software from Stanford University (http://rana.lbl.gov/EisenSoftware.htm).

Gene annotation and promoter analysis

Using BLAST search program, we annotated all ESTs encoding about 10 000 genes. Three major rice databases were used for both BLASTn and BLASTp analysis: NCBI (http://www.ncbi.nlm.nih.gov), TIGR rice genome project (www.Tigr.org) and GRAMENE (www.gramene.org). Genes were assigned only when their annotations from at least two of the three databases were consistent with each other. Whereas, cDNA sequences without alignment to any known gene of the three databases were sorted as putative genes, unknown genes or no hits (no significant homology). In addition, based on gene location information from TIGR database, a 2.0-kb sequence upstream of the 5′ UTR was found from GRAMENE as a putative promoter region. Then the 2.0-kb sequence was analyzed to search GA_3- or JA-responsive elements by use of PlantCARE software (http://intra.psb.ugent.be:8080/PlantCARE).

Reverse transcription-polymerase chain reaction (RT-PCR)

To verify the reliability of the microarray and further study gene expression patterns, RT-PCR was conducted with use of One Step RNA PCR Kit (AMV) (TaKaRa, Japan) with gene-specific primers designed by Primer 5.0 and synthesized by Sangon Company (Shanghai, China) (Table 1). Total RNA for RT-PCR was extracted from materials used for microarray. Rice *tubA* gene (gi:1136119) was used as a control (Table 1).

To investigate the expression pattern of gene y656d05, 3-week-old rice seedlings were treated for 24 h with 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} or 10^{-7} M of GA_3 and then harvested for RNA extraction. Seedlings, roots of 3-week-old plants, shoots, leaves of mature plants, spikes, meiotic anthers and mature anthers were also collected from untreated plants for RNA extraction. RT-PCR results were quantified and standardized by comparing the intensity of *tubA* with tested genes by use of BIO-1D software (Vilber Lourmat, France).

RNA in situ *hybridization*

For RNA *in situ* hybridization of gene y656d05, a probe containing a direct repeat of a 150-bp gene-specific fragment was constructed into pGEM-T-Easy vector. Use of isocaudarners, including *Nhe*I and *Spe*I, and *Xho*I and *Sa* I, allowed for the introduction of *Nhe*I and *Xho*I, respectively, into the ends of gene-specific primers (Wang *et al.*, 2004). Plasmid DNA was linearized by use of *Nde*I and *Eco*RI and then transcribed *in vitro* with T7 and SP6 RNA polymerase, respectively. The 500-bp RNA transcript corresponding to the T7 direction was used as a sense probe, while the SP6 transcript was used as an anti-sense probe. *In situ* hybridization was carried out as described (Xu *et al.*, 2002b). Images were observed and captured by microscopy (Zeiss, Germany).

Results

The reliability of cDNA microarray in profiling gene expression during anther development

To minimize the false-positive results in our microarray experiment, we performed at least four

Table 1. Primers used in RT-PCR to verify the expression pattern of differentially expressed genes from the microarray experiment.

Clone	Forward primer sequence	Reverse primer sequence	Log_2 Ratio[a]	Log_2 Ratio[b]
Y688h03[c]	5′TCC AGT ACT CCA CCA CAA GCT CTA-3′	5′-TTA GTT GAT CCT GCA TAA AA-3′	2.41	2.09
Y630g09[c]	5′-TTC CCT TCT GTC AAA TAC ATG AG-3′	5′-ATC GGG AAC AGA TCT CAA TTT GC-3′	2.38	1.77
Y697f12[c]	5′-ACG GTT GCT GCA GCT CGG CAT CAA-3′	5′-GTA GAT GAA ACG CAC GCG AAC TAT-3′	2.35	1.82
Y638f01[c]	5′-GAA TGG TGA GGA TGG ATA CAA GCC-3′	5′-CTT GTG GAT CAA TAG GCT GAG CGG-3′	−1.74	−1.06
Y689d12[c]	5′-TCA GTG CCC GCA GTT CGA TCG ATC-3′	5′-TGG TTT CGT CAG GAG GTT GCT TGC-3′	−1.89	−0.89
Y657f05[c]	5′-TTG GAG CTG TTG CAA ACC CAA AGA-3′	5′-AAG CCG AAT TAG CTT ACG CTT CAT-3′	−2.00	−0.97
P629e11[c]	5′-GTC GCG TGG ATC AAG CGC CTC CTC-3′	5′-TCT CGC ATC CAT GAC ATC GTG CAT-3′	−2.00	−1.06
y656d05[c]	5′-CCA ATG GCG TCC TCC ACC AAG ATC CCC-3′	5′-GGA GGT ATT AAT CAT ACG GTA AAA-3′	2.21	2.06
y827c11[d]	5′- GAG AAA GCG CTT GCA AAC TCC GCT-3′	5′-TGT TTT TAT ATC TCT CTG TAA AGG-3′	2.72	2.27
y775f07[d]	5′-AAG CAC ACC AAC CAG TAC CC GTT -3′	5′-GCG GTT CAG CTG TAC TGG ACG AAG-3′	1.96	1.32
y812c10[d]	5′- ACT AGC TTA GAT AGA GAT GGC TCC T-3′	5′-CCA CAA TAT TTT TAG GCG TAC TCA-3′	1.89	1.32
p803a08[e]	5′-AGA AGA TGT CTT GCT GCG GCG GCA-3′	5′-GAT AGA TTC AGT TGC AGG AGC AGC-3′	−5.06	−3.47
y670f04[e]	5′- TCA TCA GGA ATG GAG CGG CTG AGC-3′	5′-GGG TAG CAC TAC CAG CTC CAC CTC-3′	3.01	2.65
y798c05[e]	5′-GAG GTT CAA GAT GGC TTT TGA GAC-3′	5′-TTT CTC GAT TCC GAA CAT GTC AGC-3′	3.22	2.70
y616f01[e]	5′-CCT CAG CAA CCA TGT CGG CCT ACT-3′	5′-AGA CGG GCA CGC CAC TCG GCG AAG C-3′	3.29	2.70
y679c01[e]	5′-CAG CAT AAT CTT CCT GAT CAA CAA-3′	5′-CTA AAA GTT TCT ACT GAG GAT TTG-3′	3.63	3.35
Tubulin[f]	5′-TCA GAT GCC CAG TGA CAG GA-3′	5′-TTG GTG ATC TCG GCA ACA GA-3′		

[a] Log_2 Ratio from microarray experiment.
[b] Log_2 Ratio from RT-PCR.
[c] genes differentially expressed in meiotic anthers.
[d] GA_3-responsive genes.
[e] JA-responsive genes.
[f] primer sequence for rice *tubA* used as a control for RT-PCR.

replicates including two dye-exchange replicates with RNA extracted independently from different batches of plant materials. The distribution of all ESTs showed an overall balance of the two dyes in one hybridization (Figure 2A), and the expression level changes of genes detected from two replicates were superimposed (Figure 2B). Moreover, the correlation coefficients among the four replicates using one probe, for example hybridization with probes synthesized by meiotic anthers, ranged from 0.78 to 0.93 (Table 2), which suggests that the microarray hybridization results are generally producible.

To verify the results from microarray analysis, we performed RT-PCR on 16 randomly selected genes using gene-specific primers to examine their expression level changes (Table 1). The expression pattern of all 16 genes was similar to that from microarray analysis (Figure 3A and Table 1), as indicated by a high degree of concordance ($R = 0.9872$) between the two methods (Figure 3B). Furthermore, in our microarray data, four randomly scattered cDNAs showed a similar expression pattern (Figure 3C), which is consistent with three of the cDNAs representing the same gene and the fourth encoding a similar protein. In summary, our results from the microarray experiments are reliable.

Expression profiling of genes potentially involved in rice anther development

To analyze predicted biochemical function of genes involved in rice anther development, we compared gene expression between anthers in meiosis and anthers just before anthesis. In this study, the developmental stages of anther were examined by the observation of chromosome behavior using DAPI staining. The anthers undergoing meiosis were first decided by the distance

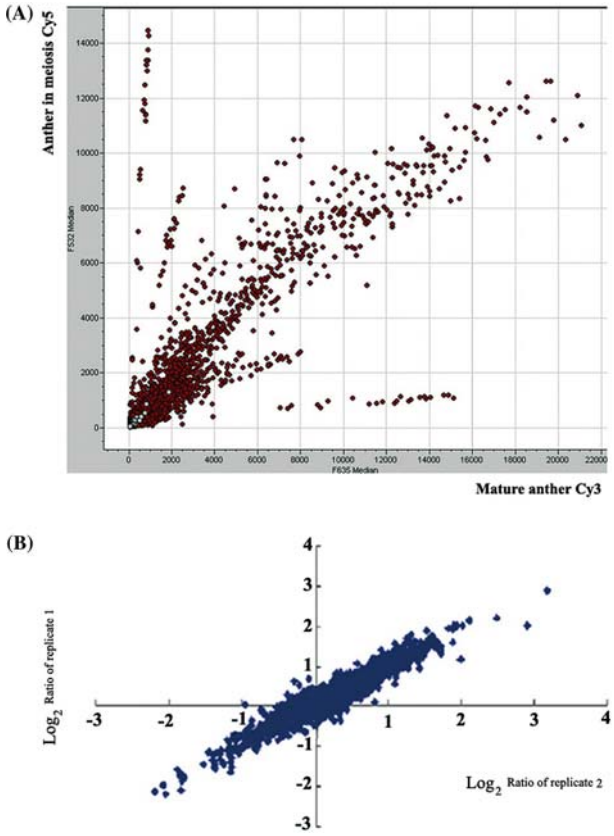

Figure 2. Reliability of the microarray experiment. (A) Distribution of about 10 000 genes in one hybridization. (B) Reproducibility analysis according to the ratio of the replicates with Ma-Cy3 and Am-Cy5.

Table 2. Correlation coefficient from four replications of Am vs. Ma[a] hybridization.

	Am-Cy5 Ma-Cy3 (1)[b]	Am-Cy5 Ma-Cy3 (2)	Am-Cy3 Ma-Cy5 (1)	Am-Cy3 Ma-Cy5 (2)
Am-Cy5 vs. Ma-Cy3 (1)[b]	/[c]	0.9300	0.8000	0.8130
Am-Cy5 vs. Ma-Cy3 (2)	0.9300	/	0.7754	0.8000
Am-Cy3 vs. Ma-Cy5 (1)	0.8000	0.7754	/	0.8737
Am-Cy3 vs. Ma-Cy5 (2)	0.8130	0.8000	0.8737	/

[a] Am: anthers in meiosis (meiotic anthers). Ma: mature anthers. Cy3: Cy3-dUTP. Cy5: Cy5-dUTP.
[b] (1) & (2) represent replicate 1 and replicate 2, respectively.
[c] the same slide.

between the last two leaf collars and then confirmed by their chromosome features in meiosis (Figure 1) (Chen *et al*., 2005).

Comparing gene expression between mature anthers and seedlings revealed that 2155 genes with a 2-fold or more changes. Furthermore, 47 genes showed expression changes between meiotic anthers and mature anthers, suggesting that they might be involved in male meiosis (Table 3 and Figure 4). These 47 genes, representing 0.47% of the 10 000 genes in the microarray, were divided into 6 main groups based on their potential functions: cell cycle and cell structure, hormone, photosynthesis, carbohydrate metabolism, stress and transportation (Table 4). Among the 47 genes y692f03 (BX900363), y638f01 (CR289672) and y759c09 (BX898653) could play some roles in meiosis based on the characteristics of their encoding proteins (Pavlova and Zakiian, 2003; Prigent and Dimitrov, 2003; Strunnikov, 2003). Interestingly, most of these anther development-related genes belong to metabolism or unknown protein group, accounting for 36.2% and 19.1% of the 47 genes, respectively.

Identification of anther development genes affected by GA_3 or JA

Both GA_3 and JA are important for anther development and pollen fertility in variety of plant species (Cheng *et al*., 2004; Kaneko *et al*., 2004). To investigate the role of GA_3 and JA in anther development genome wide, we examined gene expression of suspension cells treated with 10 μM GA_3 or JA. A total of 88 genes showed response to GA_3 treatment and 248 to JA treatment, representing 314 genes and 3.14% of genes of the array (Table 3). Among them, 24 GA_3- and 82

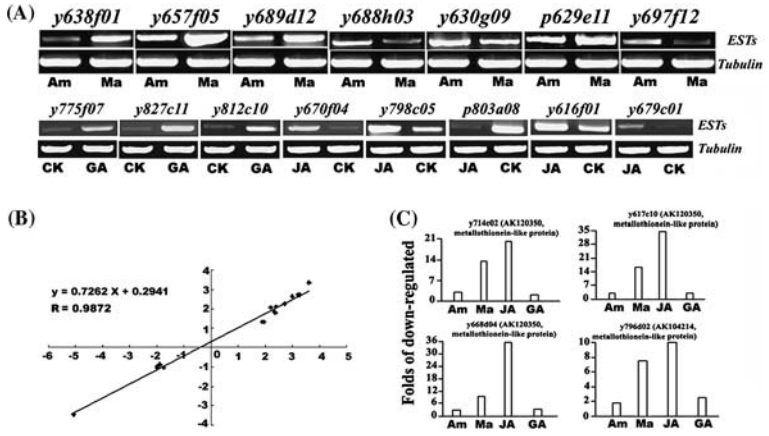

Figure 3. The reproducibility of microarray experiment assessed by RT-PCR. (A) Expression pattern of the 15 randomly selected differentially expressed genes by RT-PCR. (B) Correlation analysis of the ratio of differentially expression level from microarray experiment to that from RT-PCT. (C) Similar expression pattern of four randomly scattered genes encoding the same category of proteins. *Y*-axis represents the ratio in hybridization; ID of ESTs, their accession number and the corresponding putative proteins are on the top of each panel. Am: anthers in meiosis (meiotic anthers); Ma: mature anthers; JA: jasmonic acid treatment; GA_3: gibberellin treatment; CK: no treatment control.

JA-responsive genes showed 2-fold more expression changes between meiotic anthers and mature anthers (Figure 5). Fifteen of them have been identified as possibly involved in anther development (Table 5). Moreover, 12 of the 23 genes responsive to both GA_3 and JA displayed expression changes during anther development. Whereas, only 1 anther-preferential gene exhibited a similar expression pattern after treated by GA_3 or JA (Figure 5). In addition, clustering analysis of the 314 genes demonstrated that anther development-related genes responded differently to the 2 hormones (Figure 6). Therefore, our results suggest that GA_3 and JA could function in anther development, although most anther development involved genes possessed different expression patterns in GA_3 and JA treatments.

To seek additional evidence that these genes are responsive to GA_3 and/or JA treatments, promoter analysis was performed with PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE). Studies revealed that TGACG or CGTCA is MeJA-responsive elements in *Hordeum vulgare* and CCTTNNN or TATCNNN is gibberellin-responsive elements in *Oryza sativa* (Mason *et al*., 1993; Gomez-Maldonado *et al*., 2004). Our promoter analysis showed that 56.5% and 61.4% of JA- or GA_3-responsive genes detected in the microarray

Table 3. Number of genes differentially expressed in microarray hybridizations.

Plant part and treatment	Up-regulated genes			Down-regulated genes			Up/down	Percentage
	2–3	3–5	> 5	2–3	3–5	> 5		
Ma/Se	607	426	640	282	119	81	1673/482	21.6
Am/Ma	16	13	4	9	5	0	33/14	0.5
GA_3/CK	54	14	1	6	13	0	69/19	0.9
JA/CK	46	45	23	74	40	20	114/134	2.5

Numbers in the table represent the number of genes detected as differentially expressed in the microarray hybridizations. Ma: mature anthers. Se: seedling. Am: anthers in meiosis. GA_3: GA_3-treatment. JA: JA-treatment. CK: suspension-cultured rice cells of wild type.

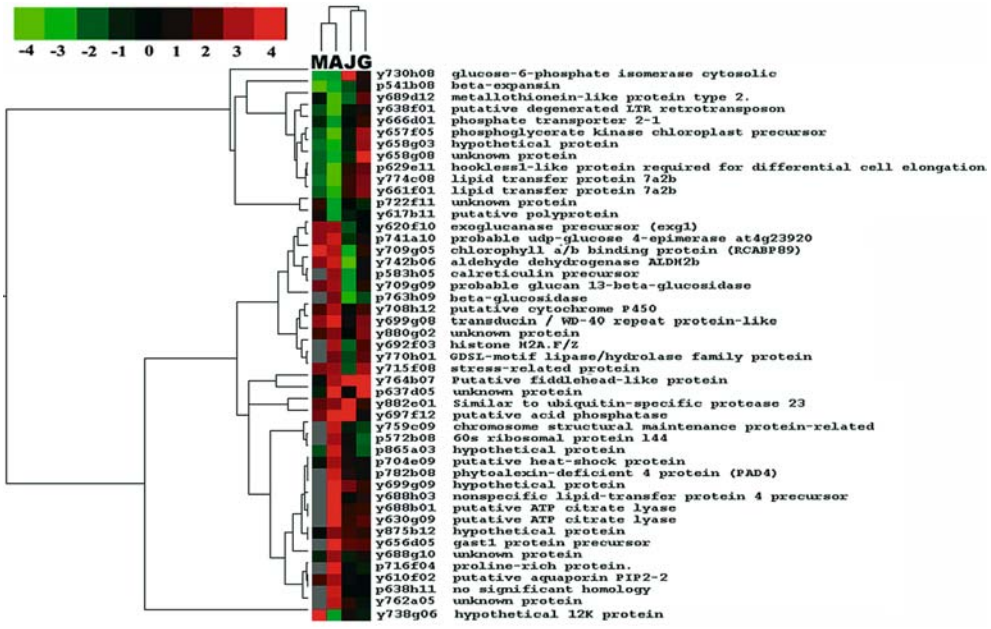

Figure 4. Clustering of the 47 genes showing expression changes in meiotic and mature anthers. J: JA-regulated genes; G: GA_3-regulated genes; M: genes differentially expressed in mature anthers compared with seedlings; A: genes differentially expressed in meiotic anthers compared with mature anthers.

Table 4. The 47 rice genes differentially expressed in anther development.

Putative function	Clone ID Description	Accession number	E-value	Log_2 M[a]	Log_2 A[b]	Log_2 J[c]	Log_2 G[d]
Cell cycle and cell structure							
	y638f01 putative chromosome condensation factor[e]	AC104285	e−143	−1.83			
	y882e01 similar to ubiquitin-specific protease 23[e]	AB117997	0.0	1.30		3.45	
	y759c09 chromosome structural maintenance protein-related[e]	AK101365	0.0	1.75			
	y699g08 transducin/WD-40 repeat protein-like[e]	AK111777	0.0	1.92	1.37		1.14
	y692f03 histone H2A[e]	AK121533	0.0	1.53			
	p572b08 60s ribosomal protein l44	AK069083	e−121	1.47			
	p541b08 beta-expansin[e]	AK061423	0.0	−1.36	−3.15		
Hormone							
	y656d05 gast1 protein precursor	AK062516	2.00E−95	2.21			
	p629e11 hookless1-like protein	AK119884	3.00E−52	−2.02			
Photosynthesis							
	y708h12 putative cytochrome P450	AP005467	0.0	1.52			
	y709g05 chlorophyll a/b binding protein (RCABP89)	AK066762	0.0	1.60	2.52	−1.44	
	y657f05 phosphoglycerate kinase chloroplast precursor	AK100371	0.0	−1.98			1.46
Carbohydrate metabolism							
	y709g09 probable glucan 13-beta-glucosidase	AK122100	1.00E−55	1.45	1.02	−1.25	
	y620f10 exoglucanase precursor (exg1)	AK065044	e−108	1.39	1.33		
	p763h09 beta-glucosidase	AK105026	0.0	1.17		−1.60	
	y730h08 glucose−6-phosphate isomerase	AK103010	0.0	−1.30	−1.32	2.24	
	y738g06 ribulose bisphosphate carboxylase large subunit	AL731605.3	e−153	−1.26	4.76		
Stress/defense							
	p716f04 proline-rich protein[e]	AC118673	e−133	1.94			
	P704e09 putative heat-shock protein	AK073732	0.0	1.50			
	y715f08 stress-related protein	AK105010	0.0	1.40	1.16		1.49
	y689d12 metallothionein-like protein type 2.	AP002540	e−153	−1.88			
	p782b08 phytoalexin-deficient 4 protein (PAD4)	AC145322	3.00E−05	1.80			
	y931e01 chitinase	T03614	2.00E−27	−0.35			
	y659a07 chitinase	L37289	4.00E−29	−0.23			
Transportation							
	y610f02 putative aquaporin PIP2-2[e]	AK102155	0.0	1.48			
	y666d01 phosphate transporter 2-1	AP004079	0.0	−1.24			
	y688h03 nonspecific lipid-transfer protein 4 precursor	AK119692	0.0	2.41			
	y661f01 lipid transfer protein 7a2b	AK064888	0.0	−1.58			1.23
	y774c08 lipid transfer protein 7a2b	BX000511	e−130	−1.94			1.15

Table 4. (Continued).

Putative function	Clone ID Description	Accession number	E-value	Log_2 M[a]	Log_2 A[b]	Log_2 J[c]	Log_2 G[d]
Other metabolism activities							
	y742b06 aldehyde dehydrogenase ALDH2b[e]	AK120296	0.0	1.81	1.26	−2.51	
	p741a10 probable udp-glucose 4-epimerase at4g23920	AB087745	0.0	1.78	1.37		
	y697f12 putative acid phosphatase	AK119190	0.0	2.36	1.12	2.01	
	y630g09 putative ATP citrate lyase[f]	NM-189189	0.0	2.40			
	y688b01 putative ATP citrate lyase[f]	NM-189189	0.0	2.21			
Unclassified							
	y764b07 putative fiddlehead-like protein[e]	AC126223	e−170	1.53		2.16	2.53
	p583h05 calreticulin precursor	AP003738	e−121	1.44		−1.20	
	y770h01 GDSL-motif lipase/hydrolase family protein	AK100958	0.0	1.11			
	y617b11 putative polyprotein	AK059510	e−139	−1.27			
Unknown protein							
	p637d05 unknown protein	AK119706	0.0	2.16			2.42
	y762a05 unknown protein	AY345599	0.0	1.65			
	y688g10 unknown protein	AK109429	0.0	1.38			
	p722f11 unknown protein	AL442115	0.0	−1.31			
	Y658g08 unknown protein	AC084818	2.00E−83	−1.37			1.97
	p865a03 hypothetical protein	AL606457	7.00E−92	1.81			1.43
	y699g09 hypothetical protein	AP001278	e−120	4.05		1.10	
	y658g03 hypothetical protein	AC135225	e−139	−1.53			
	p638h11 no significant homology	AP005535	e−132	1.77			

Clone ID: the clone identification number of cDNA sequence; Description: gene annotation obtained from sequence homology search (gene with the highest score was selected).
[a] M: Ratio of (Meiotic anthers/mature anthers).
[b] A: Ratio of (Mature anthers/seedlings).
[c] J: Ratio of (Jasmonic acid-treated/CK).
[d] G: Ratio of (Gibberellin-treated/CK).
[e] genes proven to be involved in anther development by previous researches.
[f] different clones represent the same gene.

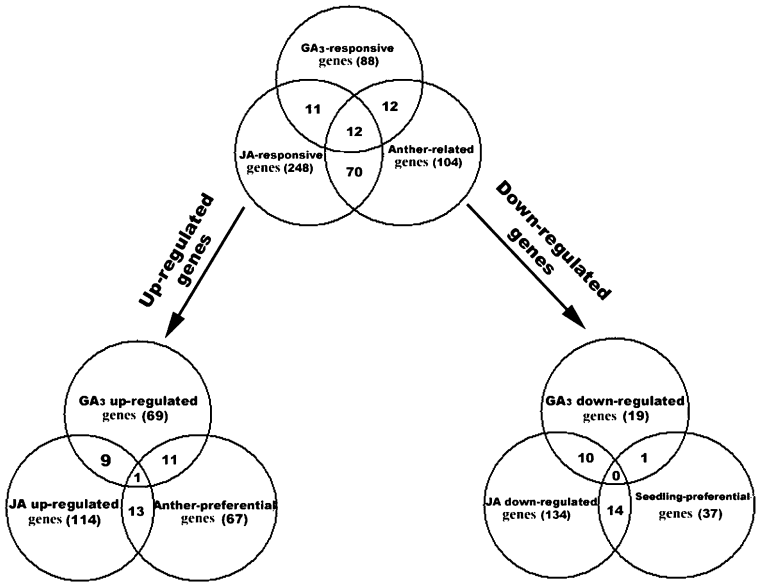

Figure 5. Analysis of the overlap between hormone-responsive genes and anther development genes. Numbers in brackets represent genes differentially expressed.

contained corresponding regulatory elements in their 2.0-kb putative promoter regions. The number of these elements varied from 2 to 7 (Table 6). In addition, related elements were also found in the promoter of 54.5% genes regulated by both GA_3 and JA (Table 6).

Gene y656d05 is a potential anther development gene and also up-regulated by GA_3

Microarray data showed that gene y656d05, encoding a putative GAST-like protein, was preferentially expressed in meiotic anthers (Table 4). RT-PCR revealed that the expression level of y656d05 was higher in meiotic anthers than in seedlings, roots, shoots, leaves, spikes and mature anthers (Figure 7A and B), thus confirming the microarray result that the expression of y656d05 was increased in meiotic anthers (Table 4). To examine the expression of y656d05 and investigate its function in anther development, we carried out *in situ* hybridization. The y656d05 RNA signal was highly present in pollen mother cells and tapetum of anthers (Figure 8). Thus, y656d05 might be involved in anther development, probably in meiosis.

Our microarray data also showed that y656d05 was responsive to GA_3 treatment. To further study its response to GA_3 treatment, we performed RT-PCR using RNA from seedlings treated with a serial concentration of GA_3 (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M). The y656d05 expression was slightly increased with the increase of GA_3 concentration (Figure 7C and D). In summary, y656d05 identified by our microarray analysis is a potential anther development gene and also affected by GA_3.

Discussion

Genes involved in anther development with various known functions

Anther development is a complex process, which is unique in producing male gametophytes in higher plants (Armstrong and Jones, 2003). Our microarray experiment using probes synthesized from mature anthers and seedlings found that 2155 differentially expressed genes, which represent approximately 21.6% genes of the array. Among them the expression of 47 genes in meiotic anthers changed their level 2-fold or more compared to their expression in mature anthers.

Anther development is required for anther-specific genes and the cooperation of genes that are essential for reproductive and vegetative development. Among the 47 genes detected as important for anther development, 10 genes involve in cell

Table 5. GA_3 or JA-responsive genes and their potential functions.

Clone	Description	Accession number	Log_2 (JA/CK)	Identity	Species	Biological function	Reference
y736a12	Putative receptor-like serine/ threonine kinase	AL606648	−1.89	51%	*A. thaliana*	Pollen and pistil interactions	Muschietti J. *et al.* (1998)
y844h01	Aquaporin	AJ224327	−1.45	93%	*Zea mays*	Pollen hydration and germination	Ikeda *et al.* (1997)
y718d06	Branched-chain amino acid aminotransferase	AK108687	−1.64	75%	*A. thaliana*	Pollen tube growth and guidance	Palanivelu *et al.* (2003)
y724b04	Similar to CER1-like protein	AK066386	1.21	56%	*A. thaliana*	Pollen fertility	Aarts *et al.* (1995)
y867e09	SCARECROW (SCR) gene	AK061050	2.34	96%	*O. sativa*	Cell division and differentiation	Sabatini *et al.* 2003.
p657c12	Homologous to putative PRP38 family	AC130610	−1.15	84%	*A. thaliana*	Pollen growth and fertility	Gibbon *et al.* (1998)
p620d07	Putative CCR4-associated factor protein	AP004703	−1.58	62%	*A. thaliana*	Cell cycle regulation	Liu *et al.* (1997)
y875h10	Chalcone synthase	AK067810	−1.92	93%	*S. bicolor*	Pollen germination and pollen tube growth	Pollak *et al.* (1993)
p585c01	b2 protein	AK106151	2.47	80%	*O. sativa*	Cell cycle	Weingartner *et al.* (2003)
			Log_2 (GA_3/CK)				
y775f07	Beta-expansin (EXPB4)[a]	NM_197701	1.96	92%	*Zea mays*	Cell division and Pollen tube growth	Cosgrove *et al.* (2002)
p723f12	Beta expansin (EXPB4)[a]	NM_197701	1.76	92%	*Zea mays*	Cell division and Pollen tube growth	Cosgrove *et al.* (2002)
y617a02	Beta-expansin (EXPB4)	NM_197698	1.65	73%	*O. sativa*	Cell division and Pollen tube growth	Cho *et al.* (1997)
y653e02	Alcohol dehydrogenase 1[a]	AK069330	1.42	88%	*A. thaliana*	Anther development	Liu *et al.* (2001)
p536h02	Histone H3	AF093633	1.10	96%	*H.sapiens*	Cell cycle and chromatin decondesation	Prigent *et al.* (2003)
p607e12	Putative F-box domain protein	AP005795	1.54	45%	*O. sativa*	Cell cycle and protein degradation	Itoh *et al.* (2003)

[a] genes regulated by both GA_3 and JA are listed only once.

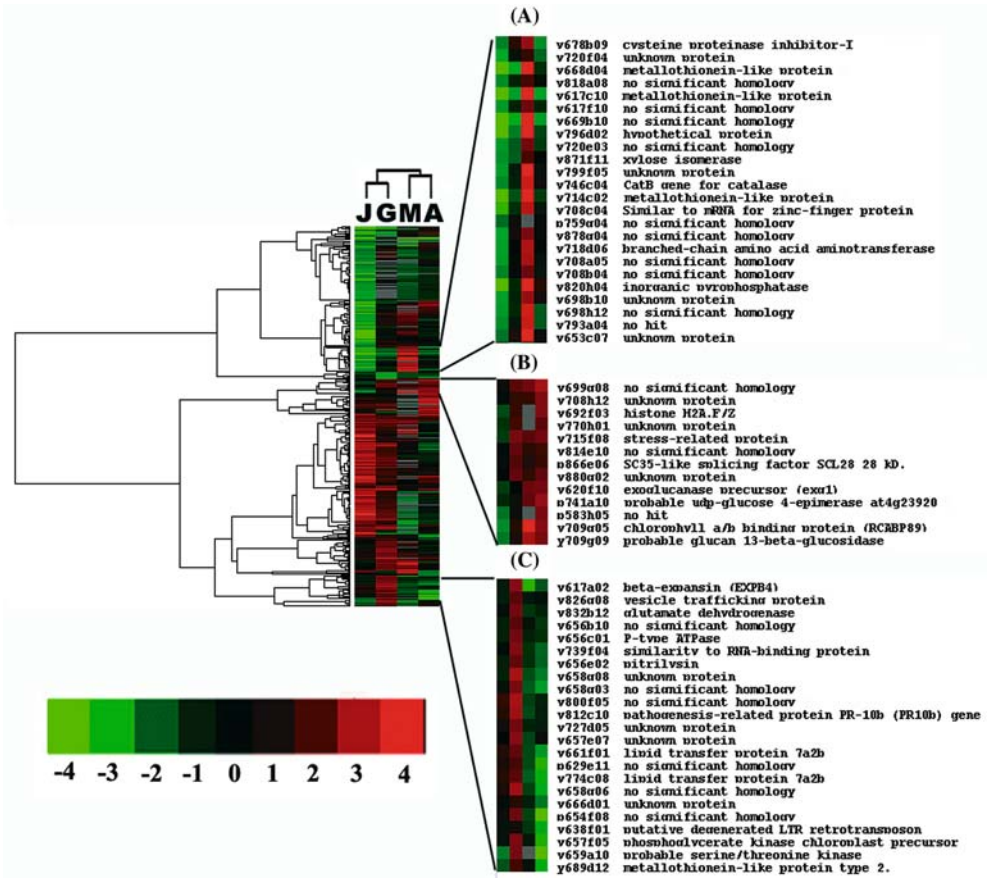

Figure 6. Hierarchical clustering of 314 genes differentially expressed. Genes with more than 2-fold change were selected in at least 1 comparison. The amount of change represents Log_2 Ratio and the result was from an average hierarchical clustering. As shown in the color scale, genes up- or down-regulated are in red and green, respectively. Gray represents missing values. (A) Sub-cluster illustrates some anther-preferential genes with decreased expression in meiosis; (B) sub-cluster exhibits the anther-preferential genes up-regulated in meiosis; (C) sub-clusters are vegetative organ-preferential genes down-regulated in meiosis. J: JA-regulated genes; G: GA_3-regulated genes; M: genes differentially expressed in mature anthers compared with seedlings; A: genes differentially expressed in meiotic anthers.

division or anther development (Table 4). Some of these genes also showed a high transcription level during pollination and fertilization in rice (Lan *et al*., 2004). The SMC (Structural Maintenance of Chromosome) protein are crucial for chromosome cohesion and condensation in both mitosis and meiosis (Strunnikov and Jessberger, 1999; Pavlova and Zakiian, 2003). Rice SMC protein detected here is highly homologous to SMC proteins in *Arabidopsis* (Table 4). Its high expression in meiotic rice anthers suggests that it might play some roles in chromosome cohesion or condensation in meiosis. Anther development is also involved in an active stage of protein and lipid metabolism for pollen wall formation. The 40.4% genes with predicted functions in carbohydrate, protein and lipid metabolism suggest that they may play roles in pollen wall formation. Moreover, 19.2% genes encode proteins responsive to environmental factors (Table 4). This might result from the fact that anther development is an environment sensitive process (Dix *et al*., 1996). Functional studies of genes detected from our genome wide screening should further our understanding of anther development in rice.

GA_3 and JA might play different roles in rice anther development

Plant hormones, such as GA_3 and JA, are critical for anther development (Zhao *et al*., 2003; Cheng *et al*., 2004). In our genome-wide screening, 314

Table 6. Analysis of regulatory elements in the promoter of phytohormone-regulated ESTs.

	No. of genes	Element in promoter	Element sequence	Organism	No. of ESTs with regulation elements	Average No. of elements in one promoter ± SD	Percentage
JA-responsive ESTs	248	MeJA-responsiveness	TGACg (CGTCa)	*Hordeum vulgare*	140	4.74 ± 2.54	56.50
GA_3-responsive ESTs	88	gibberellin-responsive element	CCTTNNN (TATCNNN)	*Oryza sativa*	54	4.59 ± 2.81	61.40
		MeJA-responsiveness	TGACg (CGTCa)	*Hordeum vulgare*			
ESTs regulated by JA and GA_3	23	gibberellin-responsive element	CCTTNNN (TATCNNN)	*Oryza sativa*	12	4 ± 3.07	54.50

genes exhibited responsiveness to GA_3 or JA treatment. Some of these hormone-responsive genes were also detected in an array of rice calli treated with GA_3 (Yazaki *et al*., 2003). Among the hormone-regulated genes detected here, approximately 29.9% genes were differentially expressed in anther development (Figure 5). Meanwhile, about 4.4% anther development-related genes were affected by GA_3 or JA. Some of these genes have been identified and found to be involved in anther development (Table 5). For example, pollen tube elongation requires *EXPB4*, which can loosen cell walls by weakening glucan–glucan binding (Cosgrove *et al*., 2002), while aquaporin-related genes regulate pollen development (Ikeda *et al*., 1997). Recently, more evidence supports the involvement of GA_3 and JA in anther development (Table 5) (Park and Lord, 2003; Prigent and Dimitrov, 2003; Weingartner *et al*., 2003; Lan *et al*., 2004).

Although both GA_3 and JA are important for anther development, they may play distinct roles. GA_3 is found to regulate the development of both male and female organs in plants (Huang *et al*., 2003), while JA regulates anther dehiscence and late pollen maturation (Devoto *et al*., 2002; Xu *et al*., 2002a). In our array, 12 anther development-related genes were found to respond to both GA_3 and JA treatments. Among them, only one showed the same regulation pattern in the two hormone treatments, while no overlap of other genes was detected. Moreover, clustering analysis suggested that most anther development involved genes responded differently to the two hormone treatments (Figure 6). Therefore, we propose that GA_3 and JA may play different roles in rice anther development.

Gene y656d05 (BX901779) is possibly involved in meiosis

Sequence analysis showed that gene y656d05 belongs to genes assigned as GA_3-stimulated transcripts (GAST). RT-PCR analysis confirmed our microarray result that y656d05 was slightly up-regulated by GA_3 treatment (Figure 7B), which is similar to the expression pattern of the family members in *Arabidopsis* and *Petunia* (Aubert *et al*., 1998; Ben-Nissan *et al*., 2004). Moreover, studies on *GIP*, a GA_3 response marker gene from GAST family of *Petunia*, indicated that *GIP* was

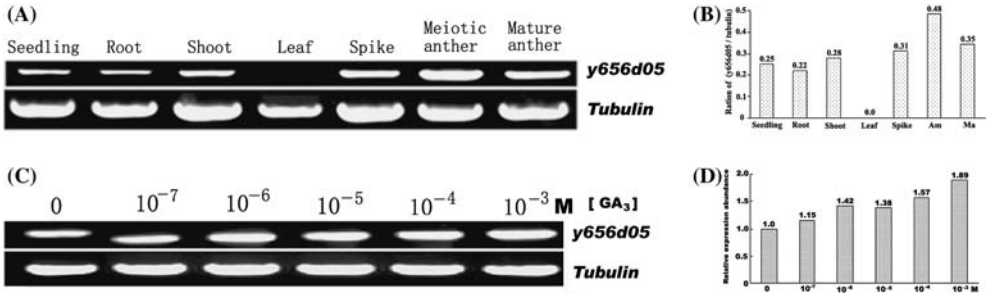

Figure 7. Expression pattern of gene y656d05 by RT-PCR. (A) Expression pattern of gene y656d05 in seven different rice organs; (B) Diagram corresponding to quantified A; (C) Expression pattern of gene y656d05 in GA_3-treated rice seedlings; (D) Diagram corresponding to quantified C.

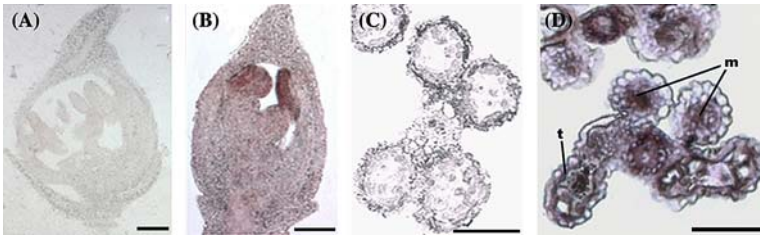

Figure 8. RNA *in situ* hybridization of y656d05 in anthers. Rice florets during meiosis were used to detect the expression of y656d05. (A) Longitudinal section of floret hybridized with sense probe as control. (B) Floret hybridized with antisense probe. (C) Transverse section of an anther at meiosis stage hybridized with sense probe as control. (D) Transverse section of an anther at meiosis stage hybridized with antisense probe (D). m: Microsporangium. t: Tapetum. Bars = 100 μm.

increased during anther development (Izhaki *et al*., 2002). Similarly, y656d05 was highly expressed in meiotic anther (Figure 7A) and strong RNA signal was detected in both pollen mother cells and the tapetum of anthers (Figure 8). Therefore, y656d05 is possibly involved in meiosis. Further functional studies will be needed to define its function in meiosis.

In summary, in this report we compared gene expression in seedlings, meiotic and mature anthers, as well as GA_3 or JA treated suspension cells using a cDNA microarray containing about 10 000 rice genes. There were 2155 genes found to be preferentially expressed in anthers and 47 genes were differentially expressed in meiotic and mature anthers. Moreover, 314 genes responded to either GA_3 or JA treatment. Among them, 24 GA_3- and 82 JA-responsive genes showed significant expression changes from meiotic anthers to mature anthers, suggesting that both GA_3 and JA play a wide range of roles in rice anther development. We also examined the expression of gene y656d05 in detail using RT-PCR and *in situ* hybridization. Further functional studies of these genes using molecular genetic and reverse genetic approach will help elucidate the mechanism of anther development.

Acknowledgements

We thank Dr L.G. Ma and Dr X.W. Deng for technical assistance, Dr W. Chen and Dr Y. Lai for helpful discussion and advice in microarray hybridization, L. Wang and H.L. Liu for the material collection, and technician R.X. Jiang for preparing suspension-cultured rice cells. We also thank Dr H. Ma and Dr H.A. Owen for critical reading of the manuscript. We are grateful for sequence annotation by C.H. Xu, Y.J. Liu and D. Li. This work was supported by the Major State Basic Research Program (China, G19990116) and an Innovation Grant of the Chinese Academy of Sciences.

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