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A Proteomic Approach to Analyze Rice Bran and Shoots of Kao Dawk Mali 105 and its Mutants

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ABSTRACT

Proteomic analysis was used to investigate bran proteins from wild-type *Oryza sativa L.* variety KDML105 and its mutant RD15. Fractionation of bran proteins by sequential solubilization showed about 1,000 total spots in 2-D gel. The 2-D gels revealed the similarity in protein patterns between KDML105 and RD15 because most expressed proteins in bran were house-keeping proteins. Consequently, rice shoots were investigated using etiolated shoots and green shoots of KDML105 and its two mutants, namely RD15 and RD6. Rice shoot proteins were extracted by 10% TCA in acetone. 2-D gels presented a greater number of etiolated proteins than green shoot proteins in all 3 varieties. Due to low expression of RuBisCO enzyme in etiolate, some proteins appeared in higher intensities in etiolated samples than in green shoot samples. As a consequence the proteins were excised for MS analysis. However, most of the different proteins among these three varieties appeared in low intensities. The result of this study revealed that KDML105, RD15 and RD6 expressed different types of salt-stress induced proteins.

Key words: proteomic, *Orysa sativa L.* ssp. *indica*, Kao Dawk Mali 105

INTRODUCTION

The phenotypic appearance is controlled by many proteins and genes. One way of getting insight into this complex biological system is to focus on the network of gene products, which can be accomplished by proteome analyses [Roberts, 2002]. Proteomic analysis is a powerful tool that can be used both to visualize and compare complex mixtures of protein and to gain a large amount of information about the individual proteins involved in specific biological responses. Proteome analysis, in general, is performed by (i) separation of proteins by 2D-PAGE, (ii) determination of peptide mass fingerprints/amino acid sequences by mass spectrometry, (iii) identification of protein/protein homologs using databases, and (iv) characterization of proteins of unknown function by amount, localization, structure, post-translational modification and enzyme activity (Komatsu *et al*., 2003; Rakwal and Agrawal, 2003). Plant proteins extracted from embryos (Woo *et al*., 2002), leaves (Wilson *et al*., 2002), bran (Trisiriroj *et al*., 2004), roots (Mang *et al*., 2004) and etiolated and green shoots (Komatsu *et al*., 1999) were previously separated and analyzed by 2D-PAGE.

Kao Dawk Mali 105 (KDML105) is the famous aromatic rice of Thailand and RD15 and RD6 are mutant rice derived from KDML105 by gamma-ray mutation. The relation of their physiological and protein profiles will provide valuable information for further functional genomic

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studying. In this study, the protein patterns of rice bran and shoots from KDML105 RD15 and RD6 were compared and the different proteins encoded by expressional genes in each tissue were identified. In addition, the global protein complements expressed under the consequence of the mutation could be investigated.

MATERIAL AND METHODS

1. Plant materials

1.1 Rice bran

Rice seeds of KDML105, RD15 and RD6 were obtained from Prachinburi Rice Research Center. Seeds were milled by laboratory-scale milling machine and seed bran was stored immediately at -80°C before use.

1.2 Rice shoot

Three cultivars of Thai rice (*Oryza sativa L*. ssp. *indica*) were used in this research: Kao Dawk Mali 105 (KDML105), RD15 and RD6. All of them were harvested from Sakolnakorn province (northeastern of Thailand) and were broken of their dormancy by incubation at 50°C for 3 days. Seeds of KDML 105, RD 15 and RD 6 were soaked in sterile tap water overnight and kept in damp cotton wool for 10 days. For etiolate, the rice seeds were planted in a dark room. After 10 days, both green shoots and etiolates were harvested and kept at –80°C before use.

2. Two dimensional electrophoresis (2-DE)

2.1 Preparation of bran proteins

Seed bran from each variety was ground in liquid nitrogen and proteins were precipitated by 10% trichloroacetic acid (TCA) in acetone (Damerval *et al*., 1986) and lyophilized. Lyophilized proteins were fractionate solubilized in 2 lysis buffers (Jacobs *et al*., 2001). One mg of lyophilized powder proteins was first solubilized in lysis buffer A, consisting of 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 65 mM dithioerythritol (DTE). The supernatant A was kept for running 2D-PAGE by centrifugation at 10,000 *g* for 10 min. The residue was re-solubilized in lysis buffer B, consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS and 65 mM DTE. The supernatant B was kept by centrifugation at 10,000 *g* for 10 min for running 2D-PAGE.

2.2 Preparation of shoot proteins

Three mg of rice shoot from each variety were ground in liquid nitrogen, and proteins were precipitated by 10% TCA in acetone and lyophilized. Lyophilized proteins were solubilized in a lysis buffer consisting of 5 M urea, 2 M thiourea, 2% CHAPS, sulfobetaine 3-10, 20 mM DTE and 5 mM Tris (2-carboxyethyl) phosphine (TCEP) (Mechin *et al*., 2003). The protein concentration in the supernatant was determined by a PlusOne protein assay kit (Amersham Biosciences).

2.3 Protein separation by 2D-PAGE

2-DE was performed three times for each sample. Approximately 500 µg of extracted rice shoot proteins in 350 µL lysis buffer was loaded into 18 cm IPG strips (Amersham Biosciences) for pH range 4-7. Strips were re-hydrated in the presence of sample solution plus 0.5% (v/v) ampholyte buffer pH 4-7 and 0.25% (v/v) ampholyte buffer pH 3-10 under constant low voltage (50 V) for 12 h. The first dimensional isoelectric focusing (IEF) was conducted at 20°C using an IPG-phore (Amersham Biosciences). It was programmed with a voltage of 100 V for 1 h, 250 V for 1 h, 1000 V for 0.5 h, 2000 V for 0.5 h, 4000 V for 1 h and 6000 V, for a total of 90 KVh. After first dimension, the strips were incubated in an equilibration buffer which contained 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) plus 65 mM DTE for 20 min, followed by incubation in the equilibration buffer plus 65 mM iodoacetamide for 20 min. The second dimension electrophoresis using PROTEAN II xi Multi-Cells (Bio-Rad Laboratories) was performed on 12.5% (w/v) linear

polyacrylamide gel at 40 mA until the buffer front line was 5 to 10 mm from the bottom of the gel. Proteins were stained with Sypro Ruby. The electrophoretic patterns of 2-D gels were compared and analyzed using PDQUEST software, version 7.0 (Bio-Rad Laboratories).

3. In-gel digestion and peptides extraction for MS analysis

Spots of interest were excised and de-stained by washing twice with 250 μL of acetonitrile/50 mM ammonium bicarbonate at the ratio of 1:1 (v/v) for 15 min. The gels were dried using a centrifugal vacuum concentrator. A reduction and alkylation process for cysteine residues was performed on samples using DTE and iodoacetamide, respectively, before adding trypsin solution. For tryptic digestion, the dried gel was rehydrated in 12.5 ng/μL modified trypsin and incubated at 37° C for at least 16 h.

Peptide mass spectra were acquired using a MALDI-Q-TOF mass spectrometer (Micromass, UK) operating in a delayed extraction reflector mode. Mass spectrometry was performed using an accelerating voltage of 20 kV. Selected peptides in the mass range of 1000-3500 Da were used for database matches.

Peptide mass fingerprint data from MALDI-Q-TOF were used to match against protein candidates in NCBI and SWISS-PROT protein databases using MASCOT (http://www.matrixscience.com). Search parameters were allowed for oxidation of methionine, carbamidomethylation of cysteine, one misscleaved site and peptide mass tolerance of 0.15 Da and MS/MS tolerance of 0.25 Da.

RESULTS AND DISCUSSION

The purpose of this work was not to identify the proteins derived from the mutated genes, but to distinguish between protein patterns of wild-type KDML105 and mutant varieties RD15 and RD6. This study provided information for further analysis of the functional genomics of rice. Different proteins expressed in bran, and in etiolated and green shoots were investigated.

Analysis of 2-D protein patterns of bran proteins of wild-type KDML 105 and mutant RD15

Bran proteins of KDML105 and RD15 were sequentially solubilized in both lysis buffers A and B. Both proteins in lysis A and B were separated by 2D-PAGE at pH range 4-7 and stained by Sypro Ruby (Figure 1). 2-D gels were analyzed by PDQUEST, which revealed similar protein patterns of KDML105 and RD15 in both lysis A and B.

Most of the different spots detected by PDQUEST were irreproducible spots. By this extraction method, it could be determined about 600 spots in lysis buffer A and 500 spots in lysis buffer B. There were about 137 spots which were common in both lysis buffers A and B. Therefore, about 1,000 spots could be detectd in rice bran by the sequential solubilization method. From the similarity of protein patterns in both KDML105 and RD15, it was likely that most of these proteins were house-keeping proteins. However, some spots to identify by MALDI-Q-TOF were chosen.

Protein identification by MALDI-Q-TOF

More than twenty spots were digested with trypsin and the resultant peptides were analyzed by peptide mass fingerprinting (Figure 2). The acquired mass spectra were searched against NCBI databases of *Oryza sativa* for protein identification (Table 2).

Table 2 shows protein identification by both MALDI-TOF and MALDI-Q-TOF. For MALDI-TOF, the proteins reported were those which matched the NCBI database with a score higher than 50, or those for which the molecular weight and pI observed of each spot was close to the calculated one. The result revealed that most of them were proteins with no known function. Some

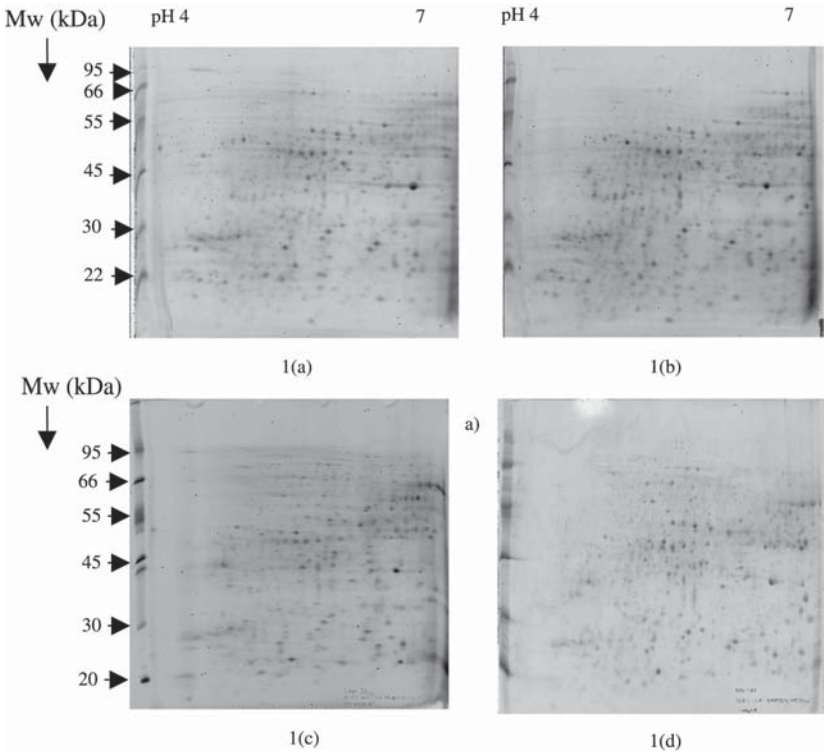

Figure 1 2-D gels of bran proteins of KDML105 and RD15 in lysis buffer A and B.
1(a) KDML105, lysis buffer A, 1(b) RD15, lysis buffer A
1(c) KDML105, lysis buffer B, 1(d) RD15, lysis buffer B

spots had no peptide peaks that matched the database, and some spots provided very low score match. These were excised and analyzed again by MALDI-Q-TOF. From MALDI-Q-TOF, three spots, 16, 19 and 20 showed low score match, and spots no. 16, 17, 19 and 20 showed low matched peaks compared with the rice protein databases.

This outcome can be attributed to two reasons: First, the protein databases do not have enough information for identification of known rice proteins. Second, it is possible that the TCA/acetone precipitate protein method may lead to poor protein identification. A similar result by Fukuda *et al*., (2003) reported that embryo proteins precipitated by TCA/acetone were difficult to identify because of low recovery of proteolytic peptide peaks and mis-matches against the NCBI database.

Figure 2 2-D gel of bran proteins of KDML105. The arrows indicate excised protein spots.

Table 2 Identification of rice bran proteins by MALDI-TOF and MALDI-Q-TOF.

Spot No.	Score	%cov	Mw/pI observe	Mw/pI calculate	Protein identification	NCBI accession
1	82	28	43000/5.6	42929/9.7	Hypothetical protein	AC018727
2	69	30	41000/5.7	43246/7.7	Unknown protein	AC091724
3	38	39	32000/6.0	31967/11.5	Hypothetical protein	AC091247
4	50	59	42000/6.2	55184/5.9	Hypothetical protein	AC79936
5	58	54	90000/5.4	174630/8.8	Putative gag-pol polyprotein	AC084831
6	132	35	90000/5.6	37438/5.9	Putative protein phosphatase	AC084406
7	37	59	46000/5.1	43002/4.8	Tubulin beta chain	D13224
8	51	80	54000/5.2	6985/4.9	P0410E01.32	BAB17111
9	41	57	58000/5.0	70205/6.0	Putative thiamin biosynthesis protein	AC084406
10	253	59	48000/4.8	35517/5.0	Putative fructokinase II	AAL26573
11	48	45	47000/5.6	29178/4.8	Putative 14-3-3 protein	AC087181
12	54	66	42000/5.5	40008/8.7	Ferredoxin-NADP reductase, leaf isozyme	P41344
13	115	42	56252/9.2	48000/8.0	Glutelin	1312296A
14	32	63	19184/6.1	11200/6.02	Hypothetical protein	BAD11618
15	14	2	55000/6.2	61450/5.3	Hypothetical protein	NP_921892
16	11	2	15000/5.5	19863/9.5	P0413G02.20	BAC07363
17	83	42	17000/6.5	18064/8.4	Prolamin	AB016505
18	344	68	15000/6.8	16900/8.8	Prolamin	D73383
19	32	12	18000/6.8	58277/8.5	Hypothetical protein	AAO17348
20	9	2	21000/5.5	36529/11.3	P0407B12.21	BAB17184

Note : Spots no. 1-14 were analyzed by MALDI-TOF and no. 15-20 by MALDI-Q-TOF.
%cov = % coverage

Detecting different proteins in etiolate and green shoots

Most proteins in the embryo and aleuronic layer were house-keeping proteins and thus had no differential expression. Therefore, the coleoptiles were selected to study the difference in expressed proteins corresponding to their different genomes. And in this study, KDML105 and its mutants, RD15 and RD6 were used as models. The purpose of using etiolate was, first, to seek the different proteins which were not parts of the photosynthetic system and second, to decrease abundant proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (RuBisCO) involved in the photosynthetic pathway. Rice shoots from 10-day planted rice were used for protein extraction. The proteins were extracted by 10% TCA/acetone and separated by 2-DE (Figure 3). The gels of each variety were replicated before matching by PDQUEST to avoid the erroneous interpretation by irreproducible spots.

RuBisCO contributes about 50% of total proteins in leaves (Schneider *et al*., 1992), so it can interfere with detection of low abundant proteins. From Figure 3a, there were no RuBisCO in etiolate, which allowed some proteins (arrows) to be

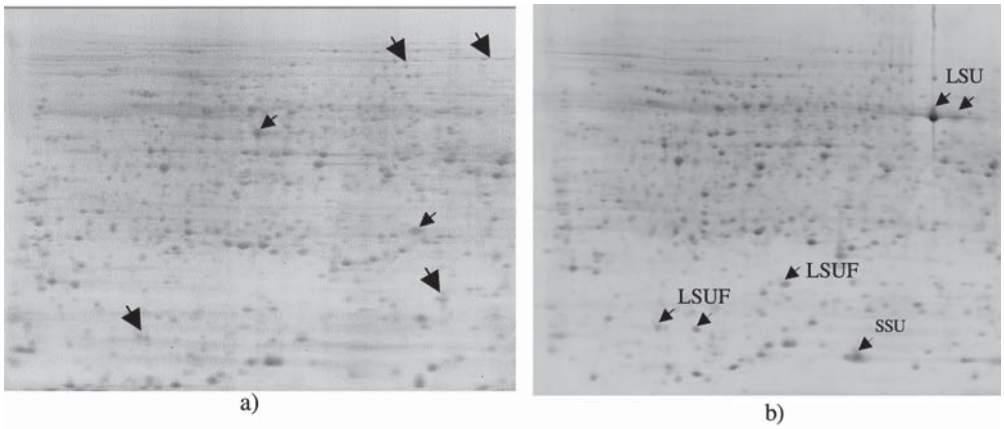

Figure 3 2-D gels of hypocotyl proteins of KDML105 a) etiolate b) green shoot. LSU = large subunit, SSU = small subunit, LSUF = large subunit fragment

detected and some proteins to increase their intensities in 2-D gel. Figure 3b shows the large subunit of RuBisCO and fragments derived from it in vivo (LSUF) and small subunit of RuBisCO, which is similar to the result reported by Salekdeh *et al*. (2002).

By this extraction method, more than 1500 spots in KDML105 etiolated shoot and 1100 spots in KDML105 green shoot could be detected (Table 3). Approximately the same number of spots were obtained from etiolated and green shoots, for both RD15 and RD6. Otherwise, it was found that about 35% of spots from both etiolated and green shoots, RD15 and RD6, were different from the KDML105 variety. However, all of the different spots were low abundant proteins. Some spots were excised to analyze further by MALDI-Q-TOF (Figure 4).

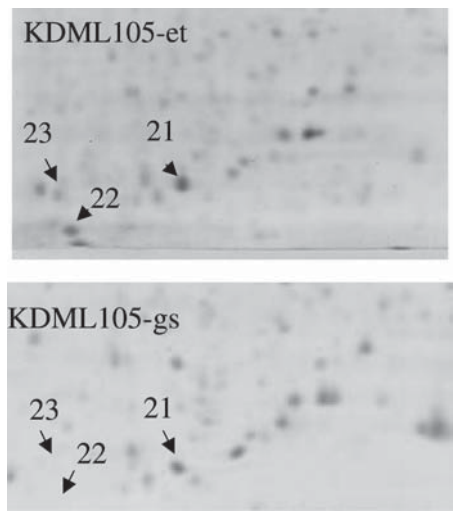

Figure 4 2-D gels showing the excised spots to analyze by MALDI-Q-TOF.

Table 3 Comparison of protein spots in etiolated and green shoots from three rice varieties by PDQUEST image analysis.

Rice variety	Etiolate (et)		Green Shoot (gs)		Match spots between et/gs
	Total spots	Match spots	Total spots	Match spots	
KDML105	1573	100%	1106	100%	74%
RD15	1033	64%	1015	65%	64%
RD6	1255	67%	1193	65%	66%

Some different spots were picked to analyze further by MALDI-Q-TOF as shown in Table 4. Spot no.21, which was absent in RD6 in both etiolated and green shoots, was identified as salt-stress induced protein, SaLT gene product and mannose binding rice lectin. Spot no. 22 could not be detected in KDML105 green shoot, but it could be detected in the etiolate. It suggested that this protein was low abundant protein in KDML105, and it could be detected when RuBisCO was not expressed. Spot no. 23 was identified as P044D10.21. Its amino acid sequences were similar to putative mannose binding rice lectin, (a blast search NCBI at score 105 bits in first hit) and SaLT gene product (with a score of 102 bits for second hit). Interestingly, RD6 did not show spots no. 21 or 23, but KDML105 and RD15 had all three spots. Also, KDML105 had spot no. 22 in lower concentration than RD15 and RD6, as it could be detected only in etiolated shoot but not in green shoot.

CONCLUSION

Proteomics is a powerful technique to determine the global proteins for each physiological characterization. This study was the first step to discovering the functional genomics of the rice plant. In this study, more than one thousand spots in rice bran proteins by sequential fractionation could be detected and almost two thousand spots of etiolate proteins. Although there were few different proteins in rice bran among the wild type and mutant varieties, the result showed many bran proteins that have not been investigated. Therefore, there were many proteins of unknown function found in this study. Between etiolated and green shoot, different proteins which occurred as the consequence of its mutation could be found. The result revealed that RD6, the mutant derived from KDML105, had different types of salt-stress induced proteins from those of KDML105. The expression of salt-stress induced proteins was associated with water deficit or defense responses. Their regulation in leaves is still unclear. Therefore, further study in SaLT gene product of these rice varieties will provide more understanding about their regulation and different actions in specific tissues.

Table 4 Protein identification of variable proteins among etiolated and green shoots using MALDI-Q-TOF.

Spot No.	Etiolate	Green shoot					Mr(kDa)/pI observe	Mr(kDa)/pI calculate	Score	Protein Identification
	KDML 105	RD15	RD6	KDML 105	RD15	RD6				
21	Yes	Yes	No	Yes	Yes	No	14/5.5	15/5.19	235	Salt-stress induced protein
								15/5.0		SaLT gene product
								15/5.0		Mannose binding rice lectin
22	Yes	Yes	Yes	No	Yes	Yes	13.5/4.5	15/5.19	278	Salt-stress induced protein
								15/5.0		SaLT gene product
								15/5.0		Mannose binding rice lectin
23	Yes	Yes	No	No	Yes	No	14/4.2	13/5.0	62	P044D10.21

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