Rice Anther Protein Identification by Shotgun Proteomic Analysis

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ABSTRACT Rice anthers from Nipponbare in the flowering stage were collected, proteins extracted and shotgun proteomic analysis conducted. From three biological replications, total 3,198 non-redundant rice anther proteins were identified. There was no bias of physiochemical properties in identified proteins. Proteins showing wide pI value range and molecular weight were identified. The lowest pI value was pH 3.93 (LOC_Os07g41694.1) and the highest was pH 12.48 (LOC_Os01g69020.1). Molecular weights of the identified proteins ranged from 5.2kDa (LOC_Os02g27769.1) to 486.0kDa (LOC_Os09g07300.1). Gene ontology enrichment analysis revealed that proteins associated with cellular and metabolic processes, catalytic activity, cell, cell parts, and organelles were enriched in rice anther, suggesting the status of proteins in rice anther were associated with pollen germination and pollen tube elongation. The highly abundant proteins in rice anther were pollen allergens, ATP synthase, glyceraldehyde-3-phosphate dehydrogenase, cupin domain containing proteins, and ascorbate peroxidase.

Keywords Rice, Anther, Shotgun proteomics

INTRODUCTION

Rice is an important crop because more than three billion people in Asia consume it. It is a model plant for genomics due to its small genome size. Its genome was revealed in the early stage of genomic research because of importance as a food and model crop. Rice is a staple diet for over half of the people in the world and its consumption in 2014/2015 is over 484,592 thousand metric tons (http://www.statista.com/statistics/255977/total-global-rice-consumption).

Unlike structural genomic studies where genomic information in all of cell for a certain individual plant is identical, proteomic information is dynamically different among cells of various tissues, environments, and growth stages. Thus, continued proteome identification is required to construct a rice proteome database.

In rice proteome studies, mainly leaves were analyzed. In general, rice leaves are the main tissue responding to abiotic and biotic stresses (Lee et al. 2007a; Zhao et al. 2005). Even though the half of rice plant is root, proteome studies for roots have not intensively conducted compared to those in leaves (Mirzaei et al. 2012; Yan et al. 2005; Yang et al. 2007b). This is due to difficulties in studying roots.

Some of rice grain proteome studies were conducted as an important part of seeds (Yang et al. 2007a). Pollen is a male gamete which is highly sensitive to the environment (Anwar et al. 2012; Julia and Dingkuhn 2013), such as cold (Satake and Hayase 1974) and hot (Jagadish et al. 2007; Prasad et al. 2006) climates. After one or two days of heat treatment at the flowering stage, the ability of pollen grains for be germinating were decreased due to inhibition of thecae dehiscence (Matsui et al. 2000). Less than 50% of pollen grains germinated on their stigmas under heat stress conditions (Mackill et al. 1982; Matsui et al. 2001). Rice anther under the cold stress results in 34.2% empty grains (Oliver et al. 2005). As a male gamete, pollen is important for the spikelet fertility, which eventually is associated with crop yield.

Since the physical size of pollen is so small, protein
extraction from the pollen is impossible and anthers were analyzed instead. The anther is located on a stamen. It is composed of archespores, which are surrounded by tapetum. The archesporium turn into a pollen sac. In a pollen sac, there are pollen mother cells, which transform into pollen after passing through two rounds of meiosis. Because of the difficulty in collecting rice anther and extracting proteins for proteomic analysis, limited numbers of rice anther proteome studies are reported.

The 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Mahmud et al. 2012; O’Farrell 1975) is a technique widely used in proteomic analysis (S. Komatsu 1993) because of its simplicity and fast results. Currently, an advanced proteomics system has emerged, called the ‘shotgun proteomic method’ (Haynes and Roberts 2007). The shotgun proteomic method is also called multidimensional protein identification technology (MudPIT). The benefits of this method is that it can analyze large-scale proteins for identification and it is high-throughput (Agrawal et al. 2009). This method can be used complementary to 2D-PAGE and can get results in a faster time-frame (Lee and Cooper 2006). For these reasons, MudPIT is of interest.

Previous proteome research for rice pollen focuses on the stress response (Imin et al. 2004; Nijat Imin 2006) using 2D-PAGE to reveal differentially expressed proteins. However, the identification of proteins was limited to differentially expressed proteins. In this work, we conducted shotgun proteomics analysis using rice anthers to identify the rice anther proteome.

MATERIALS AND METHODS

Plant materials

The seeds of rice, *Oryza sativa* L. Nipponbare were germinated and grown in 15 × 30 cm rows at the Seoul National University experimental field, Suwon, Republic of Korea. Rice anthers in the flowering stage were collected. To unify the developing stages of the collected anthers, anthers were collected from only one panicle and the upper part of the spikelet, where the anther exerts from the spikelet, was not used. Only 3 cm width anthers inside in the spikelet located in the middle of the panicle were collected.

Protein extraction

The samples were bottled into 1.5 ml Eppendorf tubes with 2-3 of small ball bearings. The tubes were stored in liquid nitrogen then ground to break the cell wall. Next, the proteins were extracted by adding extraction buffer (8 M Urea/5 mM DTT/1% LDS/100 mM Tris pH 8.5) to the ground powder. The suspension was incubated at room temperature for 30 minutes followed by centrifugation at 14,000 g for 15 minutes. The protein concentration of the supernatant was measured. The extracted proteins were precipitated overnight with 20% (v/v) trichloroacetic acid (TCA), washed several times with cold acetone until chlorophyll or other pigments removed. Finally, the extracted proteins were resolubilized in 8 M Urea/Tris-HCl pH 8.5. Protein concentration was assayed by the 2D-Protein Quant Kit (GE Healthcare, Piscataway, NJ, USA) with the method by Lee et al. (2007b).

1D LDS-PAGE and In-gel Digestion by Trypsin

Five-hundred μg of protein from pollen powder was prepared with NuPage® LDS Sample Buffer (4X)/NuPAGE® Reducing Agent (10X)/Deionized Water (Invitrogen, Carlsbad, CA, USA). The proteins were loaded into 4-12% NuPAGE® Novex Bis-Tris Mini Gels (Life Technologies, Invitrogen, Carlsbad, CA, USA), then separated by electrophoresis. After electrophoresis, the gels were fixed in 100 mL of fixing solution (Deionized Water 40 ml/Methanol 50 ml/Acetic Acid 10 ml) for 10 minutes and the fixing solution drained. To stain the protein bands, staining solution (Deionized Water 55 ml/Methanol 20 ml/Stainer A 20 ml, NOVEX® Colloidal Blue Stain Kit (Invitrogen)/Stainer B 5 ml, NOVEX® Colloidal Blue Stain Kit (Invitrogen)) was used.

Each lane of stained protein bands was sliced into seven equal pieces. Each piece was further divided into smaller regular hexahedral cubes (approximate size 1 mm³) and gathered into a 1.5 ml Eppendorf® tube. In each tube, destaining buffer (50% acetonitrile (ACN) in 50 mM ammonium bicarbonate (ABC) pH 7.8) was added to remove the Coomassie blue stain. The tube contents were dehydrated in a SpeedVac, then reduced with reduction
buffer (10 mM DTT in 25 mM NH₄HCO₃) for 45 minutes, at 56°C and alkylated for 30 minutes with alkylation buffer (55 mM iodoacetamide in 25 mM NH₄HCO₃) at room temperature in a dark environment. The gels pieces were dried by SpeedVac and digested with trypsin (trypsin 12.5 ng/μl in 50 mM NH₄HCO₃). Finally, the gel fragments in digestion solution were incubated at 36°C overnight. Tryptic peptides were harvested from the gel using harvest buffer (5% formic acid in 50% acetonitrile). Following vortex, the solution was spun down for few seconds and kept at room temperature for 20 minutes. Supernatants were dehydrated by the SpeedVac, and the samples were desalted by Pierce® C₁₈ spin columns (Thermo Scientific, Rockford, IL, USA). Each sample was then ready to analyze with LC MS/MS.

**LC MS/MS Analysis with Q Exactive**

A Nanoflow HPLC instrument (Easy nLC, Thermo Fisher Scientific, San Jose, CA, USA) was connected on-line to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The columns for analysis (12 cm, 75 μm inner diameter) were packed in-house with Alltima C₁₈-AQ 5 μm resin. There are two buffers (0.1% formic acid, Buffer A and acetonitrile in 0.1% formic acid, buffer B) for reverse phase chromatography. The samples were separated with a binary buffer system in a linear gradient of 3-50% buffer B at a flow rate of 270 nL/min. The total run time for the LC MS/MS is 120 minutes.

MS data was acquired using a data-dependent top 8 method, which dynamically chooses the most abundant precursor ions among the survey scan (300-2,000 Da) for higher-energy collisional dissociation (HCD) fragmentation. Dynamic exclusion duration was 15s HCD fragmentation. Survey scans were acquired at a resolution of 70,000 m/z 200 and resolution for HCD spectra was set to 17,500 m/z 200.

**Bioinformatics Analysis of Proteomic Data**

The whole rice genomic protein MW and pI value were calculated by EMBoss Pepstats < Sequence Statistics < EMBL-EBI (http://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/) that using EMBoss Pepstats http://emboss.bioinformatics.nl/cgi-bin/emboss/pepstats) algorithm. Pantherdb (http://pantherdb.org) categorized gene ontology (GO) categories. The GO of the identified proteins were retrieved from the TIGR Rice Pseudomolecule protein database Release V7.0. GO singular enrichment analyses were performed in agriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) (Du et al. 2010).
RESULTS AND DISCUSSION

Identified proteins in rice anther

Rice anther proteome were identified with three biological replications. A total of 3,198 non-redundant proteins were identified by merging all three replications’ MudPIT runs. The number of each identified protein replications were 2,645 (Replication 1), 2,662 (Replication 2), 2,702 (Replication 3). All 3,198 proteins were not identified through the three replications reproducibly. This resulted from the analytical incompleteness phenomenon, which happens when identifying highly complex mixtures of peptides. A single analytical run may only recognize a fraction of associated peptides when analyzing a highly complex peptide mixture (Wilkins et al. 2006). Since proteins exist in a cell, in huge dynamic ranges, and the physiochemical properties of the proteins vary, a single analysis of proteome identification is not enough to reveal the entire proteome. In this study, ~2,600 proteins were identified from a single MudPIT run. 2D-PAGE analysis generally resolves ~1,000 protein spots in a single 2D-gel, whereas a single MudPIT run showed a higher resolving power.

The physiochemical properties of the 3,198 identified proteins were analyzed. The proportion of the distribution of pI values and MWs for the 3,198 proteins were compared to those of entire rice genome (Fig. 1). The lowest pI value was pH 3.93 (LOC_Os07g41694.1) and the highest was pH 12.48 (LOC_Os01g69020.1). The overall distribution of the pI value of the identified proteins was similar to that of entire rice genome even though the identification of the proteins ranging of pH 5-6 and pH 6-7 are slightly higher (Fig. 1A). In the plant proteome study with 2D-PAGE, proteins were generally resolved in the pI value range of pH 4 to 7.

We identified a wide distribution of acidic and basic
proteins; of note, ~40% of the proteins were basic proteins, implying that the protein deification was unbiased by pI value. The MWs of the identified proteins from pollen ranged from 5.2 kDa (LOC_Os02g27769.1) to 486.0 kDa (LOC_Os09g07300.1). The distribution of the MW was quite similar with entire rice genome (Fig. 1B). Proteins having less than 20 kDa were less likely to be identified. This may be due to MudPIT analysis relying on peptide detection. Peptides originate from proteins and proteins having a low MW can produce a small number of peptides decreasing chances of mass spectrometry detection in comparison to high MW proteins.

Gene ontology (GO) category analysis
To represent the overall trends of the existing proteins mature anthers, the GO categories of the identified 3,198 proteins were analyzed. The identified proteins were categorized into 22 groups by their protein class (Fig. 2). There were four categories consisting over 46.5% of GO. The category of hydrolase (12.8%) and transferase (12.8%) were the highest, followed by the categories of oxidoreductase (10.7%) and nucleic acid binding proteins (10.2%).

GO enrichment analysis was conducted with total 3,198 rice anther proteins to reveal that 16 GO terms of biological processes (Fig. 3a), five GO terms of molecular functions (Fig. 3b), and six GO terms of cellular components were enriched in rice anther compared to the proportion of those proteins in the whole rice genome (Fig. 3c).

Proteins associated with biological processes, specifically, proteins associated with cellular and metabolic processes were present in high proportions. Concerning molecular function, the proteins associated with catalytic activity were enriched. In relation to cellular components, proteins associated with the cell, cell part and organelle were enriched. These results imply that the mature anthers were preparing for cell nucleus division, pollen tube germination and elongation. The proteins for catalytic activity imply that starch in pollen is a material that supports pollen germination and pollen tube elongation. Possibly, proteins associated with metabolism utilize stored starch in pollen as energy/carbon skeleton-building material (Dai et al. 2006).
Fig. 3. Enriched GO terms of rice anther proteins: (a): biological processes, (b): molecular functions and (c): cellular components.
During pollen tube germination and pollen tube elongation, cell wall and cellular organelle synthesis is required. Thus, the enriched proteins associated with cell, cell parts, and organelles suggest that those proteins exist in mature pollen for pollen germination and pollen tube elongation (Drøbak et al. 2004; Suen et al. 2003). This rice anther proteome database was constructed from rice anthers in natural field conditions, thus the enrichment of the proteins associated with response to stimulus were unexpected. Generally, the roles of those proteins are tolerant of environmental stressors. Pollen is very vulnerable to the environment, such as high temperatures (Jagadish et al. 2007; Prasad et al. 2006), Thus enrichment of pollens and mature pollens represent preparation to respond to an unfavorable environment (Dai et al. 2006; Dietz 2003; Foyer and Noctor 2005; Sheoran et al. 2007).

**Table 1.** The 20 most abundant proteins in the rice anther genome.

| Accession     | Description                                                                 | AAs | MW [kDa] | calc. pI |
|---------------|-----------------------------------------------------------------------------|-----|----------|----------|
| LOC_Os09g23999.1 protein | pollen allergen Cyn d 23, putative, expressed                              | 134 | 14.0    | 8.07     |
| LOC_Os09g23899.1 protein | pollen allergen Cyn d 23, putative, expressed                              | 134 | 14.0    | 6.76     |
| LOC_Os04g26220.1 protein | pollen allergen, putative, expressed                                      | 117 | 12.4    | 6.06     |
| LOC_Os05g02520.1 protein | cupin domain containing protein, expressed                               | 359 | 38.2    | 6.16     |
| LOC_Os06g45180.1 protein | pollen allergen, putative, expressed                                      | 117 | 12.3    | 6.68     |
| LOC_Os01g74480.1 protein | cupin domain containing protein, expressed                               | 377 | 40.2    | 5.94     |
| LOC_Os04g26230.1 protein | pollen allergen, putative, expressed                                      | 117 | 12.4    | 5.48     |
| LOC_Os08g12520.1 protein | expressed protein                                                          | 226 | 24.8    | 5.69     |
| LOC_Os04g25150.1 protein | pollen allergen, putative, expressed                                      | 117 | 12.4    | 5.48     |
| LOC_Os04g40950.1 protein | glyceraldehyde-3-phosphate dehydrogenase, putative, expressed             | 337 | 36.7    | 6.81     |
| LOC_Os05g47980.1 protein | ATP synthase, putative, expressed                                          | 552 | 58.9    | 6.37     |
| LOC_Os01g49190.1 protein | ATP synthase, putative, expressed                                          | 557 | 59.4    | 6.55     |
| LOC_Os08g03290.1 protein | glyceraldehyde-3-phosphate dehydrogenase, putative, expressed             | 337 | 36.4    | 7.11     |
| LOC_Os02g38920.1 protein | glyceraldehyde-3-phosphate dehydrogenase, putative, expressed             | 356 | 39.0    | 6.95     |
| LOC_Os04g25190.1 protein | pollen allergen, putative, expressed                                      | 117 | 12.3    | 8.10     |
| LOC_Os03g17690.1 protein | OsAPx1 - Cytosolic Ascorbate Peroxidase encoding gene 1-8, expressed     | 250 | 27.1    | 5.66     |
| LOC_Os03g50885.1 protein | actin, putative, expressed                                                | 377 | 41.8    | 5.49     |
| LOC_Os07g49400.2 protein | OsAPx2 - Cytosolic Ascorbate Peroxidase encoding gene 4,5,6,8, expressed   | 251 | 27.1    | 5.36     |
| LOC_Os03g50290.1 protein | 14-3-3 protein, putative, expressed                                      | 260 | 29.2    | 4.88     |
| LOC_Os10g08550.1 protein | enolase, putative, expressed                                              | 480 | 51.6    | 5.99     |

*AAs is the number of amino acids*
With the relative protein amount for all of the identified proteins, 20 highly expressed proteins in rice anther are listed in Table 1. For general rice leaf proteome studies, the highly abundant proteins are RuBisCO proteins. In rice anther proteins, we detected several pollen allergens and they were present in high amounts. It is known that pollen allergen proteins are allergenic to humans, however in plants, pollen allergen proteins are present in high amounts suggesting that these proteins have physiological roles required in pollen germination and growth (Songnuan 2013).

In addition, ATP synthase and glyceraldehyde-3-phosphate dehydrogenase were present in high amounts, suggesting that in mature pollen proteins that supply energy for pollen germination and tube elongation are present in high amounts. Interestingly, two cupin domain-containing proteins were identified in high amounts. Cupins are expressed in the early phase of wheat embryo germination. This study suggests the possible role of the cupins in rice pollen germination. As discussed in the previous section, stress resistant protein, ascorbate peroxidase was present in high amounts. Ascorbate peroxidase is an enzyme that detoxifies peroxides, such as hydrogen peroxide. A previous tomato pollen proteome study reported the role of ascorbate peroxide in detoxification (Sheoran et al. 2007). One protein of unknown function (LOC_Os08g12520) was present in high amounts. In the BLSAT search, all of the homologue proteins are uncharacterized. Based on the high amount of this protein in rice anther, it may have a role in pollen germination and tube elongation; however, further study of this protein is required to reveal its role in rice pollen.

CONCLUSION

In summary, with high-throughput proteomic analysis method of shotgun proteomics, we could identified total 3,198 non-redundant rice anther proteins. There was no bias of physiochemical properties in identified proteins showing wide pI value range and molecular weight. Gene ontolgy enrichment analysis revealed that proteins associated with cellular and metabolic processes, catalytic activity, cell, cell parts, and organelles were enriched in rice anther, suggesting the status of proteins in rice anther were associated with pollen germination and pollen tube elongation. With estimated relative amount of the total identified proteins, we could detect highly abundant proteins in rice anther. There were pollen allergens, ATP synthase, glyceraldehyde-3-phosphate dehydrogenase, cupin domain containing proteins, and ascorbate peroxidase.

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