Development of Protein Biomarkers for the Authentication of Organic Rice

Ju-Young Lee · Jinkyu Lim*

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Abstract  The rice protein profiles of Oryza sativa L (Koshihikari) grown under organic and conventional cultivation regimes were compared on 2-D gels to develop diagnostic marker proteins for organic rice. The selected proteins, differentially expressed between organic and conventional rice, were compared with the differentially expressed proteins of another organic and conventional rice pairing, produced at a different location. In the first comparison among conventional, no-chemical, and organic rice grown in the same region, Korea, 13 proteins exhibiting differential expression in organic and conventionally grown plants were selected. Eight of the 13 proteins were down-regulated, and the 5 remaining proteins were up-regulated from conventional to organic rice. The second comparison pairing from Kyungju, revealed 12 differentially expressed proteins, with 8 down-regulated and 4 up-regulated proteins. Ten of the differentially expressed proteins that overlapped between the two comparison sets could not be clustered into any functional group using a functional annotation clustering tool. Further comparisons using another set of conventional and organic rice, belonging to a different variety of Oryza sativa L and produced in Sanchung, revealed 8 differentially expressed proteins, 5 of which were down-regulated and 3 of which were up-regulated in the organic rice. Overall, 3 differentially expressed proteins were commonly found in all three organic rice crops. These 3 proteins, along with other overlapping differentially expressed proteins, can provide a good starting point for the development of signature proteins that can be used for the authentication of organic rice with a follow-up studies with more comparison sets.

Keywords  2D gel · authentication · conventional · differential expression · endosperm · organic · Oryza sativa L · rice soluble protein

Introduction

According to the United States Department of Agriculture (USDA) definition, organic food is made with more than 95% certified organic ingredients, and using organic methods (USDA, 2015). The organic food market in Korea grew at an average rate of 50% from 2006 to 2011, and is expected to expand to $6 billion by 2020 (Organic Trade Association, 2015). Consumers are becoming more and more interested in organic agricultural products, mainly because of food safety and nutritional quality concerns; however, there are no data that support the public opinion that organic food is of a higher nutritional quality than conventionally produced food (Bourn and Prescott, 2002). In addition, consumers of organic produce contribute to the preservation of natural resources and biodiversity, and to the support of animal health and welfare, as they favor these values, regardless of their association with higher food prices. Thus, farmers attempt to enter the organic farming industry, attracted by the prospect of selling their products at premium prices and in increasingly expansive markets, in spite of the industry’s strict standards and regulations and the lower yields seen in the production of organic agriculture products. However, the genuine efforts made by organic rice producers to preserve the environment and provide healthy food to consumers can easily be discouraged by the adulteration of organic rice with conventional rice, and the use of falsified labels. Conventional produce falsely labeled as organic has also discouraged consumers from purchasing organically grown products. For this reason, differentiation and certification methods developed for the authentication of organic products can promote and improve organic farming.

There have been many attempts to develop methods for identifying organic produce: developing “diagnostic” genes from
Materials and Methods

Sample collection and protein preparation. Rice sample collection: The rice samples used in this study were collected from three different organic rice farms located in Kyungju, Yeouj, and Sanchung, which are located in the south-eastern, middle, and south-western regions of Korea. Three commercial Ministry of Agriculture, Food and Rural Affairs (MAFRA), Korea certified organic and conventional rice samples (cultivar Koshihikari) were purchased directly from the producers.

Identification of proteins via peptide mass fingerprinting. Using automatic spot detection and matching and semiautomatic spot editing, differentially expressed proteins exhibiting greater than twofold change in abundance in at least one gel were considered differentially expressed. Identification of proteins via peptide mass fingerprinting.
than two fold differences in expression between conventionally and organically grown plants were selected and manually excised from gels using scalpels. The proteins in the gel pieces were de-stained and trypsinized as described by Lee et al. (2012). The resulting peptides were extracted, dried, and dissolved in a mixture of deionized water:acetonitrile:trifluoroacetic acid (TFA) (93:5:2). A 1:1 mixture of peptides and a matrix solution (10% α-cyano-4-hydroxycinnamic acid, 50% methanol, 0.1% TFA) spiked with internal standards (bradykinin, m/z=904.4681 and angiotensin 1, m/z=1296.6853) was spotted and crystalized on a MALDI plate. The mass values of the peptides at each protein spot were measured using a MALDI-Time-of-Flight (TOF) MS (Voyager-DE STR, PerSeptive Biosystems, USA) with a range of 850–3000 Da. Proteins were identified by searching their mass values against NCBI and Swiss-Prot public databases using the search engines MS-Fit (http://prospector.ucsf.edu/prospector/mshome.htm) and Mascot (Matrix Science, UK) for confirmations, respectively. Information about the proteins’ functions was obtained by searching databases with the accession numbers of the proteins using UniProt (http://www.uniprot.org/uniprot/). The functional groupings of the selected proteins were analyzed using the DAVID Annotation Clustering Tool ver. 6.7 (Huang and Lempicki, 2009a; 2009b) (http://david.abcc.ncifcrf.gov/).

**Results and Discussion**

**Agricultural regime and rice protein profile changes.** Assayed by micro-Kjeldahl analysis (Miller and Houghton, 1945), the total protein content of the samples from Yeoju, Kyungju, and Sanchung were determined to be 6.21, 6.01, and 6.38% for organic rice, and 6.34, 6.22, and 6.75% for conventional rice, respectively. The water soluble protein content of the samples was determined via the Bradford method (Bradford, 1976), and found to make up approximately 40% of the total protein content. These results suggest that agricultural regimes, including nitrogen input, soil, and temperature changes affect the expression levels of proteins in the endosperm of rice, lowering the protein content of organic rice compared to that of conventional rice (Zorb et al., 2009). However, although the total protein content of conventional rice was higher than that of organic rice, the overall protein profiles as visualized on the 2-D gels were not significantly different (Fig. 1, 3). Thus, the gel image sets used for the selection of organic rice-specific protein markers were comparable. This phenomenon was also observed in rice grown under two different conventional regimes which included different temperature fluctuations and N inputs during the cultivation period (Lee et al., 2012).

The representative protein profiles of rice grown under different regimes (conventional, no-chemical, and organic farming) from Yeoju, revealed approximately 450 proteins on the 2D gels (Fig. 1). Functional analysis via peptide mass-fingerprinting using MALDI-TOF MS identified 111 proteins out of the 450 protein spots visible on the gels (Fig. 2 and Supplementary Table 1). The differentially expressed proteins were labeled using an alphabetical initial denoting the region in which the samples were grown was assigned, along with a number to allow differentiation among spots in samples from each region (Fig. 2). For example, protein spot number 19 from Yeoju rice was designated Y19, while Kyungju rice spot number 19 was labeled K19, etc. Prior to the comparison of the individual proteins with those on the other 2-D gels, the loading amount of the proteins on each gel should be normalized. Thus, in addition to adjusting the spot intensities by calibrating gaining functions, the spot intensities of the proteins were normalized by adjusting the intensity of spot #73, which is consistently expressed in similar amount in all samples, using the image-analysis software PDQuest. The functions of the differentially expressed proteins were annotated by searching databases using the search engines, Uniprot (www.uniprot.org) and DAVID (see Materials and Methods) (Supplementary Table 1). Among a set of proteins whose expression levels differed between conventional

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**Fig. 1** Comparison of rice proteins from conventional, no chemical, and organic rice on 2-D gels. Proteins extracted from rice from three different cultivation regimes (conventional, no chemical, and organic) in the Yeoju area were separated on 2-D gels. The spot intensities on different gels were normalized via the equalization of the intensity of protein spot #73 on each gel, using the image-analysis software program PDQuest. Differentially expressed proteins, either down-regulated or up-regulated in organic rice, were selected using painstaking image analysis comparison procedures and marked with arrows and numbers preceded with a Y to designate the samples’ cultivation area.
and organic samples from Yeoju by a factor of >2, 8 proteins underwent a gradual decrease in expression from that observed in conventionally grown rice, to that of no-chemical rice, and finally to that of organic rice. Conversely, the differential expression of 5 proteins increased from conventionally grown rice, to organic rice (Fig. 1, 4A). The group of proteins whose expression levels decreased from conventional to organic rice is comprised of Poly [ADP-ribose] polymerase 1, Protein disulfide isomerase-like 1-1, cellulose synthase A catalytic subunit 9, laccase 21, ketol-acid reductoisomerase, malate dehydrogenase, 60S acidic ribosomal protein, and seed allergen protein. The group of proteins whose expression levels increased is made up of a B3 domain-containing DNA binding protein, potassium channel AKT1, a xylanase inhibitor, probable cellulose synthase A subunit 3, and 1-cys peroxiredoxin A (Table 1).

Table 1 List of differentially expressed proteins selected via comparisons of organic and conventional rice

| Spot # | MOWSE Score | % Cov Accession Number | Protein Name | Protein Function |
|--------|-------------|------------------------|--------------|-----------------|
| 4      | 66901       | 12.8 Q7EYV7            | Poly [ADP-ribose] polymerase | DNA repair |
| 5      | 5225        | 9.7 Q7EYV7             | Poly [ADP-ribose] polymerase | DNA repair |
| 8      | 11927       | 7.1 Q7Y001             | PINHEAD      | translational regulation miRNA binding |
| 13     | 124130      | 12.2 A2ZIC8            | Cellulose synthase A catalytic subunit | cell wall biosynthesis |
| 19     | 81254       | 13.4 Q2QZ0             | Laccase-21   | lignin degradation |
| 22     | 4.15E+13    | 46.5 Q5QLQ0            | Protein disulfide isomerase-like 1-1 | protein folding |
| 26     | 2.39E+11    | 26.8 Q65XK0            | Ketal-acid reductoisomerase | amino acid metabolism |
| 31     | 2.86E+15    | 52 Q42971              | Enolase      | glycolysis |
| 45     | 2.22E+07    | 35.2 Q7XDC8            | Malate dehydrogenase, cytoplasmic | metabolism |
| 47     | 38289       | 21.3 P41095            | 60S acidic ribosomal protein P0 | translation |
| 51     | 26521       | 17.7 Q5JNA1            | B3 domain-containing protein | DNA repair |
| 58     | 3.90E+08    | 41.2 Q948T6            | Lactoylglutathione lyase | conversion of hemimercaptal, S-lactoylglutathione |
| 59     | 4488        | 21 Q948T6              | Lactoylglutathione lyase | conversion of hemimercaptal, S-lactoylglutathione |
| 63     | 23082       | 8.4 Q0JKV1             | Potassium channel AKT1 | ion transport |
| 70     | 1.66E+08    | 49.7 Q53NL5            | Xylanase inhibitor protein | defense against secreted fungal pathogen xylanases |
| 80     | 3406        | 11.7 Q10MH8            | DEAD-box ATP-dependent RNA helicase | hydrolylase |
| 88     | 2.32E+05    | 43.6 Q69V23            | Cellulose synthase A catalytic subunit | cell wall biosynthesis |
| 92     | 393150      | 25.8 P29835            | 19 kDa globulin | storage |
| 93     | 3.10E+06    | 43.6 P0C5C9            | 1-Cys peroxiredoxin A | antioxidant protein |
| 94     | 19945       | 25.8 P29835            | 19 kDa globulin | storage |
| 102    | 5747        | 24.7 Q01882            | Seed allergenic protein RAG2 | allergen |
| 110    | 42908       | 25 Q0JMY1              | Poly synthetase 2-B | DNA repair |
of the 8 down-regulated proteins are as follows: poly [ADP-ribose] polymerase, cellulose synthase A catalytic subunit, lactoyl-glutathione lyase, DEAD-box ATP-dependent RNA helicase, probable cellulose synthase A catalytic subunit, 1-Cys peroxiredoxin A, and poly [ADP-ribose] polymerase 2-B. The functions of the 4 up-regulated proteins are as follows: protein argonaute, protein disulfide isomerase-like 1-1, B3 domain-containing protein, and potassium channel AKT1. There are 7 overlapping differentially expressed proteins between the Yeoju and Kyungju rice comparison sets. Two of the overlapping proteins down-regulated in organic rice are #4, a poly [ADP-ribose] polymerase, which is responsible for base excision repair, and #13, a cellulose synthase A catalytic subunit, which is involved in cell wall synthesis or non-cellulosic polysaccharide synthesis. Two overlapping differentially expressed proteins that are up-regulated in organic rice are #51, a B3 domain-containing protein, and #63, a potassium channel AKT1. These proteins are involved in transcription regulation and ion transport, respectively. The other two inversely overlapping proteins, #88, cellulose synthethase A, and #93, 1-cys peroxiredoxin A, which are active in non-cellulosic polysaccharide synthesis and growth regulation during anti-oxidative stress, respectively, are down-regulated in organic rice grown in Kyungju. However, in the Yeoju comparison set, these proteins are up-regulated in organic rice. Conversely, Y22 and K23, which are protein disulfide isomerase-like 1-1, are down-regulated in organic rice grown in Yeoju, but up-regulated in Kyungju organic rice. As mature grain is the final product of cultivation, which is influenced by multiple environmental factors, cis- and/or trans-acting elements can reciprocally change the expression level of specific genes (Guo et al., 2004). Thus, it is possible that the expression levels of Y88 and Y93 in organic rice are down-regulated, while Y23 is up-regulated compared to expression levels observed in conventional rice. However, in organic Kyungju rice, the expression patterns of K88, K93, and K22 is the opposite of those observed in rice from Yeoju (Fig. 4A, B). The seven overlaps between 12 and 13 differentially expressed proteins found in rice grown under different cultivation regimes strongly support our attempts to identify proteins suitable for use in identifying organically produced rice. In addition, we investigated whether the selected differentially expressed proteins from Yeoju and Kyungju rice were also differentially expressed in a different variety of Oryza sativa L., produced under a third cultivation regime.
Differentially expressed proteins from a different cultivar of *Oryza sativa* L. cultivated under different regimes. To evaluate the overlapping proteins from the two different cultivation regimes, at Yeoju and Kyungju, for the suitability of their use in the authentication of organic rice, proteins from a different variety of *Oryza sativa* L., cultivated under organic and conventional regimes in Sanchung, (200 km south from Yeoju and 120 km west from Kyungju), were compared on 2-D gels. Eight proteins were found to exhibit more than 2-fold difference in expression between the conventional and organic cultivation conditions. These proteins were then selected and identified in Sanchung rice, and compared to the differentially expressed proteins from the Yeoju and Kyungju comparison sets. Four of the 8 differentially expressed proteins from Sanchung organic rice overlapped with the differentially expressed proteins found in Kyungju rice. In organic rice, S57, S88, and S93 are down-regulated, while S51 is up-regulated. Meanwhile, 4 overlapping differentially expressed proteins from Yeoju and Sanchung rice are down-regulated at #26 and up-regulated at #51, and inversely regulated at #88 and #93 (i.e., up-regulated in Yeoju but down-regulated in Sanchung organic rice).

Overall, there are 3 commonly overlapping proteins found in all three comparison sets: #51, #88, and #93, a B3 domain-containing protein, cellulose synthase A catalytic subunit 3, and 1-cys peroxiredoxin A, respectively. Plus, S26, a Ketal-acid reductoisomerase, overlaps with Y26 while S57 (lactoylglutathione lyase) overlaps with K58. According to the functional analysis, these proteins seem to be differentially expressed during the early stages of endosperm formation, because only actively dividing cells require cell wall synthesis, DNA repair, amino acid synthesis, and anti-oxidation. This suggests that although differential protein expression is influenced by multi-factorial inputs during the low-temperature stress, *Proteomics* 11, 2839–50.

Gao B, Lu Y, Sheng Y, Chen P, and Yu LL (2013) Differentiating organic and conventional rice using metabolite analysis via gas chromatography-tandem MS (Wang et al., 2013b) or even an electronic nose (Peng et al., 2015) can also complement each other and together confirm the suitability of the selected diagnostic markers.

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