Plant Physiology Preview. Published on June 7, 2007, as DOI:10.1104/pp.107.101600

Running head: Molecular Functions of Stigma-Specific Genes in Rice

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Research area: Genome Analysis

Keywords: rice, stigma, molecular functions, signaling, genomics

Genome-Wide Gene Expression Profiling Reveals Conserved and Novel Molecular Functions of the Stigma in Rice (*Oryza sativa* L.) [1]

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Footnotes:

[1]This work was supported by grants from the Ministry of Science and Technology of China (2005CB120800) and the Chinese Academy of Sciences.

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ABSTRACT

In angiosperms, stigma provides initial nutrients and guidance cues for pollen grain germination and tube growth. However, little is known about the genes that regulate these processes in rice. Here we generate rice stigma-specific or -preferential gene expression profiles through comparing genome-wide expression patterns of hand dissected unpollinated stigma at anthesis with seven tissues including seedling shoot, seedling root, mature anther, ovary at anthesis, seeds of five days after pollination, 10-day-old embryo, 10-day-old endosperm as well as suspension cultured cells by using both 57K Affymetrix rice whole genome array and 10K rice cDNA microarray. A high reproducibility of the microarray results was detected between the two different technology platforms. In total, we identified 548 genes to be expressed specifically or predominantly in the stigma papillar cells of rice. Real-Time quantitative RT-PCR analysis of 34 selected genes all confirmed their stigma-specific expression. The expression of five selected genes was further validated by RNA *in situ* hybridization. Gene Ontology (GO) analysis shows that several auxin-signaling components, transcription and stress-related genes are significantly overrepresented in the rice stigma gene set. Interestingly, most of them also share several *cis*-regulatory elements with known stress-responsive genes, supporting the notion of an overlap of genetic programs regulating pollination and stress/defense responses. We also found that genes involved in cell wall metabolism and cellular communication appear to be conserved in the stigma between rice and *Arabidopsis*. Our results indicate that the stigmas appear to have conserved and novel molecular functions between rice and *Arabidopsis*.

INTRODUCTION

In flowering plants, pollination is the first major reproduction process that results in the production of seeds. The process begins with the adhesion of pollen grains to the stigmatic tissue of style. Then the highly desiccated pollen grains are rehydrated to reach a certain water content that allows them to regain active metabolisms. Once the pollen grain cell has established its internal polarity relative to an external signal, the pollen germinates and breaches the exine wall to emit a pollen tube. The newly formed pollen tube subsequently penetrates the stigma, grows down through the transmitting tissue of the style and ultimately reaches an ovule allowing fertilization to take place (Lord, 2003; Sanchez et al., 2004; Swanson et al., 2004; Boavida et al., 2005).

Successful pollination requires continued communication and coordination between the pollen and stigma and is essential to maximize the seed set. The stigma, the uppermost part of the pistil, is generally considered to be a passive structure for pollen grain capture and reception, germination and initial growth of the pollen tubes. There are two major types of stigma, the “dry” and the “wet” types, which differ by the presence or absence of the secreting exudate compounds (Edlund et al., 2004). The adhesion and germination of pollen on the “wet” stigma is facilitated by the presence of the exudate, but its ingredients are highly variable among species (Edlund et al., 2004; Boavida et al., 2005). Tobacco (*Nicotiana sylvestris*) is a wet stigma-type plant and its stigma has three distinct zones: an epidermis with papillae, a sub-epidermal secretory zone and a zone of parenchyma ground tissue (Kandasamy and Kristen, 1987). Elimination of the secretory zone involved in producing the exudates in tobacco (*N. tabacum*) by expressing a chimeric stigma-specific cytotoxic gene results in female sterility but fertility could be restored upon application of the stigma exudate of wild-type tobacco plants (Goldman et al., 1994). In species with the “dry” stigma, including *Arabidopsis thaliana*, *Brassica oleracea* and rice (*Oryza sativa*), the epidermis is composed of large specialized papillae cells that interact directly with the surface of pollen and the surface of each papilla is covered with a waxy cuticle overlaid with a distinct proteinaceous pellicle layer (Gaude and Dumas, 1986; Zinkl et

al., 1999; Ciampolini et al., 2001). In *Brassica*, the stigmatic papillae cells completely lose their ability to capture the pollen grains when using acetone to strip off both the waxy cuticle and proteinaceous stigma pellicle (Heizmann et al., 2000). In addition, protease digestion of pellicles from kale (*B. oleracea*) also adversely affects pollen adhesion (Stead et al., 1980; Luu et al., 1997). These studies clearly demonstrate that the stigma plays crucial roles in pollination.

Nevertheless, the molecular roles of the stigma in pollination are largely unknown. Several stigma-specific expressed proteins have been identified and a few of which are found to be directly involved in the pollen-stigma interaction. *SRK* (*S*-locus receptor kinase) encoding a glycoprotein localizes to the stigmatic plasma membrane and interacts with the pollen coat S-locus cysteine-rich protein (SCR) in the self-incompatibility (SI) response in *Brassica* (Nasrallah, 2000; Takayama and Isogai, 2005). Both *SLG* (*S*-locus glycoprotein) and *SLR* (*S*-locus related protein) are expressed in stigma papillae cells and their products localize to the stigma cell wall in *Brassica* (Kandasamy et al., 1989; Lalonde et al., 1989; Umbach et al., 1990). Although there is some controversy as to whether SLG and SLR are required for the SI response in *Brassica*, both of them are shown to interact with several small pollen coat proteins and these interactions are believed to generate adhesive forces of the pollen-stigma interaction (Luu et al., 1999; Takayama and Isogai, 2005). The *pis63* gene encoding an unknown protein in *B. napus* is expressed in the stigma papillae cells and a reduction in its transcript level results in reduced pollen germination and seed set (Kang and Nasrallah, 2001). Aquaporin-like proteins in the *Brassica* stigma may act as water channels in controlling water flow into the pollen grain from the stigma (Dixit et al., 2001). An ABC transporter gene, designated as *NtWBC1* (*Nicotiana tabacum* ABC transporter of the White-Brown Complex subfamily), is highly expressed in the stigmatic secretary zone, suggesting its important role in the reproductive process (Otsu et al., 2004). Recently, a stigma-specific peroxidase (SSP) from the ragwort *Senecio squalidus* is identified and its expression is confined to the specialized epidermal cells of the stigma (McInnis et al., 2005). The activity of stigma peroxidases has long been used to determine the pistil receptivity for pollen grains

and the high levels of the peroxidase activity show that the stigma is “ripe” for pollination (McInnis et al., 2006). But, the function of the peroxidase in pollination still is not clear.

Additional factors have been found to be involved in pollination in several species. Auxin has been proposed to operate as pollination signal and promotes the differentiation and development of ovules in orchid flowers (O'Neill, 1997). In Petunia (*Petunia hybrida* L.) the interaction of the male gametophyte with the stigmatic tissues is accompanied by a threefold increase in the ethylene production and a 1.5-fold increase in the auxin (IAA) content in the pollen–pistil system within 0–4 hour after compatible pollination and the growth of pollen tubes in the stylar tissues is accompanied by a further increase in IAA content and a decrease in the ethylene production by stigmatic tissues (Kovaleva et al., 2002). The ethylene/auxin status of the stigma has been suggested to control the processes of adhesion, hydration, and germination of pollen grains during pollination in Petunia (Kovaleva et al., 2002). In our previous study, we found that an extensive set of genes are shared between abiotic stress responses and pollination/fertilization in rice and nearly half of the genes expressed preferentially in the un-pollinated pistils are responsive to dehydration and the majority of them are dehydration-inducible, suggesting an overlap in the genetic programs controlling between these processes (Lan et al., 2005). Temperature is a major physical factor influencing the plant reproductive phase and limits geographical distributions of plant species. High temperature has been found to cause the loss of the sporophytic self-incompatibility response in *Ipomoea fistulosa* (Prabha et al., 1982). The stigmatic receptivity of peach loses the capacities to sustain the pollen tube penetration into the transmitting tissue, pollen germination and adhesion as the temperature increases during pollination (Hedhly et al., 2005).

However, the molecular mechanisms of these events involved in pollination remain obscure. It is anticipated that, given recent rapid developments in functional genomics, genome-wide identification of stigma-specific genes could provide important initial steps in dissecting molecular control of pollination. Recently, two groups have carried out the stigma-specific gene profiling of *Arabidopsis* using whole–genome microarray

analysis (Swanson et al., 2005; Tung et al., 2005). Tung et al. (2005) have identified 115 genes predicted to be expressed specifically in the stigma epidermis comparing transcriptional profiles of the stigmas isolated from wild-type *Arabidopsis* and from the transgenic plants in which cells of the stigma epidermis were specifically ablated by expression of a cellular toxin. Swanson et al (2005) have identified 317 genes that are highly expressed in the stigma after comparing wild-type stigma, ovary and seedling transcriptional profiles and the cDNA library subtractive hybridization has further confirmed the results. Both the stigma gene data sets are significantly enriched in those genes involved the cell wall metabolism, cellular communication and signal transduction related proteins. They are thought to play roles in the adhesion, hydration and germination of pollen grain.

Cultivated rice is by far one of the most important food crops and also considered the model monocot plant for molecular and genetic studies. Rice is primarily an autogamous, self-pollinating plant and has the typical dry stigma. But, very little is known about stigma-specific genes that are required for ensuring successful pollination in rice. In this study, we generate rice stigma-specific or -preferential gene expression profiles by using both the commercially available 57K Affymetrix rice whole genome array and 10K rice cDNA microarray (Lan et al., 2004). Gene Ontology (GO) analysis shows that several auxin-signaling components, transcription and stress-related genes are significantly overrepresented in the rice stigma gene set. We also found that the genes involved in cell wall metabolism and cellular communication appear to be conserved in the stigmas between rice and *Arabidopsis*.

RESULTS

Gene Expression Profiles of Stigma *vs*. Ovary by Two Independent Microarray Platforms

To generate stigma-specific and ovary-specific gene expression profiles, two independent microarrays were applied in this study. The stigma samples were hand collected by cutting the pistil just below the base of the plumose stigma, and the remainder of the pistil containing the style was used as the ovary samples. The

important criterion for the dissection was that the stigma samples had the papillar cells but not the ovary samples.

To identify the genes preferentially expressed in the stigma, 57K Affymetrix rice whole genome array was used to generate the genome expression patterns across eight representative organs or tissues and suspension cultured cells. They included seedling shoots (Sh), seedling roots (Rt), mature anthers (An), unpollinated stigmas at anthesis (St), ovaries at anthesis (Ov), seeds of 5 days after pollination (5DAP), 10-day-old embryos (10EM) and 10-day-old endosperms (10EN). We performed three biological replicates for the stigma and ovary samples, respectively. The correlation coefficient value of each experiment was large than 0.99. By using *p*-value $\leq$ 0.05 and fold change $\geq$ 2 as cut-off to identify differentially expressed genes, 3300 probe sets were up-regulated in the stigma compared to the ovary and 4125 probe sets were up-regulated in the ovary compared to the stigma (Supplemental Table I). All microarray data are available online (http://www.ncbi.nlm.nih.gov/geo) and (http://plantbiol.genetics.ac.cn).

We also used the 10K rice cDNA microarray constructed previously (Lan et al., 2004) to compare gene expression profiles of the stigma with the ovary. The gene expression profiles of the rest organs were generated before (Lan et al., 2004 and 2005). Three biological and one dye-swapped replicates were performed for the stigma *vs.* ovary hybridizations. By using the same cut-off (fold change $\geq$ 2, $p < 0.05$), in total, 453 cDNAs had more abundant expressions in the stigma compared to the ovary (Supplemental Table II) and 257 cDNAs were identified as enriched in the ovary compared to the stigma (Supplemental Table III).

The microarray results showed a high reproducibility using the two different platforms (cDNA microarray *vs.* Affymetrix oligo array). For the 453 cDNAs preferentially expressed in the stigma identified using the 10K rice cDNA microarray, 201 cDNAs' (using E-value=1e-20 and identity > 80% as cut-off) expression patterns were confirmed by the Affymetrix GeneChip results (Supplemental Table IV), with about 44.37% match. For the 257 cDNA clones highly expressed in the ovary, 109 cDNAs' expression patterns (using E-value=1e-20 and identity > 80% as cut-off)

were confirmed by the Affymetrix GeneChip results, with about 42.41% match (Supplemental Table V).

Identification and Functional Classification of the Stigma-Specific/-Preferential Genes

To identify rice stigma preferentially expressed genes, two statistical methods were conducted for data processing. First, SAM analysis (Significance Analysis of Microarrays software package) was conducted for three biological samples replicates between the stigma and ovary. Using q-value ≤ 0.05 and fold change ≥ 2 as cut-off, 3300 probe sets were subsequently picked out and their expression levels were significantly up-regulated in the stigma compared to the ovary. We also did normal *t*-test and added *p*-value for these probe sets. Second, we used *Z*-score transformation normalization method to compare the gene expression levels in stigma with other tested organs or tissues and suspension cultured cells and directly calculated significant changes in the gene expression levels between them. For *Z*-score transformation normalization, we used average values of the stigma and ovary, separately. In total, we identified 548 genes (665 probe sets) preferentially expressed in the stigma using *Z*-score ≥ 2.32 and *p*-value ≤ 0.01(Supplemental Table VI).

Among the identified 548 genes preferentially expressed in the stigma, 410 genes had putative functions, and 103 genes were assigned as expressed proteins and 23 genes as hypothetical proteins and 12 genes had no hits when blasted against the TIGR rice genome annotation database (http://www.tigr.org). The 410 genes were classified into eleven groups according to their annotations and one unclassified group: transcription, cell-wall-related, stress/defense, signal transduction, lipid metabolism, transport, hormone-related, protein metabolism, carbohydrate and energy metabolism, nucleic acid metabolism, amino acid metabolism and unclassified. The categories are listed in the order of gene numbers in each group (Supplemental Table VII). We list 149 genes derived from 173 probe sets with putative annotated functions and their ratios of the stigma/ovary of more than 10 (p <0.05) in Table I. The largest category was transcription related proteins including two MADS box proteins, four zinc finger

proteins and six Myb- or Myb-like transcription factors. These proteins accounted nearly for 15% of the 410 putative functional genes. In the second category, including putative cell wall-localized enzymes (e.g. α-expansin, pectinesterase and peroxidase), cellulose synthase, glycerol-3-phosphate acyltransferase and UDP-glucosyltransferase, the third category consisted of proteins that were potentially involved in stress/defence responses, such as glutathione s-transferase, phospholipase D and wound-induced protein WI12. Many signal transduction and transport related proteins were enriched in the stigma. There were a large percentage of genes that may be related to lipid metabolism including lipid binding protein, triacylglycerol lipase and fatty acid elongase. The auxin signaling related proteins were identified to be highly expressed in the stigma including auxin responsive factor (ARF), five members of auxin-responsive SAUR gene family and the highly expressed indole-3-acetic acid-amido synthetase *GH3.1* gene (see Table I and Supplemental Table VII).

Gene Ontology (GO) analysis of the rice stigma-preferential genes was performed and the biological process term enrichment status and hierarchy were shown in Supplemental Figure S1. The results showed that the DNA-dependant transcription may play an important role in the stigma and the biological processes that respond to hormone stimuli, especially to auxin, were overrepresented and seemed to be more significant in the rice stigma-specific or –preferential gene data set.

Comparison of the Stigma Preferentially Expressed Genes in Rice and Arabidopsis

We compared the stigma preferentially expressed genes in rice with that identified in *Arabidopsis* (Tung et al., 2005). We extracted the protein sequences for 115 Arabidopsis genes, blasted them against rice Affymetrix consensus sequence database (https://www.affymetrix.com/analysis/netaffx/index.affx) and filtered 3504 probe sets using E-value ≤1e-4. We found that 45 Arabidopsis genes hitting 92 rice probe sets with the stigma preferentially expression pattern (Supplemental Table VIII). Meanwhile, we also blasted the sequences of the 665 rice probe sets against Arabidopsis whole genome protein sequence database

(http://www.arabidopsis.org/Blast/index.jsp) and found that 90 rice probe sets (83 genes) were similar to 42 Arabidopsis stigma specific/ preferentially expression genes (Supplemental Table VIII). From both blast results, we found that they matched to each other well. The gene annotation showed that the majorities of them encoded the cell-wall related and signal transduction related proteins. We also listed the number of the stigma-preferential genes unique to rice (465) and Arabidopsis (70).

Validation of the Microarray Results

Real-Time Quantitative RT-PCR (qRT-PCR) analysis was employed to validate the candidate genes. In total, 34 of the identified genes preferentially expressed in stigma were selected including 30 putative function genes belonging to different classification groups, two hypothetical proteins, one expressed protein and one unknown function gene. The signal intensity range of the selected genes was from 107.8 to 28284.3 and the ratios (stigma *vs.* ovary) from 2.6 to 2188.5 (see Supplemental Table I). To confirm whether these genes were stigma-specific by qRT-PCR analysis, the expression of the 34 candidate genes were compared between the stigma and the ovary, anther, seeding-shoot or flag leaf (heading stage) samples. (Table II). The results showed that their expression patterns detected by 57K Affymetrix rice whole genome array were in good correlation with those obtained by qRT-PCR.

Confirmation of the Candidate Stigma-specific Genes by RNA *in situ* Hybridization

To further examine the expression of the candidate stigma-specific genes, we selected five genes to perform RNA *in situ* hybridization, including a serine carboxypeptidase 1 precursor gene (LOC_Os02g46260), an extracellular ribonuclease LE precursor gene (LOC_Os09g36700), a putative CER1 gene (LOC_Os10g33250), a metal tolerance gene C3 (LOC_Os01g62070) and an unknown function gene (AK071040). They exhibited relatively high hybridization signals in the stigma microarray data sets, ensuring that their transcripts could be detected by the RNA *in*

situ hybridization technology. A CCCH-type zinc finger protein gene (LOC_Os01g09620) expressed in the entire mature pistil according to the microarray results was used as a reference. Longitudinal sections through the center of pistil just before pollination were used for all the hybridizations. The results showed that the five selected genes exhibited unique expression patterns in the stigma papilla cells (Figure 1), and that the hybridization signals of the examined genes were in good correlation with their intensities in 57K Affymetrix rice GeneChip. Taken together, our results showed that the 548 genes preferentially expressed in the stigma thus identified represented good candidates for the stigma-specific genes in rice.

Cis-Acting Regulatory Element Analyses of the Candidate Stigma-specific Genes

To identify possible *cis*-acting regulatory elements responsible for the transcription regulation of the candidate stigma-specific genes, we first did the hierarchical clustering analysis and found that nearly 35% of the identified genes (193) were mainly located in three clades (Supplemental Figure S2). The common features were that all the genes in the three clades were specific or highly expressed in the stigma and the correlation coefficients of the three clusters were 0.9277, 0.9389 and 0.8263, respectively. We named the three clades as Cluster I, II and III, respectively. Then, 1,000-bp regions located in the upstream of the start codons of the genes from the three clusters were used for analysis. Several conserved motifs were subsequently identified using the MEME/MAST system (Bailey and Elkan, 1994; Bailey and Gribskov, 1998). Figure 2 shows the top three motifs identified for each cluster. The E-value of the three motifs in Cluster I are 3.5e+004, 3.3e+006, 7.3e+001, respectively; in Cluster II 1.1e+006, 2.8e+003, 2.2e+005, respectively; in Cluster III 7.5e+005, 3.3e+006, 1.2e+007, respectively. The common features of the *cis*-acting regulatory elements we identified from the conserved motifs using PLACE database were mostly related to stress- or defense- responses (Prestridge, 1991; Higo et al., 1999). A large percentage of the genes in Cluster I had the same motif named GCC-box (Brown et al., 2003; Chakravarthy et al., 2003). Most of genes in Cluster III had Myb-transcription factor recognition sites which are usually found in the

promoters of the dehydration-responsive genes. One of the motifs (Figure 2C, bottom) in Cluster III appeared to have unknown function, indicating that they might represent new *cis*-acting elements in regulating the transcription of the stigma-specific genes.

DISCUSSION

In this study, we have identified 548 genes expressed specifically or predominantly in the stigma papillar cells of rice by using 57K Affymetrix rice whole genome array and 10K rice cDNA microarray. It is highly likely that they represent good candidates for the stigma-specific genes in rice. First, for the two different technological platforms we used, there existed the good correlations and the variables observed were reasonable according to several recent reports (Maruyama et al., 2004; Yauk et al., 2004; Hannah et al., 2005). Second, the qRT-PCR and RNA *in situ* hybridization all confirmed the microarray results (Table II and Figure 1). Third, the rice stigma gene data set is similar to that identified in *Arabidopsis* (Tung et al., 2005). Thus, the careful selection of the starting tissues is an effective approach to identify the stigma-specific genes using the whole genome and cDNA microarrays.

The Stigma-Specific Genes have Conservative Roles in Plants

The functional annotation of both rice and *Arabidopsis* genes specifically or preferentially expressed in stigma suggest that several groups of the genes appear to play conserved roles in the stigma. Forty-two *Arabidopsis* stigma-specific genes representing 36% of the gene set are highly similar to 83 genes representing about 15% of the rice stigma gene data-set (Supplemental Table VIII). The majorities of them belong to the cell-wall related and signal transduction groups, indicating that these two classes of genes have conserved functions in the stigma. Swanson et al. (2005) also found that these two functional categories were over-represented in contrast to the distributions of the total stigma message RNA. The cell-wall-related group represents the second largest group of the genes identified in the rice stigma gene data set, which include the genes encoding pectinesterase, peroxidase and α-expansin. They are putative cell wall-localized enzymes and may be the most

suitable candidates for the papilla cell wall loosening or expansion (Wu et al., 1996; Schopfer, 2001). The cellulose synthase, glycosyl transferase and endoglucanase may be involved in the cell wall synthesis required for the pollen tube growth (Hong et al., 2001; Goubet et al., 2003; Aspeborg et al., 2005; Yokoyama and Nishitani, 2006). Consistent with the cell-cell communication in the pollen-pistil interaction, the rice stigma gene data-set also contained a significant proportion of signal transduction related genes (8.9%). Protein kinases play crucial roles in a wide variety of cellular functions including proliferation and differentiation by adding phosphate groups to theronine, tyrosine and serine residues of specific target proteins. The plant receptor-like kinases (RLKs) likely are transmembrane proteins that transduce external messages into cell through their extracellular domains and intracellular kinase domains. Previously, this protein category was identified and implicated in the pollen-pistil interaction and pollen tube growth (Muschietti et al., 1998; Tang et al., 2002). In *Brassica*, the stigma-expressed SRK interacts with SCR and initiates a signal transduction cascade that inhibits the pollen rehydration, and consequently the growth of self-pollen, while allowing non-self pollen to grow (Kachroo et al., 2002; Takayama and Isogai, 2005). The putative protein kinases and putative protein receptor-like kinase identified in the rice stigma gene sets are likely involved in the pollen-stigma interaction and the early steps of pollination. The three genes confirmed by whole-mount RNA *in situ* hybridizations in the *Arabidopsis* study all had the homologous genes in our stigma-specific gene list (Tung et al., 2005). At5g59810 is related to LOC_Os02g44590 encoding an expressed subtilisin-like protease precursor protein, At5g19880 to LOC_Os06g48030 encoding an expressed putative peroxidase 16 precursor and At2g02850 to LOC_Os03g02400 encoding an expressed early nodulin-like protein 1 precursor protein. Thus, our results and that of *Arabidopsis* suggest that the stigma involves the genes with the highly conserved functions.

The Phytohormone Auxin and Stigma Function during Pollination

Auxin is the central growth regulator of a myriad of aspects of plant growth and developmental processes and appears to be actively transported throughout the plant

to control cell division, extension, and differentiation (Benjamins et al., 2005). Several roles have been suggested for auxin in the pollination process. In orchid, it has been shown that the pollination and auxin regulate the ethylene production and ovary development because when inhibitors of ethylene were used the pollination- or auxin-induced ovary development were inhibited (Zhang and O'Neill, 1993). During *Arabidopsis* flower development, the concentration of free auxin increased gradually starting at the floral-organ tip visualized by immunolocalization with polyclonal antibodies against auxin and accumulated in pollen grains and stigma before fertilization (Aloni et al., 2006). In maize, the augmentation of auxin in the pistil tissues is important for egg cell differentiation (Mol et al., 2004).

However, it is unclear whether auxin is involved directly in the interaction of the pollen and stigma. In the hormone-related genes group from our data-set, the auxin-related genes appear to be over-represented (Table 1and Supplemental Table VII) and the results of the Gene Ontology (GO) analysis of the rice stigma also suggested that the auxin signaling plays a significant role in the rice stigma function (Supplemental Figure S1). The five of the identified auxin-related genes are *SAURs* (small auxin-up RNA) (Jain et al., 2006). They are LOC_Os04g56690**,** LOC_Os09g37430, LOC_Os09g37440, LOC_Os09g37490 and LOC_Os09g37500, namely *OsSAUR23*, *OsSAUR48*, *OsSAUR49*, *OsSAUR54* and *OsSAUR55*, respectively. Interestingly, four of them belong to a big gene cluster that contains 17 *OsSAURs* contiguously arranged in the chromosome 9 (Jain et al., 2006). The *SAURs* encode short-lived nuclear proteins that are induced within minutes after auxin application and may play a role in auxin-mediated cell elongation (McClure and Guilfoyle, 1989; Franco et al., 1990; Gee et al., 1991; Knauss et al., 2003). It was reported that a suitable amount of auxin could lead to loosening of the cell wall and promote cell growth through inducing H (+)-secretion and activating cellulose synthesis (Fry et al., 1990). The ARF (auxin response factor) seems to control the expression of the *OsSAURs* through the auxin responsive elements (*AuxREs*) present in their promoter regions (Ulmasov et al., 1997). These studies indicate that auxin may be involved in loosening the stigma papillar cell wall and promote the pollen

tube growth through inducing the expression of *OsSAURs*. The indole-3-acetic acid-amido synthetase *GH3.1* gene (LOC_Os01g57610) is involved in the auxin homeostasis through the conjugation of indolacetic to amino acids (Terol et al., 2006). But it is unclear where the auxin comes from. Could it be synthesized in the stigma papillar cell or transported from other organs (see below)? Further analyses of these candidate genes will shed light on this aspect of the auxin signaling in the stigma during pollination.

Transport Function in the Stigma

The transport-related proteins are mostly abundant in the rice stigma gene data-set and appear to be actively involved in the stigma function. The rice pollen is not fully dehydrated and metabolically active when shed from the anthers. They likely require the stigma to be fully developed to supply all the nutrition and metabolites for the pollen germination and tube growth. The different kinds of transporters appear to be involved in the exchange of materials and information between pollen and stigma. The ABC (ATP binding cassette) transporter is one of the active transport systems of the cell, which is widespread in prokaryotes and eukaryotes. In tobacco, the gene *NtWBC1* is developmentally regulated in the stigma/style, with mRNA accumulation increasing toward anthesis (Otsu et al., 2004). RNA *in situ* hybridization experiments demonstrated that *NtWBC1* is predominately expressed in the stigmatic secretory zone. There are four ABC transporters expressed in the stigma papillar cells of rice (see Table I and Supplemental Table VII). Two recent reports indicate that plant ABC transporters mediate the cellular and long-distance transport of the plant hormone auxin (Sidler et al., 1998; Multani et al., 2003). The functional study of the four ABC transporters may help to clarify the mechanism of the auxin signaling in the stigma during pollination. The potassium (K) uptake gene expression was highly enriched in the stigma. Recently, it has been reported that K may regulate anther dehiscence, pollen imbibition and papilla hydration (Rehman and Yun, 2006). In *Brassica*, aquaporin-like proteins in the stigma may act as water channels to regulate water flow into the pollen grain from the stigma (Dixit et al., 2001). There are 33 genes annotated

for aquaporins in the rice genome which are predicted to enable the fast and controlled translocation of water across the membrane and most of them have been identified and characterized recently (Sakurai et al., 2005; Guo et al., 2006). From our data-set, one aquaporin protein encoding gene, *PIP2.2* (LOC_Os02g41860), was identified to have a specific and enriched expression in the rice stigma papilla cells, suggesting that it may function in the stigma during pollination. However, it remains an important task to address their roles in pollination.

Possible Cross-talks of Stress Responses and Pollination

Previously, we detected that an extensive overlap of the genes involved in pollination and abiotic stress responses (Lan et al. 2005). In consistent, many stigma-specific genes also were related to stress and defense genes, such as those encoding MLO-like proteins, heat shock protein binding proteins, disease resistance proteins, Bowman-Birk type bran trypsin inhibitor precursor proteins and wound-induced protein WI12 containing protein (Table I and Supplemental Table VII). Defense-related compounds have been found to accumulate specifically in the stigma, the proteinase inhibitor (PI) proteins and chitinase are highly enriched in stigmas of solanaceous plants (Leung, 1992; Atkinson et al., 1993). It has been shown that the pollination and wounding induced nearly identical flavonol kinetics in Petunia and patterns of the accumulation in the outer cell layers and exudates of the stigma, suggesting that they share elements of a common signal transduction pathway (Vogt et al., 1994). In *P. hybrida* flowers, both pollination and stigma wounding induced a transient increase in ethylene production and hastened corolla senescence (Woltering et al., 1997). Further, it has been reported that the *SPP2* (*Solanum pollinated pistil*) dioxygenase from self-incompatible wild potato *Solanum chacoense* Bitt. is predominantly expressed in the pistil and could be induced by wounding of the style as well as pollination/ fertilization (Lantin et al., 1999). The SPP2 dioxygenase could be involved in the biosynthesis of deterrent alkaloids in reproductive tissues or in generating chemical signals involved in pollen tube guidance (Lantin et al., 1999). One lectin domain-encoding gene belonged to the stress-related group. A b-lectin

receptor kinase gene has been shown to confer the rice blast resistance (Chen et al., 2006). Recently, Chu et al. (2005) have demonstrated that *R* gene *xa13,* a recessive allele conferring disease resistance against bacterial blight plays a key role in both disease resistance and pollen development and there exist a trade-off between fertility and pathogen defense, depending on the levels of expression of the *xa13* gene.

Our analysis also revealed that the stigma-specific genes share some common *cis*-regulatory elements with the stress-responsive genes. A large percentage of genes in Cluster I possess the same motif named GCC-box (Figure 2). The GCC-box has been found in many pathogen-responsive genes (Brown et al., 2003; Chakravarthy et al., 2003). The GbERF belongs to the ERF (ethylene responsive factor) family of transcription factors and regulates the GCC-box containing pathogen-related (PR) genes (Qin et al., 2006). The GCC-box is not only found in the pathogen-responsive genes but also existed in some abiotic stress-related genes. In *Arabidopsis,* AtERF7 binds to the GCC box and overexpressing *AtERF7* showed a reduced sensitivity to drought stress (Song et al., 2005). The rice OsBIERF3 could bind specifically to the GCC box sequence and was induced by salt, cold, drought, wounding and the rice blast fungus (Cao et al., 2006). Further work is required to dissect the molecular basis of these possible cross-talks.

In conclusion, we have identified a large set of genes that are specifically or highly enriched in the rice stigma. In addition to the conserved roles of the cell wall metabolism and cellular communication in the stigma, the identification of genes involved in the auxin signaling, transcription functions and a possible cross-talk between pollination and stress/defense responses provide new insights into the molecular functions of the stigma in rice.

MATERIALS AND METHODS

Plant Materials

Rice (*O. sativa* L. cv. Nipponbare) seeds were germinated and grown in a growth container for two weeks (28℃, 16 h light, 8 h dark and photo intensity 240 μm photos

$m^{-2}s^{-1}$). Then, shoot and root were harvested for seedling shoot (Sh) and seedling root (Rt), respectively. Other plant materials used in this study described below were harvested from rice plant grown under natural conditions in the field of the Institute of Genetics and Developmental biology, Beijing during May to October and Hainan, China, during December to next April. Mature anthers (An) were collected at a stage 1–2 days before floret flowering; unpollinated stigmas at anthesis (St) and ovaries at anthesis (Ov) were dissected at a stage 0-1 days before floret flowering; pistils of 5 days after pollination (5DAP) were dissected out five days after anthesis, 10-day-old embryos (10EM) and endosperms (10EN) were dissected from grains 10 days after flowering, respectively.

Suspension Cell Culture

The method used for suspension cell culture was described by Wang et al. (2005).

10K cDNA Microarray Hybridization and Data Analysis

Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany). Double strand cDNA was synthesized from five microgram of total RNA using cDNA Synthesis Kit (TaKaRa, Dalian, China). In Vitro Transcription (IVT) from cDNA to cRNA was performed using T_7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, USA). Then the cRNA was converted to DNA by using Superscript II reverse transcription kit (Invitrogen, CA, USA) and random primers. One microgram DNA product and random primers (9-mers) were annealed to denatured DNA template and extended by Klenow fragment (TaKaRa, Dalian, China) in the presence of Cy5-dCTP/Cy3-dCTP (Amersham Pharmacia, Buckinghamshire, UK) for target preparation in microarray analysis. Hybridization and washing were performed as described in CyScribe™ Post- Labeling Kit (Amersham Pharmacia) and CMTTM Hybridization Chamber (Corning, NY, USA) user manuals. Scanning and data acquisition were performed on a GenePix 4000B scanner using GENEPIX 4.0 software (Axon Instruments, CA, USA). 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, 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. 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).

Affymetrix GeneChip Hybridization and Data Analysis

Total RNA was isolated using TRizol reagent (Invitrogen, CA, USA) and purified by using Qiagen RNeasy columns (Qiagen, Hilden, Germany). For Affymetrix GeneChip (Affymetrix, CA, USA) analysis, eight microgram of total RNA was used for making biotin-labeled cRNA targets. All the processes for cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning, were conducted according to the GeneChip Standard Protocol (Eukaryotic Target Preparation, Affymetrix). Poly-A RNA Control Kit and the One-Cycle cDNA Synthesis kit were used in this experiment as described in the website: http://www.affymetrix.com/products/ arrays/specific/rice.affx. The information about GeneChip® Rice Genome Array (MAS 5.0) could be accessed from Affymetrix website: http://www.affymetrix.com/products/arrays/specific/rice.affx. GCOS software (Affymetrix GeneChip Operating Software) was used for data collection and normalization. The overall intensity of all probe sets of each array was scaled to 500 to guaranty that hybridization intensity of all arrays was equivalent, each probe set was assigned with "P", "A" and "M" and p-value from algorithm in GCOS. To identify differentially expressed genes, $\log_2$ transformed signal ratio of each gene was calculated using GCOS baseline tool, and $\log_2$ (ratio) $\geq$1.33 (2.5 fold change) was used as cut-off.

Two statistical methods were used for data processing. First, SAM analysis (Significance Analysis of Microarrays software package) was conducted for rice triplicate samples between stigma and ovary using q-value ≤ 0.05 and fold change ≥ 2-fold change as cut-off, and 7425 probe sets were subsequently picked out and their expression levels were significantly different between stigma and ovary. We also did normal *t*-test for each genes and added p-value for them. Second, we used *Z*-score transformation normalization method to compare expression levels from stigma and other organs or tissues and suspension cultured cells, and directly calculated significant changes in gene expression between different samples. Z-scores were calculated by taking the difference between the averages of stigma (X_i) and other test samples (μ, mean) and divided by the standard deviation (SD) of all of the other samples using the following equation:

$$Zi = \frac{Xi - \mu}{SD}$$

In total, we identified 665 probe sets preferentially expressed in stigma using *Z*-score ≥ 2.32 and *p*-value ≤ 0.01. For *Z*-score transformation normalization, we used average value from stigma and ovary, separately. The test samples included seedling shoots (Sh), seedling roots (Rt), mature anthers (An), seeds of 5 days after pollination (5DAP), 10-day-old embryos (10EM), 10-day-old endosperms (10EN) and suspension cultured cell. In addition, we measured the relative ratio between stigma and other samples using the following equation:

$$Ratio = \frac{\overline{S}}{Max(\overline{O}, T1, T2, ..., Tn)}$$

$\overline{S}$ is the average of expression level in stigma tissue sample, $\overline{O}$ is the average of expression level in ovary tissue sample, *T1, T2, …, Tn* are the expression levels in other tissue samples. The ratios of all 665 probe sets were more than 2 fold change.

GO (Gene Ontology) Analysis

We searched GO information for the 665 probe sets using EasyGO software (http://bioinformatics.cau.edu.cn/easygo/category_treeBrowse.html). Using the rice

Japonica gene database, we applied χ^2 tests for biological process search and cutoff for FDR was adjusted and *p*-value was 0.0001.

Real-time PCR

Total RNA preparation and real-time PCR were performed as previously described (Lan et al., 2004). In brief, two microgram of total RNA was used for cDNA synthesis with SuperScript III First-Strand Synthesis kit (Invitrogen, CA, USA). The cDNA samples were diluted to 8 and 2ng/μl. Triplicate quantitative assays were performed on 1 μl of each cDNA dilution using the SYBR Green Master Mix (Applied Biosystems, CA, USA) with an ABI 7900 sequence detection system according to the manufacture's protocol (Applied Biosystems). Gene-specific primers were designed by using PRIMEREXPRESS software (Applied Biosystems). The relative quantification method (DDCT) was used to evaluate quantitative variation between replicates examined. Amplification of 18S rRNA was used as an internal control to normalize all data.

RNA *in situ* Hybridization

RNA *in situ* hybridization was performed as previously described (Lai et al., 2002). Mature rice pistils at a stage 0-1 days before floret flowering were fixed with formalin–acetic acid–alcohol (FAA) fixative solution at 4 ˚C overnight followed by dehydration steps and then embedded in paraffin (Paraplast Plus; Sigma, St Louis, USA). The tissues were sliced into 8-μm sections with a microtome (Leica RM2145; Leica Microsystems, Nussloch, Germany), affixed to Poly-Prep slides (Sigma). The tissues were stained with Fluorescent Brightener 28 (Sigma) after hybridization. Images were observed under bright- and fluorescence-field at the same time through a microscope (Olympus BX51; Olympus Optical, Tokyo, Japan) and photographed using a Micro Color charge-coupled device (CCD) camera (Apogee Instruments Inc., CA, USA).

Promoter Analysis

1,000-bp regions located upstream of the start codons of genes of interest were used for analysis with the MEME/MAST system (http://meme.sdsc.edu/meme/meme.html) and PLACE (http://www.dna.affrc.go.jp/PLACE). Sequence logos of *cis*-acting regulatory elements were created by weblogo (http://weblogo.berkeley.edu/logo.cgi).

Supplemental Materials

Supplemental Table I Significantly expressed probe sets of stigma compared to ovary identified by 57K Affymetix rice whole genome array

Supplemental Table II Candidate cDNA clones highly expressed in stigma identified by 10K cDNAmicroarray

Supplemental Table III Candidate cDNA clones highly expressed in ovary identified by 10K cDNAmicroarray

Supplemental Table IV Candidate genes highly expressed in stigma identified by both the two microarrays

Supplemental Table V Candidate genes highly expressed in ovary identified by both the two microarrays

Supplemental Table VI Candidate probe sets highly expressed in stigma identified by 57K Affymetix rice whole genome array

Supplemental Table VII Classifications of candidate genes highly expressed in stigma identified by 57K Affymetix rice whole genome array

Supplemental Table VIII Candidate stigma-specific genes conserved in rice and *Arabidopsis*

ACKNOWLEDGMENTS

We thank Kang Chong and Lingfeng Chen (Institute of Botany, Chinese Academy of Sciences) for technical assistance in microarray scanning; Zhen Su and Xin Zhou (China Agricultural University) for assisting on microarray analysis.

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FIGURE LEGENDS:

Figure 1. Expression of five stigma-preferential genes by RNA *in situ* hybridization. A, A rice pistil before pollination. B, D, E, F, G and H show the hybridization by the antisense probes of LOC_Os02g46260, LOC_Os09g36700, LOC_Os10g33250, LOC_Os01g62070, AK071040 and LOC_Os01g09620, respectively. C, I, J, K, L and M indicate the hybridization by their respective sense probes. Among them, the gene LOC_Os01g09620 (H and M) was used as a reference because the microarray result showed that it was expressed in the entire mature pistil. The longitudinal section through the pistil before pollination was used for all the hybridizations. All of the slices were stained with Fluorescent Brightener 28 after hybridization. The hybridization signals are shown in dark purple. The structures of the pistil are illustrated in A and B. Bar: 100μm.

Figure 2. Putative *cis*-acting regulatory elements enriched in the stigma-specific or preferential genes. A, B and C represent the top three candidate motifs of Cluster I, Cluster II and Cluster III, respectively. The overall height of each stack indicates the sequence conservation at that position (measured in bits), whereas the heights of symbols within the stack reflect the relative frequencies of the corresponding nucleic acids at that position.

SUPPLEMENTAL FIGURE LEGENDS:

Supplemental Figure S1. Gene Ontology (GO) term enrichment status for the putative rice stigma-specific genes. The graph displayed term enrichment levels along with the GO term hierarchy within the "biological process" branch, and the analysis was performed using EasyGO. The classification terms and their serial numbers are represented as rectangles. The numbers in brackets represent the total number of genes that may be involved in the corresponding biological processes. The graph displays the classification term enrichment status and term hierarchy. The color scale shows the *p*-value cutoff levels for each biological process. The deeper colors represent the more significant biological processes in the putative stigma pathway.

Supplemental Figure S2. Hierarchical clustering of the stigma-preferential genes from the three clusters with the eight tested organs or tissues and suspension culture cells. A, Cluster I. B, Cluster II. C, Cluster III. The genes in the three clusters were preferentially and/or highly expressed in stigma and the correlation coefficients of the three clusters were 0.9277, 0.9389 and 0.8263, respectively. 1: Unpollinated stigmas at anthesis. 2: Mature anthers. 3: Seedling shoots. 4: Seedling roots. 5: Seeds of 5 days after pollination. 6: Endosperms derived from 10-day-old seed. 7: Ovaries at anthesis. 8: Embryos derived from 10-day-old seed. 9: Suspension culture cells. The color scale shows the gene signal intensities in 57K Affymetrix rice GeneChip.

Tables

Table 1. Candidate genes preferentially expressed in rice stigma

Probe Set ID[a]	Gene ID	Description	Stigma-S[b]	Ovary-S[c]	Ratio[d]	*P*-value
Transcription						
Os.3386.1.S1_x_at	LOC_Os06g10350	anthocyanin regulatory C1 protein	2087.6	90.4	23.1	1.93E-04
Os.2233.1.S1_at	LOC_Os04g47080	anthocyanin regulatory Lc protein	7912.47	75.93	104.2	1.98E-04
Os.38982.1.S1_at	LOC_Os02g43820	AP2 domain containing protein	1498.93	72.97	20.5	1.67E-02
Os.49746.1.S1_at	LOC_Os01g74020	ARR1 protein-like	13525.53	908.27	14.9	3.05E-04
Os.31989.1.S1_at	LOC_Os03g02900	B3 DNA binding domain containing protein	3014.57	260.47	11.6	2.72E-03
OsAffx.27272.1.S1_at	LOC_Os05g41070	bZIP transcription factor	267.53	23.2	11.5	4.17E-02
Os.46006.1.S1_at	LOC_Os07g47140	CCT motif family protein	10524.93	313.83	33.5	8.37E-04
Os.37618.1.S1_at	LOC_Os06g04850	homeobox-leucine zipper protein ATHB-4	1608.2	93.23	17.2	2.78E-03
Os.2365.1.S1_at	LOC_Os06g04870	homeobox-leucine zipper protein HAT1	9315.13	352.67	26.4	1.31E-04
Os.49761.1.S1_at	LOC_Os03g54170	MADS-box transcription factor 34	3492.13	276.37	12.6	6.24E-03
OsAffx.21941.1.S1_s_at	LOC_Os01g19970	myb-like transcription factor	1679.7	165.77	10.1	1.98E-04
OsAffx.14605.1.S1_at	LOC_Os05g09020	OsWRKY67	3840.1	229.9	16.7	1.96E-03
OsAffx.14605.1.S1_s_at	LOC_Os05g09020	OsWRKY67	3244.13	225.6	14.4	3.32E-07
OsAffx.2905.1.S1_at	LOC_Os02g38320	protein binding protein	1157.37	63.13	18.3	2.91E-04
OsAffx.11799.1.S1_at	LOC_Os01g69200	regulatory protein	2144.53	2.9	739.5	2.25E-03
Os.56298.1.S1_at	LOC_Os03g63810	WRKY transcription factor 14	2149.4	21.93	98	5.89E-04
OsAffx.17417.1.S1_x_at	LOC_Os09g31140	zinc finger protein F15B8.140 - Arabidopsis thaliana	374.63	16.3	23	8.56E-03
Os.49270.1.S1_at	LOC_Os06g40960	zinc finger protein	5652.4	172.57	32.8	1.37E-03
Cell-wall-related						
Os.53291.1.S1_at	LOC_Os03g64280	1-aminocyclopropane-1-carboxylate oxidase	861.17	85.33	10.1	7.57E-04
Os.27155.1.S1_at	LOC_Os06g15170	3-ketoacyl-CoA synthase	2845.73	76.87	37	1.19E-02
Os.2367.1.S1_at	LOC_Os03g21820	alpha-expansin 10 precursor	11840.7	658.43	18	5.25E-04
Os.31669.1.S1_at	LOC_Os01g53370	anthocyanidin 5,3-O-glucosyltransferase	475.63	5.57	85.4	6.27E-03
Os.31669.1.S1_x_at	LOC_Os01g53370	anthocyanidin 5,3-O-glucosyltransferase	667.57	10.43	64	9.77E-03
Os.8842.1.S1_at	LOC_Os07g32620	anthocyanidin 5,3-O-glucosyltransferase	15546.97	288.1	54	2.47E-04
Os.57078.1.S1_at	LOC_Os05g45200	anthocyanidin 5,3-O-glucosyltransferase	1105.83	47.2	23.4	1.24E-02
OsAffx.23995.2.S1_x_at	LOC_Os01g71094	basic 7S globulin 2 precursor	349.03	4.27	81.8	3.36E-02
Os.27968.1.S1_x_at	LOC_Os01g71094	basic 7S globulin 2 precursor	2661.17	184.47	14.4	1.16E-03
Os.27968.1.S1_at	LOC_Os01g71094	basic 7S globulin 2 precursor	3755.6	271.67	13.8	5.83E-03
Os.2075.1.S1_at	LOC_Os01g19220	beta-D-xylosidase	6110.4	567.4	10.8	1.70E-04
Os.14358.4.S1_x_at	LOC_Os02g12730	beta-galactosidase precursor	6569.7	243.7	27	7.94E-04
Os.14358.1.S1_at	LOC_Os02g12730	beta-galactosidase precursor	1490.43	71.67	20.8	3.07E-04
Os.2322.1.S1_at	LOC_Os10g33250	CER1	28284.37	469.33	60.3	4.07E-03
Os.2322.2.S1_at	LOC_Os10g33250	CER1	1049.97	32.73	32.1	2.42E-02
OsAffx.15853.1.S1_x_at	LOC_Os06g39970	CESA11 - cellulose synthase	252.67	15	16.8	1.77E-02
OsAffx.15853.1.S1_at	LOC_Os06g39970	CESA11 - cellulose synthase	200.13	15.33	13.1	5.18E-03
OsAffx.22999.1.S1_at	LOC_Os07g32710	d-genomic expressed protein	19698.87	292.03	67.5	4.54E-03
OsAffx.4968.1.S1_at	LOC_Os06g30090	DNA binding protein	325.67	2.43	133.8	4.58E-03
Os.5804.1.S1_at	LOC_Os11g40210	DNA binding protein	1662.97	129.93	12.8	6.56E-04
OsAffx.2049.2.S1_x_at	LOC_Os01g21310	dTDP-glucose 4-6-dehydratase-like protein	534.87	33.43	16	4.85E-02
Os.26486.1.S1_at	LOC_Os09g25850	gl1 protein	19971.97	353.67	56.5	2.77E-03
OsAffx.30743.1.S1_at	LOC_Os01g14900	glycerol-3-phosphate acyltransferase 1	135.2	5.87	23	5.01E-03
Os.32123.1.S1_at	LOC_Os01g02930	glycosyltransferase	7300.53	480.7	15.2	1.54E-03
Os.53164.1.S1_at	LOC_Os06g18010	hydroquinone glucosyltransferase	7375.97	115	64.1	6.97E-04
Os.15570.1.S1_at	LOC_Os07g32630	hydroquinone glucosyltransferase	10598	395.8	26.8	1.99E-04
OsAffx.31322.1.S1_at	LOC_Os11g36240	pectinesterase precursor	6228.2	1.53	4061.9	9.86E-04
Os.6192.1.S1_at	LOC_Os03g02460	retinol dehydrogenase 12	10156.47	721.63	14.1	1.60E-06
Stress/defence						
Os.4377.1.S1_at	LOC_Os02g46970	4-coumarate--CoA ligase 2	4725.17	28.87	163.7	1.03E-03
Os.46537.1.S1_at	LOC_Os10g04429	alcohol acyl transferase	336.03	6.07	55.4	2.42E-03
Os.23215.1.S1_at	LOC_Os06g24404	anther-specific proline-rich protein APG precursor	4965.7	247.37	20.1	2.43E-03
Os.22594.2.S1_at	LOC_Os01g03390	Bowman-Birk type bran trypsin inhibitor precursor	206.1	9.63	21.4	1.29E-02
Os.22594.1.S1_at	LOC_Os01g03390	Bowman-Birk type bran trypsin inhibitor precursor	20176.87	1726.13	11.7	4.64E-03
Os.3121.1.S1_at	LOC_Os10g28050	chitinase 2	11909.67	287.67	41.4	7.30E-03
Os.47136.1.A1_x_at	LOC_Os01g27360	d-genomic glutathione S-transferase GSTF1	1140.33	36.13	31.6	2.45E-02
Os.14539.1.S1_at	LOC_Os07g01600	disease resistance response protein 206	10803.27	662.5	16.3	2.94E-03
Os.12922.1.S1_at	LOC_Os09g36700	extracellular ribonuclease LE precursor	9753.43	146.17	66.7	1.47E-04
Os.46551.1.S1_at	LOC_Os10g17260	flavonoid 3-monooxygenase	3769.07	16.27	231.7	6.04E-03
Os.25651.1.S1_at	LOC_Os01g27360	glutathione S-transferase GSTF1	2437.67	59.23	41.2	7.15E-03
OsAffx.13105.1.S1_at	LOC_Os03g29250	ids4-like protein	134.93	9.43	14.3	6.16E-03
Os.51747.1.S1_at	LOC_Os03g29250	ids4-like protein	1188.83	94.13	12.6	2.91E-03
OsAffx.14039.1.S1_s_at	LOC_Os04g30040	jacalin-like lectin domain containing protein	348.73	5.33	65.4	7.62E-03
OsAffx.25096.1.S1_at	LOC_Os03g15360	leucoanthocyanidin reductase	4015.07	294.57	13.6	9.87E-04
Os.42490.1.S1_at	LOC_Os01g62070	metal tolerance protein C3	28270.2	199.37	141.8	4.92E-03
Os.42490.1.S1_x_at	LOC_Os01g62070	metal tolerance protein C3	27456.03	206.4	133	3.13E-03
OsAffx.10363.1.S1_at	LOC_Os06g03500	NBS-LRR disease resistance protein	225.27	17.27	13	2.59E-02

Os.12851.1.S1_at	LOC_Os01g14670	nectarin-1 precursor	6357.33	141.37	45	6.85E-04
Os.12761.1.S1_at	LOC_Os03g46060	osmotin-like protein OSML13 precursor	19564.23	702.03	27.9	2.60E-03
Os.52536.1.S1_at	LOC_Os04g43800	phenylalanine ammonia-lyase	6313.3	20.2	312.5	2.92E-03
Os.50455.1.S1_at	LOC_Os06g40170	phospholipase D alpha 2	3267.17	166.77	19.6	9.06E-05
Os.11575.1.S1_a_at	LOC_Os03g46440	regulatory protein NPR1	1400.97	120.6	11.6	9.44E-03
OsAffx.13793.1.S1_at	LOC_Os04g12840	resistance protein	1537.77	142.13	10.8	4.86E-05
Os.10600.1.S1_a_at	LOC_Os01g25820	respiratory burst oxidase 2	12428.1	1231.33	10.1	2.68E-03
Os.57186.1.S1_at	LOC_Os05g27590	wound-induced protein WI12 containing protein	1239.17	45.27	27.4	3.73E-02
Signal transduction						
OsAffx.13408.1.S1_at	LOC_Os03g48560	BGGP Beta-1-3-galactosyl-O-glycosyl-glycoprotein	350.6	24.93	14.1	7.61E-03
OsAffx.13408.1.S1_x_at	LOC_Os03g48560	BGGP Beta-1-3-galactosyl-O-glycosyl-glycoprotein	224.77	19.97	11.3	1.68E-03
OsAffx.24314.1.S1_at	LOC_Os02g18930	calcineurin B-like protein 4	2628.87	24.77	106.1	4.50E-03
Os.15639.1.S1_at	LOC_Os06g40370	CBL-interacting serine/threonine-protein kinase 24	7217.23	666.6	10.8	3.66E-03
Os.48846.1.S1_at	LOC_Os09g24840	GAST1 protein precursor	3067.53	136.57	22.5	1.28E-04
Os.17201.1.S1_at	LOC_Os07g38810	lectin receptor-type protein kinase	3123.2	82.57	37.8	3.28E-03
Os.6360.1.S1_at	LOC_Os08g02996	lectin-like receptor kinase 1	4453.83	85.53	52.1	1.53E-03
Os.6360.2.S1_x_at	LOC_Os08g02996	lectin-like receptor kinase 1	4304.07	162.17	26.5	2.10E-03
OsAffx.30716.1.S1_x_at	LOC_Os10g38960	lectin-like receptor kinase 7	3306.8	244.23	13.5	3.84E-03
Os.12660.1.S2_at	LOC_Os07g38800	lectin-like receptor kinase 7	2745.87	27.73	99	2.88E-03
Os.12660.1.S1_at	LOC_Os07g38800	lectin-like receptor kinase 7	938.63	16.2	57.9	1.14E-02
Os.26943.1.S1_at	LOC_Os09g32840	nucleotide pyrophosphatase/phosphodiesterase	3333.23	205.1	16.3	1.96E-03
OsAffx.29386.1.S1_x_at	LOC_Os08g27810	OsWAK115	1312.67	121.3	10.8	1.52E-02
OsAffx.6309.1.S1_at	LOC_Os09g16980	OsWAK86	2528.73	9.33	270.9	2.87E-03
Os.54625.1.S1_at	LOC_Os12g42020	protein kinase PVPK-1	7055.77	441.67	16	2.73E-04
Os.44814.1.S1_at	LOC_Os06g03610	protein kinase	85.97	4.37	19.7	1.29E-02
OsAffx.17892.1.S1_at	LOC_Os09g25540	receptor-like protein kinase precursor	124.07	6.3	19.7	3.54E-02
OsAffx.14892.1.S1_at	LOC_Os05g30740	SRC2	823.27	54.3	15.2	6.54E-04
Lipid metabolism						
Os.5295.1.S1_at	LOC_Os08g10010	acyl-desaturase, chloroplast precursor	12530.77	158.63	79	4.74E-03
Os.30537.1.S1_at	LOC_Os03g01820	C-4 methylsterol oxidase	7759.1	306.77	25.3	1.48E-04
Os.57035.1.S1_at	LOC_Os01g21300	catalytic/ hydrolase	605.6	45.2	13.4	1.21E-02
Os.54454.1.S1_at	LOC_Os11g32650	chalcone synthase	765.67	4.03	189.8	1.61E-02
Os.57449.1.S1_x_at	LOC_Os11g32650	chalcone synthase	10288	150.9	68.2	3.56E-03
Os.37295.1.S1_at	LOC_Os11g02440	chalcone--flavonone isomerase	2854.53	13.1	217.9	7.51E-03
Os.6354.1.S1_s_at	LOC_Os12g02370	chalcone--flavonone isomerase	13196.17	228.1	57.9	1.56E-04
Os.6354.1.S1_at	LOC_Os12g02370	chalcone--flavonone isomerase	5023.07	118.8	42.3	4.68E-04
Os.53034.1.A1_at	LOC_Os11g18194	cycloartenol synthase	10955.6	23.7	462.3	4.83E-03
Os.52235.1.S1_at	LOC_Os02g21810	cytochrome P450	2250.9	4.37	515.5	6.50E-03
Os.25621.2.S1_at	LOC_Os12g16720	cytochrome P450	16932.33	546.27	31	2.06E-03
Os.53717.1.S1_at	LOC_Os06g01250	cytochrome P450	13610.93	88.73	153.4	2.18E-04
Os.39637.1.A1_s_at	LOC_Os09g17000	glycerophosphoryl diester phosphodiesterase family	6529.73	75.93	86	3.88E-03
Os.39637.1.A1_at	LOC_Os09g17000	glycerophosphoryl diester phosphodiesterase family	4584.1	73.8	62.1	2.93E-03
Os.47906.1.A1_at	LOC_Os06g49770	lipid binding protein	7864.5	210.5	37.4	3.39E-03
OsAffx.32067.1.S1_x_at	LOC_Os12g37320	lipoxygenase 2.2, chloroplast precursor	194.63	7.5	26	4.50E-03
Os.41251.1.A1_at	LOC_Os01g43140	triacylglycerol lipase	2613.6	244.57	10.7	3.00E-04
OsAffx.14502.1.S1_at	LOC_Os05g02350	type I inositol-1,4,5-trisphosphate 5-phosphatase CVP2	393.47	12.6	31.2	1.50E-03
Os.29290.1.S1_at	LOC_Os01g08780	type I inositol-1,4,5-trisphosphate 5-phosphatase CVP2	4496.2	65.27	68.9	3.62E-03
Transport						
Os.17440.1.S1_at	LOC_Os04g44610	ABC transporter C05D10.3 in chromosome III	9093.1	674.9	13.5	5.79E-04
Os.55221.1.S1_at	LOC_Os08g43120	ABC transporter	11133.57	462.03	24.1	1.16E-02
OsAffx.3427.1.S1_at	LOC_Os03g37960	acyl-CoA-binding protein	782.93	58.17	13.5	4.37E-03
Os.26802.2.S1_at	LOC_Os12g08090	amino acid transporter	18660.77	1722.8	10.8	4.13E-04
OsAffx.19991.1.S1_at	LOC_Os12g36660	antiporter/ drug transporter/ transporter	1091.07	41.33	26.4	5.61E-03
OsAffx.24678.3.S1_at	LOC_Os02g41860	aquaporin PIP2.2	1547.47	138.83	11.1	2.14E-03
OsAffx.24678.2.S1_at	LOC_Os02g41860	aquaporin PIP2.2	790.03	73.83	10.7	8.10E-03
Os.49307.1.S1_at	LOC_Os12g22284	ATP-binding cassette sub-family G member 2	3085.93	7.1	434.6	6.65E-04
Os.52570.1.S1_at	LOC_Os12g44110	ligA	12413.13	851.5	14.6	1.50E-02
OsAffx.3105.1.S1_at	LOC_Os02g58530	major facilitator superfamily protein	237.23	11.8	20.1	2.45E-02
Os.27833.1.S1_at	LOC_Os12g32940	major myo-inositol transporter iolT	2600.73	98.1	26.5	5.41E-03
OsAffx.20797.1.S1_at	LOC_Os10g02340	peptide transporter PTR2	275	12.63	21.8	4.68E-03
Os.46447.1.S1_at	LOC_Os10g33210	peptide transporter PTR2	1797.1	113.97	15.8	5.30E-03
Os.11547.1.S1_s_at	LOC_Os06g48030	peroxidase 16 precursor	9654.5	622.3	15.5	5.94E-05
Os.5843.1.S1_at	LOC_Os06g46799	peroxidase 39 precursor	11243.27	277.83	40.5	3.39E-03
Os.53303.1.S1_at	LOC_Os06g46799	peroxidase 39 precursor	1034.43	56.8	18.2	5.73E-03
Os.10754.1.S1_at	LOC_Os03g06520	sulfate transporter 3.1	7211.23	668.47	10.8	3.14E-03
Hormone-related						
Os.27822.2.S1_at	LOC_Os04g47520	auxin-independent growth promoter	1048.63	101.87	10.3	2.04E-02
OsAffx.21458.1.S1_at	LOC_Os01g46030	Brassinosteroid insensitive 1-associated receptor kinase 1 precursor	278.63	17.87	15.6	6.22E-03
OsAffx.28262.1.S1_at	LOC_Os07g04520	Brassinosteroid insensitive 1-associated receptor kinase 1 precursor	939.93	8.37	112.3	1.19E-02
OsAffx.15447.1.S1_at	LOC_Os06g16000	cytokinin-O-glucosyltransferase 3	2037.37	186.13	10.9	6.74E-04

Os.36449.1.S1_at	LOC_Os01g57610	indole-3-acetic acid-amido synthetase GH3.1	10154.47	533.07	19	4.14E-04
OsAffx.32069.1.S1_at	LOC_Os12g37490	N-acetyltransferase	405.6	22.13	18.3	8.10E-04
Os.55720.1.S1_at	LOC_Os04g56690	OsSAUR23	997.73	47.67	20.9	3.74E-03
OsAffx.30169.1.S1_at	LOC_Os09g37430	OsSAUR48	781.37	51.53	15.2	1.61E-03
Os.51408.1.S1_at	LOC_Os09g37490	OsSAUR54	1076.63	64.17	16.8	1.47E-02
Os.50785.1.S1_at	LOC_Os09g37500	OsSAUR55	5390.27	317.77	17	4.96E-03
Protein metabolism						
Os.54875.1.S1_at	LOC_Os05g04584	3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase	337.53	19.3	17.5	8.62E-04
OsAffx.24010.1.S1_x_at	LOC_Os01g72020	BOP2	806.33	30.57	26.4	2.00E-03
Os.49276.1.S1_at	LOC_Os01g72020	BOP2	1908.9	162.2	11.8	1.03E-03
Os.52793.1.S1_at	LOC_Os04g43840	carboxy-lyase	5289.97	30.97	170.8	2.02E-03
Os.10714.1.S1_s_at	LOC_Os04g38450	gamma-glutamyltranspeptidase 1 precursor	10921.73	330.83	33	6.63E-04
Os.10728.1.S1_at	LOC_Os02g46260	serine carboxypeptidase 1 precursor	23058.93	857.67	26.9	3.13E-03
OsAffx.10889.1.S1_x_at	LOC_Os01g03170	seven in absentia protein family protein	4477.57	409.87	10.9	2.57E-04
Os.3768.1.S1_at	LOC_Os02g44590	subtilisin-like protease precursor	11949.97	366.4	32.6	5.07E-03
Os.8271.2.S1_x_at	LOC_Os01g67500	ubiquitin-protein ligase	1791.4	174.73	10.3	8.17E-03
OsAffx.13747.1.S1_at	LOC_Os04g10360	xylem serine proteinase 1 precursor	4292.53	12.23	350.9	6.60E-04
Carbohydrate and energy metabolism						
Os.53178.2.S1_at	LOC_Os05g47840	ATIPT1	124.5	10.23	12.2	9.57E-03
OsAffx.28287.1.S1_at	LOC_Os07g05380	ATPase	1148.87	33.53	34.3	6.85E-03
OsAffx.5436.1.S1_at	LOC_Os07g25540	disulfide oxidoreductase/ monooxygenase/ oxidoreductase	197.67	8.37	23.6	1.74E-03
OsAffx.31989.1.S1_at	LOC_Os12g32750	disulfide oxidoreductase/ monooxygenase/ oxidoreductase	2854.47	149.03	19.2	6.31E-04
Os.18223.1.S1_s_at	LOC_Os12g32750	disulfide oxidoreductase/ monooxygenase/ oxidoreductase	7562.97	747.77	10.1	3.81E-04
OsAffx.13016.1.S1_at	LOC_Os03g25150	flavonoid 3,5-hydroxylase 2	1361.83	57.23	23.8	4.06E-02
OsAffx.13016.1.S1_x_at	LOC_Os03g25150	flavonoid 3,5-hydroxylase 2	1470.4	78.63	18.7	4.62E-02
OsAffx.27142.1.S1_at	LOC_Os05g33644	hydrolase	1714.27	93.2	18.4	2.38E-02
Os.46268.1.S1_at	LOC_Os10g02390	NAD(P)H-dependent oxidoreductase	7951.73	3.63	2188.6	6.30E-03
Nucleic acid metabolism						
Os.855.1.S1_at	LOC_Os01g03730	nuclease PA3	5603.9	85.67	65.4	1.12E-02
Os.55424.2.S1_at	LOC_Os02g50740	nucleotide binding protein	2223.23	164.1	13.5	8.77E-05
Amino acid metabolism						
Os.27876.1.S1_x_at	LOC_Os08g04540	aromatic-L-amino-acid decarboxylase	3587.43	19.3	185.9	1.49E-02
OsAffx.5702.1.S1_s_at	LOC_Os08g04560	aromatic-L-amino-acid decarboxylase	23981.57	192.23	124.8	9.93E-04
Unclassified						
Os.48933.1.S1_at	LOC_Os05g37880	axi 1 like protein	12167.7	1092.03	11.1	1.32E-03
OsAffx.3289.1.S1_at	LOC_Os03g21450	bromodomain containing protein	433.77	23.43	18.5	2.21E-02
Os.28450.1.S1_at	LOC_Os01g70730	FLP1	3929.67	103.73	37.9	1.54E-04
Os.6003.1.S1_x_at	LOC_Os09g26310	hypro1	3628.77	109.37	33.2	4.80E-05
Os.51638.1.S1_s_at	LOC_Os09g26310	hypro1	4551.53	161	28.3	4.31E-04
Os.49681.1.S1_at	LOC_Os03g07140	male sterility protein 2	2714.93	42.37	64.1	4.57E-02
Os.24786.1.S1_s_at	LOC_Os04g48870	nitrilase-associated protein	4117.03	126.8	32.5	2.12E-02
OsAffx.26533.1.S1_at	LOC_Os04g48870	nitrilase-associated protein	4745.7	152.2	31.2	1.16E-02
OsAffx.12915.1.S1_at	LOC_Os03g18300	pherophorin like protein	570.47	21.47	26.6	1.84E-03
Os.46488.1.S1_at	LOC_Os10g05790	proline-rich protein	28988.2	512.93	56.5	1.96E-03
OsAffx.16391.1.S1_at	LOC_Os07g24100	retrotransposon protein	707.07	5.73	123.3	1.28E-04
Os.28346.5.S1_x_at	LOC_Os03g63124	retrotransposon protein, unclassified	242	23.33	10.4	1.57E-02
Os.50793.1.S1_at	LOC_Os12g01900	retrotransposon protein, unclassified	621.7	6.53	95.2	7.10E-03
Os.55132.1.S1_at	LOC_Os03g26990	VQ motif family protein	222.07	20.03	11.1	1.21E-02

The total number of the preferentially expressed genes is 548(665 probe sets). Expressed protein, hypothetical protein and no hit genes are 103, 23 and 12, respectively. The table lists 149(173 probe sets) of them with putative annotated functions and their ratios of stigma/ovary are more than 10 ($p < 0.05$). [a]The gene probes of 57K Affymetrix rice GeneChip. [b]The gene's average signal intensity in stigma derived from three biological replicates of 57K Affymetrix rice GeneChip. [c]The gene's average signal intensity in ovary derived from three biological replicates of 57K Affymetrix rice GeneChip. [d]The ratios of stigma/ovary.

Table 2. Verification of microarray results by Real-Time Quantitative RT-PCR

Probe Set ID[a]	Gene ID	Description	Stigma/Ovary[b]	Stigma/Anther[c]	Stigma/Shoot[d]	Stigma/Leaf[e]
Os.12922.1.S1_at	LOC_Os08g10010	acyl-desaturase, chloroplast precursor	182.34±26.76	165.43±23.822	2.29±0.053	4239.26±1003.77
Os.2696.1.S1_at	LOC_Os04g15840	alpha-expansin 1 precursor	1.46±0.033	3.43±0.25	10.18±1.399	2.07±0.089
Os.31669.1.S1_at	LOC_Os01g53370	anthocyanidin 5,3-O-glucosyltransferase	86.66±1.7	21.05±0.282	23.54±0.327	482.61±13.111
OsAffx.5702.1.S1_s_at	LOC_Os08g04560	aromatic-L-amino-acid decarboxylase	2087.29±265.908	369.34±36.352	68.17±4.788	69.3±4.887
OsAffx.20044.1.S1_at	LOC_Os12g40080	B3 DNA binding domain containing protein	12823.5±1586.625	1647.15±159.426	1204.98±111.692	689.49±58.868
OsAffx.23995.2.S1_x_at	LOC_Os01g71094	basic 7S globulin 2 precursor	32.59±0.873	7.53±0.117	25.4±0.632	6.65±0.097
Os.22594.2.S1_at	LOC_Os01g03390	Bowman-Birk type bran trypsin inhibitor precursor	24.59±1.408	11.7±0.514	14.42±0.688	67.8±5.111
Os.2322.1.S1_at	LOC_Os10g33250	CER1	319.88±40.499	38.1±3.04	1362.35±216.136	1308.77±206.473
Os.27065.1.S1_a_at	LOC_Os01g59180	cyclin-like F-box	4.77±0.059	14±0.295	6.53±0.098	25.4±0.657
Os.51106.1.S1_at	LOC_Os06g14540	endoglucanase 1 precursor	252.24±25.059	188.66±17.754	130.53±11.419	34.2±2.167
OsAffx.28068.1.S1_at	LOC_Os06g42730	esterase/lipase/thioesterase	4.03±0.053	2.26±0.017	2.3±0.018	6.33±0.111
OsAffx.21178.1.S1_x_at	LOC_Os01g68140	expressed protein	97.87±9.342	1921.16±303.246	4341.75±759.941	21884.94±4580.042
Os.5295.1.S1_at	LOC_Os09g36700	extracellular ribonuclease LE precursor	1.34±0.002	10.64±0.161	1.53±0.004	16.49±0.296
Os.29814.1.S1_at	LOC_Os03g62860	glucan endo-1,3-beta-glucosidase A6 precursor	119.47±6.068	53.08±2.238	134.61±7.007	52.28±2.196
Os.50860.1.S1_at	LOC_Os09g27680	hypothetical protein	19.23±0.535	2.44±0.02	9.97±0.215	1.68±0.008
Os.55613.1.S1_at	LOC_Os01g65692	hypothetical protein	96.45±3.506	55.1±1.758	55.83±1.787	50.6±1.58
Os.12660.1.S2_at	LOC_Os07g38800	lectin-like receptor kinase 7	9.95±0.143	6.95±0.085	10.67±0.158	3.35±0.025
Os.47906.1.A1_at	LOC_Os06g49770	lipid binding protein	117.27±8.537	46.61±2.735	16.09±0.683	2.74±0.042
Os.31652.1.S1_at	LOC_Os01g06320	myb-like DNA-binding omain protein	37.96±0.79	6.3±0.066	148.69±4.255	79.49±1.99
Os.46268.1.S1_at	LOC_Os10g02390	NAD(P)H-dependent oxidoreductase	51.29±0.696	4.37±0.022	5.15±0.029	4.95±0.027
Os.10675.1.A1_at	LOC_Os03g46060	osmotin-like protein OSML13 precursor	11.46±0.017	26.72±0.054	34.75±0.076	52.39±0.129
OsAffx.30169.1.S1_at	LOC_Os09g37430	OsSAUR48	15.39±1.334	43.55±5.216	19.91±1.889	53.37±6.738
Os.51408.1.S1_at	LOC_Os09g37490	OsSAUR54	105.91±2.819	2.21±0.01	2.79±0.016	15.13±0.235
OsAffx.3726.1.S1_at	LOC_Os04g05050	pectate lyase 8 precursor	14.62±0.457	5.22±0.101	6.46±0.141	608.27±45.45
Os.53102.1.S1_s_at	LOC_Os09g21000	potassium transporter 12	38.62±0.749	8.77±0.101	12.95±0.176	12.75±0.172
Os.10728.1.S1_at	LOC_Os02g46260	serine carboxypeptidase 1 precursor	42.35±0.681	43.97±0.714	8.72±0.081	49.59±0.831
Os.12761.1.S1_at	LOC_Os02g46260	serine carboxypeptidase 1 precursor	125.09±21.094	500.45±108.954	71.65±10.678	184.39±33.617
Os.41415.1.S1_at	LOC_Os01g40499	serine/threonine-protein kinase receptor precursor	14.03±1.286	5.34±0.31	11.06±0.921	4.3±0.217
OsAffx.14892.1.S1_at	LOC_Os05g30740	SRC2	931.33±5.402	3901.73±27.372	111.49±0.446	126.66±0.52
Os.189.1.S1_at	LOC_Os08g43160	TCP family transcription factor containing protein	1.99±0.006	1.87±0.005	5.43±0.042	2.79±0.013
Os.52592.1.S1_at	LOC_Os02g49480	transcription factor BIM1	8.67±0.452	7.61±0.373	6.02±0.261	9.38±0.507
Os.8271.2.S1_x_at	LOC_Os01g67500	ubiquitin-protein ligase	11.65±0.081	25.74±0.236	26.56±0.246	45.2±0.486
Os.56298.1.S1_at	LOC_Os03g63810	WRKY transcription factor 14	296.26±15.558	95.22±4.002	5.54±0.087	5.51±0.087
Os.23805.1.S1_at	AK071040	unknown	63.34±1.016	77.33±1.3	301.67±6.661	1212.9±33.31

[a]The gene probes of 57K Affymetrix rice GeneChip. [b, c, d] and [e] indicate the real-time RT-PCR results plus/minus the standard deviations for stigma/ovary, stigma/anther, stigma/shoot(seedling-shoot) and stigma/leaf(mature leaf), respectively.

Figure 1

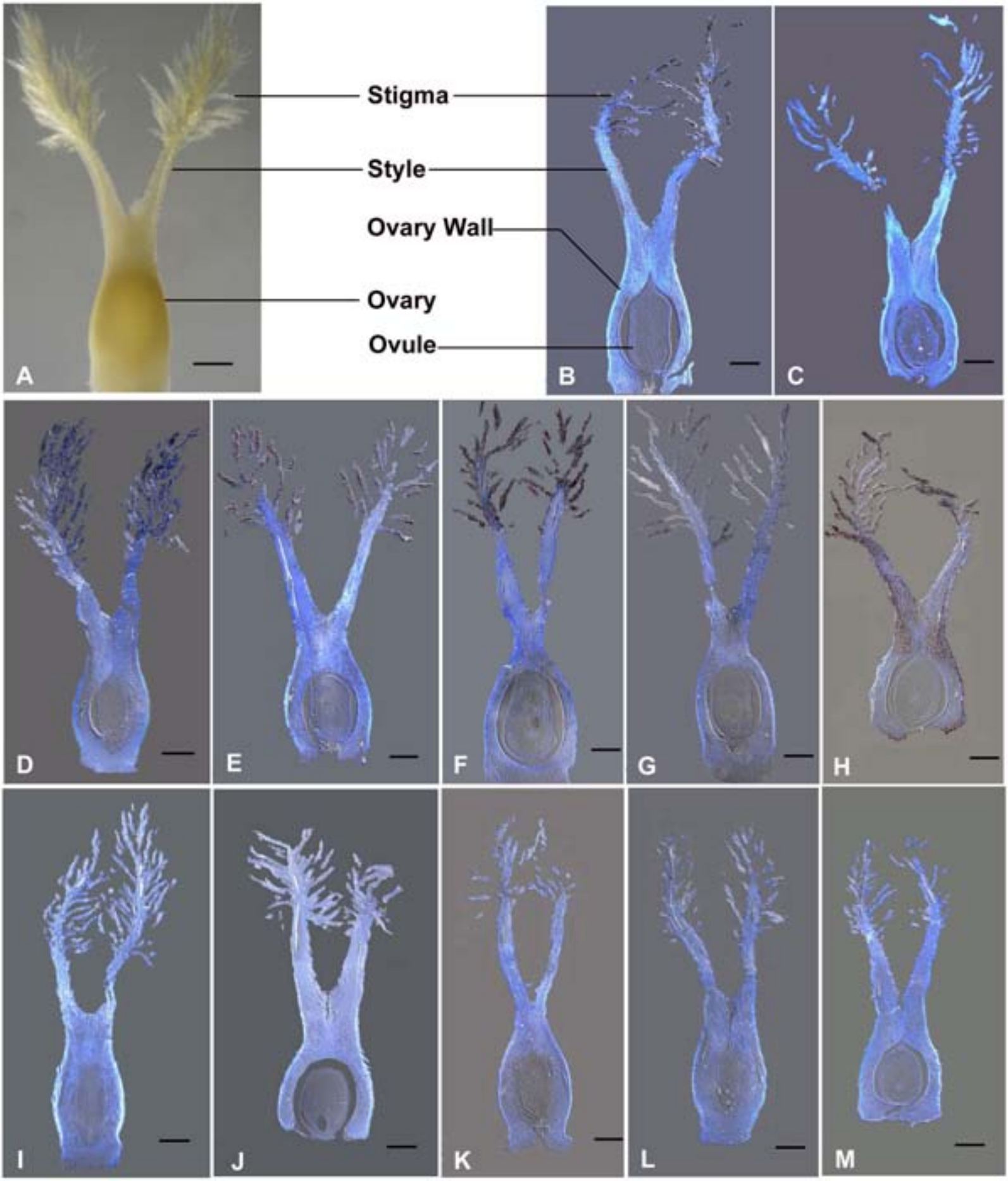

Figure 2