

# Proteomic Analysis of Drought Stress-Responsive Proteins in Rice Endosperm Affecting Grain Quality

Roohi Mushtaq<sup>1,2\*</sup>, Sanjay Katiyar<sup>2</sup>, John Bennett<sup>1</sup>

<sup>1</sup> International Rice Research Institute, Manila, Philippines

<sup>2</sup> Indira Gandhi Agricultural University, Raipur, India

<Received September 29, 2008 / Accepted November 3, 2008>

## Abstract

Drought stress is one of the major abiotic stresses in agriculture worldwide. We report here a proteomic approach to investigate the impact of post-fertilization drought on grain quality in rice seed endosperm (*Oryza sativa* cv. IR-64). Plants were stressed for 4 days at 3 days before heading. Total proteins of endosperm were extracted and separated by two-dimensional gel electrophoresis. Not many protein spots showed differential accumulation in drought-stressed samples. More than 400 protein spots were reproducibly detected, including three that were up-regulated and five down-regulated. Mass spectrometry analysis and database searching helped us to identify six spots representing different proteins. Functionally, the identified proteins were related to protein synthesis and carbohydrate metabolism, such as Granule-Bound Starch Synthase (GBSS, Wx protein), which is thought to play a very important role in starch biosynthesis and quality, a very crucial factor in determining rice grain quality.

Key words: *Oryza sativa*, Drought stress, 2DGE, Mass spectrometry

## Introduction

Rice is a representative model of cereal food crops with an immense socio-economic impact on human civilization because its genome is smaller than those of other cereals (Devos and Gale 2000). Drought is a major limitation on rice production in rainfed areas worldwide. Globally, 154 million hectares (mha) of land is under rice cultivation, 45% of which is grown in rainfed ecosystems (IRRI 2002). Any shortfall in the major rice-growing countries could be a disaster for food security. Improving grain quality has been a major objective of rice cultural management and breeding (Lin et al. 2005). Since varieties are selected by farmers on the basis of high yield and good grain quality, breeders should aim to enhance yield and maintain grain quality under drought. Very little attention has been given to the impact of post-fertilization drought on grain quality and none to the impact of drought at flowering on

grain quality. Therefore, it was proposed to use grain proteome to examine the impact of drought at flowering as a surrogate for grain quality. Water deficit affects many complex and phenologically interacting biochemical and physiological events between panicle initiation and grain filling. Several complex and interlinked processes are involved in cereal seed development (Bewley and Black 1987). The starchy endosperm and aleurone layer are formed and storage proteins, lipids, and polysaccharides are deposited in the endosperm (Olsen 2001). The storage proteins deposited during endosperm development includes different globulins, prolamins, and glutelins which are likely to be affected by drought (Yates et al. 2003). Water deficit at early post-pollination stage in cereal grains decreases endosperm cell division and in turn decrease the capacity for storage accumulation (Setter et al. 2001). Understanding the effect of drought on rice seed endosperm development should benefit the improvement of grain quality.

---

### \*To whom correspondence should be addressed

Dr. Roohi Mushtaq

E-mail: rohikhan400@rediffmail.com

Tel: +91-1942400123

---

## Materials and Methods

### Plant materials

The plant material used in this study includes IR-64 (*indica* cultivar). The *indica* type cultivar is a source of semi-dwarf gene in rice breeding programs. Plants were grown to maturity under greenhouse conditions and were subjected to 4 days of rapid drought stress starting 3 days before heading (3DBH). Properly filled seeds for proteomic analysis were collected at maturity from both well-watered and drought-stressed rice plants. Sample grains were dehusked, embryo was excised from the seed, and the remaining endosperm was used for protein extraction. The seed materials were obtained from the Genetic Resources Centre of International Rice Research Institute (IRRI), Los Banos, Laguna, Philippines.

### Protein extraction and 2-DE

Sample grains were dehusked and ground finely in a mortar cooled with liquid nitrogen and suspended in 10% (w/v) trichloroacetic acid in acetone with 0.07% (w/v) dithiothreitol (DTT) at -20 °C for 1 h, followed by centrifugation for 15 min at 35,000 g. The pellets were resuspended in 0.07% w/v DTT in acetone, incubated at -20 °C for 1 h and centrifuged for 15 min at 35,000 g. This step was repeated three times and the pellets were lyophilized. The resulting powder was solubilized in lysis buffer (9M urea, 35 mM Tris, 4% w/v CHAPS, 1% v/v pH 4-7 IPG buffer, 1% w/v DTT) followed by centrifugation for 15 min at 12,000 g. The proteins in the supernatant were precipitated by adding four volumes of ice-cold acetone, incubated at -20 °C for at least 2 h and centrifuged for 15 min at 12,000 g. The powder was solubilized in rehydration buffer (8M urea, 20 mM DTT, 2% w/v CHAPS, 0.5% v/v pH 4-7 IPG buffer). Protein concentration was determined using Bradford assay (Sangon, Shanghai, China) using bovine serum albumin as standard. For 2-DE, 120 and 1,000 µg of proteins were loaded onto analytical and preparative gels, respectively. For IEF, the Ettan IPGphor system (Amersham Biosciences, Uppsala, Sweden) and pH 4-7 IPG strips (18 cm, linear) were used according to the manufacturer's recommendations. The IPG strips were rehydrated for 12 h in 250 µl rehydration buffer containing protein samples. Focusing was performed in three steps: 500 V for 1 h, 1,000 V for 1 h, and 8,000 V for 10 h. The gel strips were equilibrated for 20 min in 10 ml equilibration buffer (50 mM Tris-HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% w/v DTT, and 0.002% w/v bromophenol blue). SDS-PAGE was performed with 12% gels using the PROTEAN II xi Cell system (Bio-Rad, Hercules, CA, USA). The gels were run at 15 mA per gel for the first 30

min and followed by 30 mA per gel. The protein spots in analytical gels were visualized by silver staining (Salekdeh et al. 2002). Preparative gels were stained with colloidal Coomassie Brilliant blue G-250 (Salekdeh et al. 2002). At least three replicates were performed for each sample.

### Image and Data analysis

Silver stained gels were scanned using GS-700 densitometer (Bio-Rad) at a resolution of 600 dots and 12-bits per inch. Data were analyzed with Melanie 3.0 software (GeneBio, Geneva, Switzerland). The abundance of each protein spot was estimated by the percentage volume (%Vol). Only those with significant and reproducible changes were considered to be differentially accumulated proteins.

### MALDI-TOF MS analysis

Protein spots were excised from preparative polyacrylamide gels that had been stained with Coomassie Brilliant Blue G-250. Proteins were digested with trypsin and MS analysis was conducted with a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA, USA) at the Australian Proteome Analysis Facility (APAF).

### Protein identification and database searching

Protein identification was performed by searching against NCBI nr and SWISS-PROT databases with Profound and Mascot software, respectively, at (<http://www.expasy.org/tools/ProteinId.html>), with *Oryza sativa* as taxonomic category.

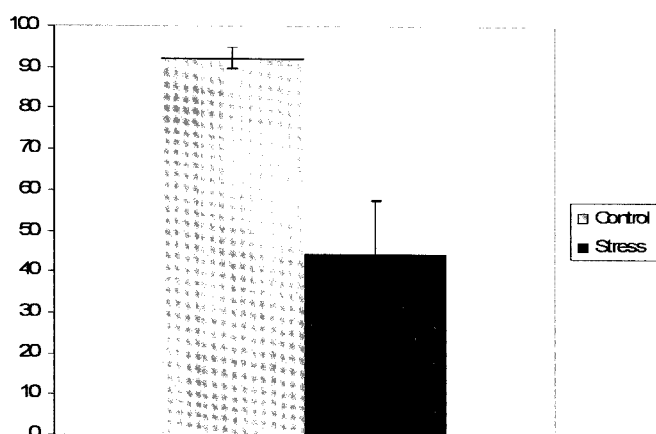
## Results

### Impact of drought stress during heading on grain yield

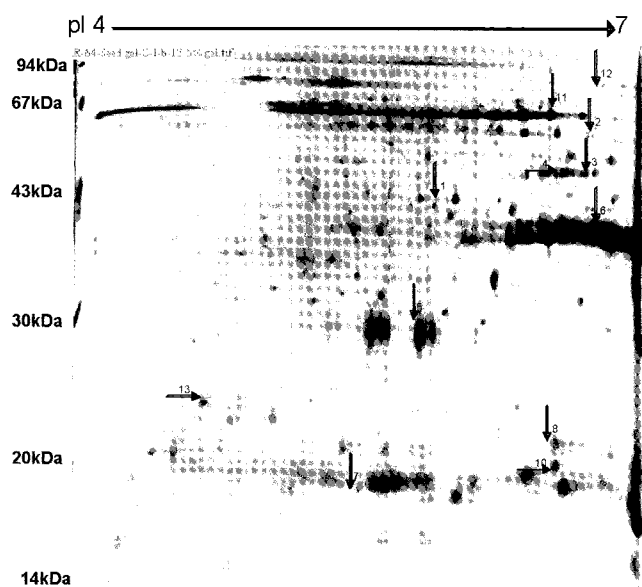
Plants of rice (*Oryza sativa* cv. IR-64) were subjected to drought stress for 4 days starting 3DBH. Brief period of drought stress resulted in the reduction of spikelet fertility from 90% in well-watered plants to 40% in drought-stressed plants (Fig. 1).

### Proteomic analysis of grain proteins in drought stressed rice plants

To counteract drought stress, plants can change their gene expression and protein accumulation. For analysis of grain proteome, seeds from IR-64 plants stressed for 4 days at 3DBH were harvested at maturity. Properly filled seeds were selected from both well-watered and drought-stressed plants. Embryo



**Fig. 1.** Effect of drought on spikelet fertility per panicle in IR-64. Purple color bar represents spikelet fertility in well watered plants and pink color bar represents spikelet fertility in drought-stressed plants.



**Fig. 2.** Two-dimensional gel protein patterns of mature seed endosperm of IR-64 plants drought stressed for 4 days at 3 days before heading. Approximately 120 µg of protein was loaded on each gel. Sizes of molecular markers and the pI range of the first dimension (pI 4-7) are indicated. Numbered spots refer to Table 1.

was excised from seed and remaining endosperm was used for protein extraction. Total proteins in endosperm were extracted and separated by 2-DE using pH 4-7 IPG strips in IEF. More than 400 protein spots were reproducibly detected on gels by Melanie 3.0 software but only those which showed significant changes (i.e., up-regulation/down-regulation) in their behavior under drought stress conditions were selected for further analysis. The representative 2-DE map is shown in Fig. 2. The differential expression of the proteins during drought is expressed in terms of abundance ratio i.e., the percentage volumes in stressed samples/the percentage volumes in control samples (Table 1). The asterisk in Table 1 identifies the abundance ratios that are significantly different from 1.00 based on triplicate extractions and analyses. Total proteins extracted from control and treated samples were separated at the same time to minimize experimental error.

In response to drought stress, nine protein spots showed significant and reproducible changes in abundance. Five spots i.e., 1, 3, 5, 8, and 10 were down-regulated in endosperm of rice seeds showing 37, 69, 58, 45, and 27% decrease in abundance, respectively (Table 1). Four (2, 4 and 7, 13) of the drought responsive spots were up-regulated showing 86%, > 2, 5-fold and 56% increase in abundance, respectively. One protein spot (9) showed qualitative changes between control and stressed samples. It was not detectable by silver staining in gels for well-watered leaves but was seen after the onset of drought stress.

**Table 1.** Abundance ratio of endosperm proteins during drought conditions.

Spot no.	pI	MW	AB ration
1	5.89	41	0.63*
2	6.75	58	1.86*
3	6.73	43	0.31*
4	6.46	43	2.31*
5	5.81	28	0.42*
6	6.84	40	NA
7	5.52	19	4.90*
8	6.55	22	0.55*
9	5.10	36	0.27/0
10	6.40	19	0.73*
11	6.51	65	NA
12	6.80	75	1.05
13	4.17	25	1.56*

NA=data not available

**Table 2.** Proteins identified by MS.

Spot no.	MP/C <sup>a</sup>	Protein name	Accession no.	Experimental Mw/pI	Subcellular localization/Probability
5	8/28	19 kDa globulin precursor	CAA45400	28/5.84	mitochondrial outer membrane / 0.850
6	6/25	Glutelin type I precursor	XP_463450	40/6.84	outside / 0.820
10	3/38	Nucleoside diphosphate kinase	XP_478187	19/6.4	microbody peroxisome / 0.640
11	15/28	Granule-bound Starch Synthase	AAC61675	65/6.51	chloroplast stroma / 0.851
12	2/47	B1160F02.9	XP_470940.1	75/6.7	Cytoplasm / 650

<sup>a</sup> the number of matched peptides/the percentage of sequence coverage.

Figure 2 displays the position of proteins under well-watered conditions. Protein spot 9 was visible only in the drought stressed samples, suggesting it as newly synthesized after drought stress treatment. Although spots 6 and 11 did not show any change under well-watered and controlled conditions, they were selected for the further analysis because of their large space occupancy on gel and their high abundance. In general, much of the changes at the protein level in endosperm of rice seeds were not observed upon drought stress.

### Protein Identified by MS in endosperm

Thirteen protein spots exhibiting differential expression on 2-DE gels of endosperm protein extract were chosen for peptide mass fingerprinting. Among the 13 selected spots, only six proteins i.e., 46.15 % (Table 2) were analyzed by MALDI-TOF. These identified proteins include 19 kDa globulin (5), Glutelin type I precursor (6), Nucleoside diphosphate kinase precursor (10), and granule-bound starch Synthase (11). Spot 12 was identified as B1160F02, located in cytoplasm. Spot 13 was identified as a hypothetical protein. Functionally, identified proteins are related to protein synthesis and carbohydrate metabolism. Psort software showed that these proteins are localized in cytoplasm, mitochondrial outer membrane, microbody peroxisome, and in chloroplast stroma. Details about these identified proteins regarding experimental/theoretical molecular weights and pI, sequence coverage, peptide matched, accession numbers, and subcellular localization are given in Table 2.

### Discussion

Many of the spots could not be resolved on 2-DE gels and very few spots were seen to show differential accumulation upon drought. Among three up-regulated protein spots (2, 7, and 4), none of the Peptide mass fingerprinting data of proteins got match in the database. Among five down-regulated protein spots two spots viz., Spots 5 and 10 were identified by MALDI-TOF analysis. These two include 19 kDa globulin (5), and Nucleoside diphosphate kinase (10). In all of the identified proteins of endosperm, their experimental pI and molecular weights (MW) did not match closely. For example, globulin had MW/ pI of 28 kDa/5.8 on 2-DE gels, but its predicted MW/pI was 21.49kDa/8.0. This apparent discrepancy might be consequence of post-translational modification. Peptide mass fingerprinting data of the rest of the down-regulated proteins did not get match in the database. It is possible that proteins which did not get match in the database might not have undergone proper trypsin digestion with the result that erroneous peptide mass fingerprint-

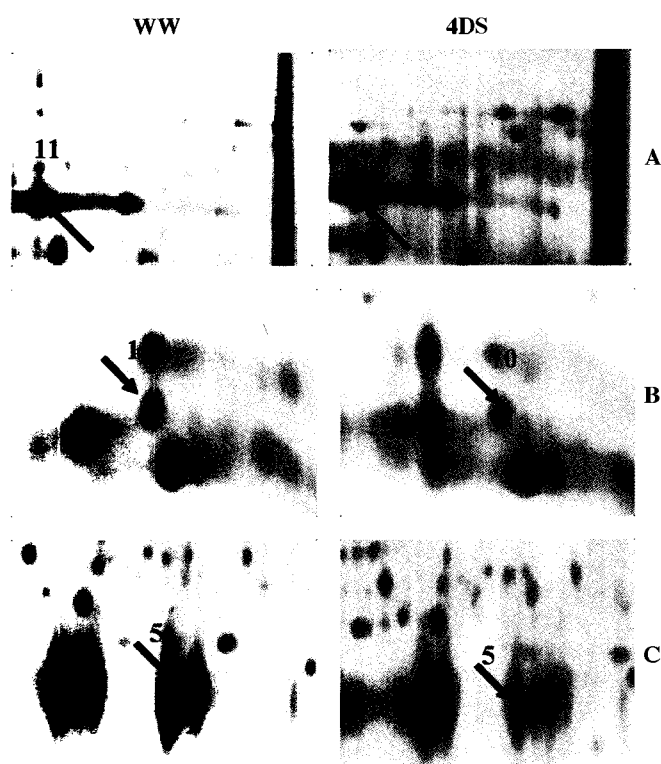


Fig. 3. Behavior of (A) Granule-Bound Starch Synthase (GBSS), (B) Nucleoside diphosphate Kinase and (C) Globulin, on 2-dimensional gels of grain proteome of rice cultivar IR-64, under well-watered (WW), and drought-stressed (4DS) conditions. Numbered spots refer to Table 1.

ing data was obtained which could not be matched in the databases.

Only two spots viz., Glutelins and Granule-bound Starch Synthase (GBSS) proteins, related to grain quality were identified which did not show any change in their behavior upon drought stress. These proteins crucially affect the grain quality (Lin et al, 2005) since majority of proteins in endosperm are storage proteins (e.g. glutenins) they may have obscured less abundant spots (Finnie et al. 2002). Proteomic analysis of wheat (*Triticum aestivum*) endosperm was conducted by Skylas et al. (2000) but again, because total protein extract was used, the protein patterns observed were dominated by the wheat storage proteins, glutenins, and gliadins.

### Nucleoside diphosphate kinase

Nucleoside diphosphate (NDP) kinase is a ubiquitous enzyme in eukaryotes and prokaryotes. NDP is involved in nitrogen metabolism (Schiltz et al. 2004). In seeds, nitrogen mainly originates from leaves and stems that mobilize more than 65% of their nitrogen content (Peoples and Dalling 1988). Our study has shown that NDP is down-regulated under drought stress conditions, showing a 27% decrease in abundance (Fig. 3 (B)).

During seed filling, the accumulation of proteins in the seed relies on the nitrogen supply from the mother plant (Schiltz et al. 2004). As NDP is involved in nitrogen metabolism, there is a possibility that its down-regulation has affected seed filling under drought stress conditions and consequently, might have affected seed yield.

### Globulins

Storage proteins account for about 50% of the total protein in mature cereal grains and have important impacts on their nutritional quality for humans and livestock and on their functional properties in food processing. Globulins are an important source of protein in seed plants and are found in minute amounts in cereals. In rice and oat, these proteins form the major endosperm storage protein fraction, accounting for about 70-80% of the total protein (Shewry and Halford 2001). The high content of globulin storage proteins in oat grain may contribute to the high nutritional value when compared with other cereals, such as barley and wheat (Shewry and Halford 2001). In the present study, globulin was down-regulated showing a 58% decrease in abundance under drought stress conditions (Fig. 3 (C)). Therefore, results signal that the nutritional quality of rice grains under drought stress conditions may have been affected.

### Granule-bound Starch Synthase (GBSS)

Starch quality is a crucial factor in determining rice grain quality. Starch is composed of two distinct polymers, amylopectin and amylose. Both the ratio of amylose to amylopectin and the fine structure of amylopectin are important factors in determining the nutritional quality of rice (Reddy et al. 1993). As in other cereals, amylose in endosperm of rice grain is synthesized by a Granule-Bound Starch Synthase (GBSS), a product of waxy gene (Sano 1984). Because amylose is a major factor affecting the physiochemical properties of starch and rice grain palatability, Wx protein has been the focus of studies on improving the quality of rice grains (Larkin et al. 2003). The amount of Wx protein is positively correlated with the amylase content of rice kernels (Sano 1984). The rice caryopses are most sensitive to environmental stresses during early development (before 15 days after anthesis (DAA), the milky stage) (Huang and Lur 2000). Since proteomic analysis of endosperm from stressed plants revealed that GBSS remained unaltered (Fig. 3(A)) in its behavior, it is therefore possible that drought stress had no effect on grain quality. This may be due to the reason that during the grain-filling period, proper irrigation was given to plants and the effect of drought 3DBH might have been on grain characters such as spikelet fertility, reduced number of filled grains, and other yield-related components. There is a pos-

sibility that grain quality proteins might show different behavior if proteins are analyzed from chaffy grains/partially filled grains. The comparison of such grains with the properly filled grains from the same drought stressed plants by proteomic analysis will give a clear picture. Therefore, this study needs to be further explored before reaching to any conclusions.

### References

- Bewley JD, Black M.** 1978. *Physiology and Biochemistry of Seeds*. Springer-Verlag, Berlin
- Devos MK, Gale DM.** 2000. Genome relationships: The grass model in current research. *Plant Cell* 12: 637-646
- Finnie C, Melchior S, Roepstorff P, Svensson B.** 2002. Proteome analysis of grain filling and seed maturation in barley. *Plant Physiol.* 129: 1-12
- IRRI (International Rice Research Institute).** 2002. *Rice Almanac*. IRRI-WARDA-CIAT-FAO. Los Banos, Philippines
- Larkin PD, McClung AM, Ayres NM, Park WD.** 2003. *Euphytica* 131: 243-253
- Lin S, Chang M, Tsai Y, Lur H.** 2005. Proteomic analysis of the expression of proteins related to rice quality during caryopsis development and the effect of high temperature on expression. *Proteomics* 5: 2140-2156
- Olsen OA.** 2001. Endosperm development: cellularization and cell fate specification. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 52: 233-267
- Peoples MB, Dalling MS.** 1988. Proteolysis and amino acid metabolism. In LD Nooden, AC Leopold, eds, *Senescence and Aging in Plants*. Academic Press, New York, pp 182-217
- Ramachandran C, Raghavan V.** 1989. *Ann. Bot.* 64: 459-468
- Reddy R, Zakiuddin Ali S, Bhattacharya K R.** 1993. The fine structure of rice-starch amylopectin and its relation to the texture of cooked rice. *Carbohydrate polymers* 22: 267-275
- Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J.** 2002. Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* 2: 1131-1145
- Sano, Y.** 1984. Differential regulation of waxy gene expression in rice endosperm. *Theor. Appl. Genet.* 68: 467-473
- Schiltz S, Gallardo K, Huart M, Negroni L, Sommerer N, Burstin J.** 2004. Proteome reference maps of vegetative tissues in pea. An investigation of nitrogen mobilization from leaves during seed filling. *Plant Physiol.* 135: 2241-2260
- Setter TL, Flannigan BA.** 2001. Water deficit inhibits cell division and expression of Transcription involved in cell proliferation and endoduplication in maize endosperm. *J. Exp. Bot.* 52: 1401-1408

- Shewry PR, Halford NG.** 2001. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* 53: 370
- Skylas DJ, Mackintosh JA, Cordwell SJ, Basseal DJ, Walsh BJ, Harry J, Blumenthal C, Copeland L, Wrigley CW, Rathmell W.** 2000. Proteome approach to the characterization of protein composition in the developing and mature wheat-grain endosperm. *J. Cereal Sci* 32: 169-188
- Tanaka K, Kasai Z, Ogawa M, Matsuo T, Kumazawa K, Ishii R, Ishihara K, Hirata H, eds,** 1995. Science of the Rice Plant, Volume Two: Physiology, Food and Agriculture Policy Research Center, Tokyo, pp 97-118
- Yates JR, Antonius AK, Michael WP, Markus B, Nancy AL, Cosmin D, Paul HA, Lara H, David S, Rayan V, Jing W, Dirk W.** 2003. Proteomic survey of metabolic pathways. *Plant Biol.* 99: 11969-11974