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Exploration of rice pistil responses during early post-pollination through a combined proteomic and transcriptomic analysis

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ABSTRACT

Pollen–stigma interaction is a multi-step and complex physiological process which contains different signaling and biochemical pathways. However, little is known about the molecular mechanism underlying this process in rice (*Oryza sativa*). In this study, the changes of gene expression were investigated through a combination study of transcriptome and proteome profiles in rice pistil during pollination. Totally, 1117 differentially expressed genes were identified, among which 962 and 167 were detected at transcriptional and protein level respectively. Functional categorization analysis showed that the genes involved in central metabolism were up-regulated, which can lead to the enhancement of these metabolisms. The reactive oxygen species (ROS) were over-accumulated in the stigma. In response to this, the proteins or transcripts involved in redox homeostasis regulation were differentially expressed. Furthermore, significant changes of protein ubiquitination and its related genes or proteins, especially some E3 ligases encoding genes, indicate that protein ubiquitination might play important roles in cell signal transduction during the pollination process. Our study sheds some lights on gene and protein expression profiles of rice pistil pollination process, and gives us a comprehensive understanding of the basic molecular mechanisms controlling pollination in rice.

Biological significance: Using RNA-seq, 2-DE and iTRAQ assays, we have generated the large-scale transcriptomic and proteomic data containing abundant information on genes involved in pollen and pistil interaction. Our results showed that ROS were significantly accumulated in stigma after pollination, and the abundance of genes involve in redox homeostasis system were changed variously. We also show that, changes of some E3 ligases encoding genes might indicate that protein ubiquitination play important roles in cell signal transduction during the pollination process. Data in this study might be helpful to deeply understand the pollination in rice.

1. Introduction

Pollination is a critical step to control seed setting, which is referred as the interaction between stigma and pollen. Stigma can capture and recognize pollen grains attached on it [1,2]. Generally, there are six steps during the interaction between stigma (some interactions occur in pistil) and pollen from pollen capture and adhesion to pollen tube enter into the ovule and discharge the sperm cells [2]. Numerous genes, proteins and signaling molecules are involved in the interaction to drive or stop pollination.

Much of early works on pollen and stigma interaction were focused on describing the mechanisms of self-compatibility and self-incompatibility [3,4]. Furthermore, the subjects in much work were either pollen or stigma. Some pollen lipids, pollen coat proteins, and pollen coat structure play important roles during pollen and stigma interaction [5–7]. An aquaporin-like protein in pistil was reported to control the water flow into pollen grains [8]. Reducing the expression level of another gene *pis63* in pistil, which encodes a protein of unknown function, could lead to a decrease in pollen germination and seed set [9]. Some other proteins, such as ATP-binding cassette (ABC) transporter in *Nicotiana tabacum* [10] and a pistil-specific peroxidase from the ragwort *Senecio squalidus* [11], may also function in the process of pollination.

Since the pollen–stigma interaction is a complex multi-step process which contains different signaling and biochemical pathways, studies focusing on single gene are far less than enough to get a full understanding about the mechanisms. It is believed that many different genes function cooperatively to ensure successful pollination. With the advent and

Abbreviations: SI, self-incompatibility; RNA-Seq, high throughput sequencing of cDNA libraries; RPKM, reads per kilo bases per million reads; FDR, false discovery rate; 2-DE, two-dimensional gel electrophoresis; TCA, tricarboxylic acid; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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development of functional genomics techniques, genome-wide profiling has been proved to be a more efficient way in identifying organ or tissue specifically expressed genes. Large amount of transcriptomic [12–20] and proteomic [6,21–27] studies have been conducted on pollen grains or tubes in different species. In recent years, several transcriptomic and proteomic studies have also been carried out to identify the stigma/pistil preferentially expressed genes in different plant species, such as *Arabidopsis* [28,29], rice [30], tobacco [31,32], Asteraceae [33], Triticeae [20] and maize [32,34]. Whereas, these studies mainly focused on the gene expression profile of stigma or pistil rather than its dynamic changes during the pollen–stigma interaction. Qin and coworkers found that penetration of pistil could change the transcriptome of pollen tube in *Arabidopsis*, which proved the reciprocal effects of pollen and pistil on their gene expression [35]. Recently, comparison of the expression profiles of pistils at different time course after pollination was conducted in *Arabidopsis*, and over 1300 differentially expressed genes were identified, which provided more information to better understand the molecular basis of plant pollination [36].

As one of the most important crops in the world especially in East Asia, rice is also a model monocot. Exploring the molecular mechanisms that underlie its sexual reproduction will not only facilitate rice breeding but also help to increase the yield. Gene expressions at RNA and protein levels are not always in consistent [37–39], and information about post-translational modification could not be acquired through RNA. Study at protein level will provide much more direct evidence. Unfortunately, except for some studies in Brassica pistil [40], tobacco and maize [32], soybean [41], *Solanum pennelii* [42], olive and lily [43], and *Triticale* [44], there are hardly any other proteomic studies about stigma or pistils. In this study, deep sequencing-based transcriptomic technique, isobaric tag for relative and absolute quantitation (iTRAQ) and two-dimensional gel electrophoresis (2-DE) via matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) based quantitative proteomic techniques were combined to analyze the dynamic changes of gene expression profile in rice pistil during pollination, which will help us to obtain a more comprehensive view into the gene expression networks and biochemical pathways during this process.

2. Materials and methods

2.1. Plant growth conditions

Rice (*Oryza sativa*) 'guang-zhan 63 S' (GZ63S) and '93-11' used in our study were planted under natural conditions in the field of Wuhan Botanical Garden, Wuhan, from April to September. Rice has typically dry stigma, which is bifurcated above the ovary and the distal parts of the two main branches are densely covered by multicellular papillae [45]. Considering rice stigma is very tiny and hard to cut off from pistil, and moreover we need to harvest large amount stigma for proteomics and transcriptomics, in this study total pistil tissue was used for proteomics and transcriptomics. The un-pollinated matured pistils (S1) were directly harvested from the floret of GZ63S at the flowering stage. For the pollinated pistils, matured pistils of GZ63S were manually pollinated with pollen grains of 93-11 and the pistils around 5 min (S2) and 15 min (S3) after pollination were harvested. Briefly, both the flowers of 'GZ63S' and '93-11' open around noon during flowering stage. The grain husks on spikelet of 'GZ63S' were half cut off. Then the flowering spikelet of '93-11' were gently cut and the pollens were gently shaken off above the flowering spikelet of 'GZ63S' immediately. After 5 min (S2) and 15 min (S3), the pistils were harvested at once. Totally, around 3 g fresh weight S1, S2, and S3 were collected in 2011 for RNA and protein assays, and around 0.5 g fresh weight S1, S2, and S3 were collected in 2012 for PCR assays. All the samples collected were immediately frozen in liquid nitrogen and kept at −80 °C.

2.2. Microscopic observation of pistil

For the pollen tube germination observation, pistils were fixed in 3:1 (v/v) ethanol/acetic acid solution for 6 h and incubated in KOH solution (1 M) at 50 °C for 60 min. The pistils were subsequently incubated in 0.1% aniline blue solution at 50 °C for 60 min and observed with fluorescent microscopy (Leica DM2500, Germany). For the ROS/H_2O_2 detection [46], fluorescent indicator dye $DCFH_2$-DA (Calbiochem Millipore, USA) was used. The pistils were immersed in 50 μM $DCFH_2$-DA in MES (2-[N-morpholino] ethanesulfonic acid)-KCl buffer (5 μM KCl, 50 μM $CaCl_2$, 10 mM MES, pH 6.15, 1% Tween) for 10 min followed by a wash step in MES-KCl buffer for 15 min and then observed at the confocal microscope (Olympus FV 1000, Japan). Negative controls were treated with MES-KCl buffer only.

2.3. Brefeldin A treatment of pistil

Before treatment, each lemma and palea of spikelet was half cut open and stigma was exposed. The 50 μM Brefeldin A solution and water (control treatment) was sprayed to spikelet respectively, and then pollinated them manually. At last we calculated the seed setting percentage when the seed maturated in bag. This experiment was conducted with three biological repeats with fifty florets in each treatment.

2.4. RNA-seq assays and data analysis

The total RNAs were extracted from 0.1 g S1, S2 and S3 samples with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions respectively. RNA concentration was measured with nanodrop Spectrophotometers (Thermo, USA) and its integrity was checked using 1% agarose gel and Agilent 2100 Bioanalyzer. After polyA mRNA isolation, the cDNA libraries for RNA-Seq were constructed using Illumina gene expression sample prep kit (Illumina, USA). The deep sequencing of the cDNA libraries were accomplished by BGI (Beijing Genome Institute at Shenzhen, China). The reference genome information was from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu). The total reads of every gene locus was counted using ERANGE software (http://woldlab.caltech.edu/gitweb/). The expression level of one gene was normalized to Reads per Kilo bases per Million reads (RPKM) value. The threshold for gene expression difference was set as "FDR ≤ 0.001 and the absolute value of $\log_2$ ratio ≥ 1".

2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR technique was used to confirm the expression profile obtained by RNA-seq and to validate the repeatability of the results in 2011 and 2012. The particular genes were selected and analyzed by qRT-PCR using CFX connect (Bio-Rad, USA) with SYBR Green I methods. The melting curves were analyzed to check the specificity of PCR products. The primers used in this study are provided in Table S1. Three technical replicates of each biological replicate were performed. The eukaryotic translation initiation factor 5B (*LOC_Os05g51500*), was taken as an internal control.

2.6. iTRAQ assays and data analysis

A portion (0.25 g) of pistils was ground in mortar with 2 mL precooled lysis buffer (7 M urea, 2 M thiourea and 1% w/v SDS) on ice. The homogenate was shifted into a centrifugal tube and centrifuged at 12,000 ×*g* for 30 min at 4 °C. The supernatant was collected in a new centrifugal tube and mixed with 3 volume of cold acetone. The tube was kept at −20 °C for 1 h, and then centrifuged at 12,000 ×*g* for 30 min at 4 °C, and the precipitate was collected and washed with cold acetone three times. After centrifugation, the pellet was vacuum-dried. The dried protein powder was stored at −80 °C. Proteins were