Biochemical and Biophysical Research Communications 379 (2009) 133–138

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential regulation of proteins and phosphoproteins in rice under drought stress

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ARTICLE INFO

Article history:
Received 30 November 2008
Available online 25 December 2008

Keywords:
Drought stress
Proteomics
Phosphoproteins
Rice

ABSTRACT

Drought is the largest constraint on rice production in Asia. Protein phosphorylation has been recognized as an important mechanism for environmental stress signaling. However, the differential expression of proteins and phosphoproteins induced by drought in rice is still largely unknown. In this paper, we report the identification of differentially expressed proteins and phosphoproteins induced by drought in rice using proteomic approaches. Three drought-responsive proteins were identified. Late embryogenesis abundant (LEA)-like protein and chloroplast Cu–Zn superoxide dismutase (SOD) were up-regulated by drought whereas Rieske Fe–S precursor protein was down-regulated. Ten drought-responsive phosphoproteins were identified: NAD-malate dehydrogenase, OSJNBa0084K20.14 protein, abscisic acid- and stress-inducible protein, ribosomal protein, drought-induced S-like ribonuclease, ethylene-inducible protein, guanine nucleotide-binding protein beta subunit-like protein, r40c1 protein, OSJNBb0039L24.13 protein and germin-like protein 1. Seven of these phosphoproteins have not previously been reported to be involved in rice drought stress. These results provide new insight into the regulatory mechanism of drought-induced proteins and implicate several previously unrecognized proteins in response to drought stress.

Rice is the dominant staple crop in Asia, providing more than 30% of the calories consumed in the region. Asia's rice production has doubled since 1961 due to breeding rice cultivars with an intensive cultivation system [1]. Nevertheless, the demand for rice production is still rising because of the continuous increase in population. As economic development and urbanization proceed in many parts of Asia, drought has become the largest constraint on rice production, with over 25 million ha affected [2]. One possible way to attain a 43% increase in rice production without a net expansion of cultivation area in order to meet the demand of population growth is to breed drought-tolerant rice, the potential of which has been enhanced by advances in stress-breeding methodology and molecular biology [3]. Considerable work has recently been undertaken to understand the genetic basis of putative drought-adaptive traits in rice [4–8], but it has been difficult to identify genetic segments with clear and repeatable effects on yield under stress.

Proteomics is a recent addition to the molecular tools used to analyze plants affected by drought [3]. Salekdeh et al. [3] identified 16 drought-responsive proteins in rice by proteomic approaches; of these, S-like RNase homolog, actin depolymerizing factor, and rubisco activase were shown to be up-regulated by drought, whereas isoflavone reductase-like protein was down-regulated.

Ali and Komatsu [9] also found that the protein levels of actin depolymerizing factor, light-harvesting complex chain II, superoxidase dismutase, and salt-induced protein in rice sheath were altered by drought and osmotic stresses. Riccardi et al. [10] identified 19 proteins in maize induced upon water stress in growth cabinet and/or greenhouse conditions.

Unlike protein expression levels, phosphoproteins in rice have not yet been analyzed in detail. Phosphorylation is one of the most important post-translational protein modifications that modulate protein activity, protein–protein interactions and cellular localization [11,12]. Owing to the importance of protein phosphorylation in regulating cellular signaling, a major goal of current proteomic efforts is to identify phosphoproteins in higher organisms and determine their definite functions. It is well known that hundreds of proteins are phosphorylated during normal growth in plants. Only a very small number of the thousands of protein kinases and phosphatases in rice have been studied experimentally to date.

A more direct approach to the identification of phosphoproteins is to screen specifically for proteins whose phosphorylation status is altered in response to a stimulus [11]. Since the sequencing of

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0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.067

the complete rice genome, the identification of various signaling components has become feasible with phosphoproteomics. Khan et al. [13] detected phosphoproteins using [^{32}P] labeling and mass spectrometric identification after treating rice seedlings with various hormones and stresses. Sixty phosphoproteins were detected, and the phosphorylation of seven proteins was enhanced by gibberellin treatment. The Pro-Q Diamond Phosphoprotein Stain is a phosphoprotein-specific fluorescence stain that has been used widely for detecting phosphoproteins following separation on 2-DE gels. Chitteti and Peng [14] used this stain to study differences in the rice phosphoproteome during salt stress. Seventeen differentially up-regulated and 11 differentially down-regulated putative phosphoproteins have been identified.

Another nonradioactive method for detecting phosphoproteins is the use of antibodies specific for phosphotyrosine, phosphothreonine, or phosphoserine [15]. Unlike fluorescent staining or [^{32}P] labeling, phospho-amino acid-specific antibodies not only detect phosphoproteins but also reveal the possible type of phosphorylated amino acid (serine, threonine, or tyrosine) [16]. In our previous study, we used phosphoserine- and phosphothreonine-specific antibodies to identify six abscisic acid (ABA)-regulated phosphoproteins [16].

In this paper, the differential regulation of proteins and phosphoproteins in rice under drought stress was investigated using a combination of techniques, including two-dimensional gel electrophoresis (2-DE), phosphospecific antibody immunoblotting and mass spectrometry.

Materials and methods

Plant material and growth conditions. Rice (*Oryza sativa* L. cv. Nipponbare) seedlings were grown in a greenhouse at 28 °C during the day and 22 °C at night under natural light conditions. Seedlings were grown in large flat trays rather than individual pots to minimize variations among pots. Drought treatments were conducted on 2-week-old rice seedling according to the procedure outlined by Xiong and Yang [17]. Under the greenhouse conditions, the leaves of 2-week-old seedlings used in this experiment began to wilt on the 4th day after watering was stopped. When the rice leaves began to wilt, the samples were excised and quickly frozen in liquid nitrogen. Each treatment plot was replicated three times.

Two-dimensional electrophoresis. Total protein extracts were prepared from fresh leaves according to Damerval et al. [18]. Protein concentrations were measured according to Bradford [19]. Focusing of the first dimension took place on a Protean IEF Cell (Bio-Rad). After isoelectric focusing, the IPG strips were immediately equilibrated in equilibration buffer and then placed directly onto 12% polyacrylamide–SDS slab gels. The gels were then probed by immunoblotting using a phosphor-amino acid-specific antibody, or parallel gels were stained by Coomassie blue or silver stain.

Immunoblotting analysis. The proteins in the 2D gels were transferred onto PVDF membranes (Millipore Corporation) and then immunoblotted as described previously [16]. Membranes were blocked with 5% (w/v) BSA in TBST [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20] for 1 h at room temperature and then incubated with rabbit anti-phosphoserine or anti-phosphothreonine antibodies (Zymed Laboratories) at 1 μg/ml in TBST for 2 h. After washing with TBST, membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase at a 1:10,000 dilution for 1 h at room temperature. Immunoreactive spots were detected by enhanced chemiluminescence (Pierce) followed by exposure to X-ray film according to the manufacturer's instructions.

Protein staining and analysis of 2D gels. Stained gels and X-ray films were scanned with the Fluores-s (Bio-Rad) scanner equipped with the MagicScan version 4.5 from UMAX Data Systems Inc. Image analysis was carried out with the PDQuest 8.01 software (Bio-Rad). After spot detection and background subtraction (lowest on boundary mode), 2D gels were aligned and matched, and spot volumes were quantitatively determined (total spot volume normalization mode). Spots of interest were excised from the stained gels using a scalpel and then stored at −4 °C in destaining solution for subsequent MS analysis.

Mass spectrometry analysis and database search. Protein spots from the two-dimensional gels were digested and analyzed as described previously [16]. For a match to be considered a valid identification, a confidence interval (CI) greater than 95% was required [16].

Statistical analysis. The fold change of differentially expressed proteins and phosphoproteins was analyzed using the DPS 3.2 for Windows statistical package.

Gene ontology (GO) annotation. Functional categorization of proteins was carried out according to the GO rules using the gene ontology tools at http://www.agbase.msstate.edu. Three independent sets of ontologies were used to describe a gene product: (1) the biological process in which the gene product participates; (2) the molecular function that describes the gene product activities; and (3) the cellular compartment where the gene product can be found [14].

Results

Identification of drought-responsive proteins

The proteome of rice leaves in response to drought stress was resolved by 2-DE, and two protein profiles were acquired and visualized with Coomassie brilliant blue (Fig. 1). More than 800 protein spots could be detected on each of the 2-DE maps within the p*I* range of 4–9 and MW range of 10–90 kDa. Eighteen proteins showed more than 1.5-fold reproducible changes in abundance (Fig. 1). Fig. 2 compares the abundances of differentially expressed proteins. Twelve proteins increased and six decreased under drought stress. We used mass spectrometry to identify some of the different proteins, which showed more than threefold change in abundance. Three proteins, which are indicated in Fig. 1 by numbers 2, 11, and 18 with arrows, were identified as LEA-like protein, Rieske Fe–S precursor protein, and chloroplast Cu–Zn superoxide dismutase (SOD) (Table 1).

Identification of drought-responsive phosphoproteins

Phosphoserine- and phosphothreonine-specific antibodies from Zymed Laboratories have been widely used to identify and characterize phosphoproteins via immunoblotting analysis [16,20–22]. Our previous research has indicated that these antibodies can specifically detect phosphoproteins in rice.

We used mass spectrometry to identify the variable phosphoproteins (Fig. 3). Putative NAD-malate dehydrogenase (spot S1, Table 2), OSJNBa0084K20.14 protein (spot S2), abscisic acid- and stress-inducible protein (spot S3), putative ribosomal protein (spot S4), putative r40c1 protein (spot S5), and OSJNBb0039L24.13 protein (spot S6) were detected by the phosphoserine-specific antibody, whereas drought-induced S-like ribonuclease (spot T1), putative ethylene-inducible protein (spot T2), guanine nucleotide-binding protein (G protein), beta subunit-like protein (spot T3), and germin-like protein 5 (spot T4) were detected only by the phosphothreonine-specific antibody.

Phosphorylation sites in the identified phosphoproteins

The tryptic peptides of phosphoproteins separated by two-dimensional electrophoresis used for protein identification were also analyzed for phosphorylation sites. Only phosphopeptides of

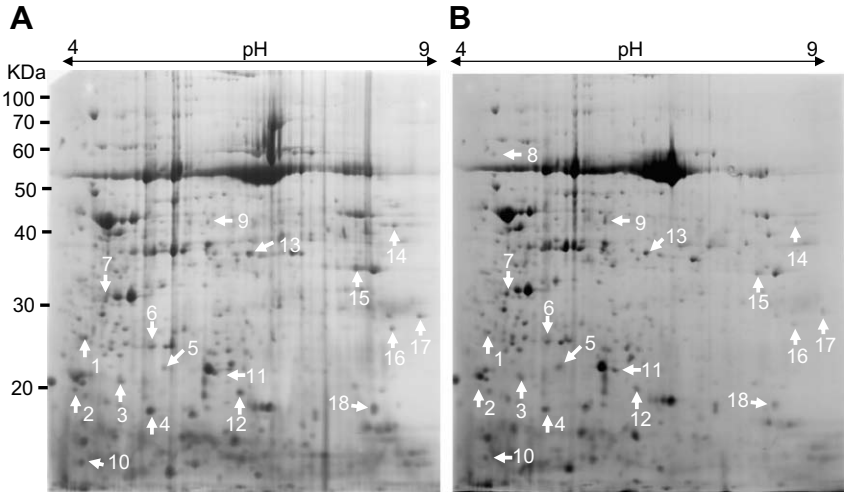


Fig. 1. Two-Dimension gel analysis of proteins extracted from leaves of rice under drought (A) and well-watered control (B) condition for 4 days. In the first dimension (IEF), 700 μg of protein was loaded on a 17 cm IPG strip with a nolinear gradient of pH 3–11. In the second dimension, 12% SDS–PAGE gels were used, with a well for *M*r standards. Proteins were visualized by Coomassie blue staining. The arrows indicate 18 proteins that changed reproducibly and significantly in drought-stressed plants compared with well-watered controls.

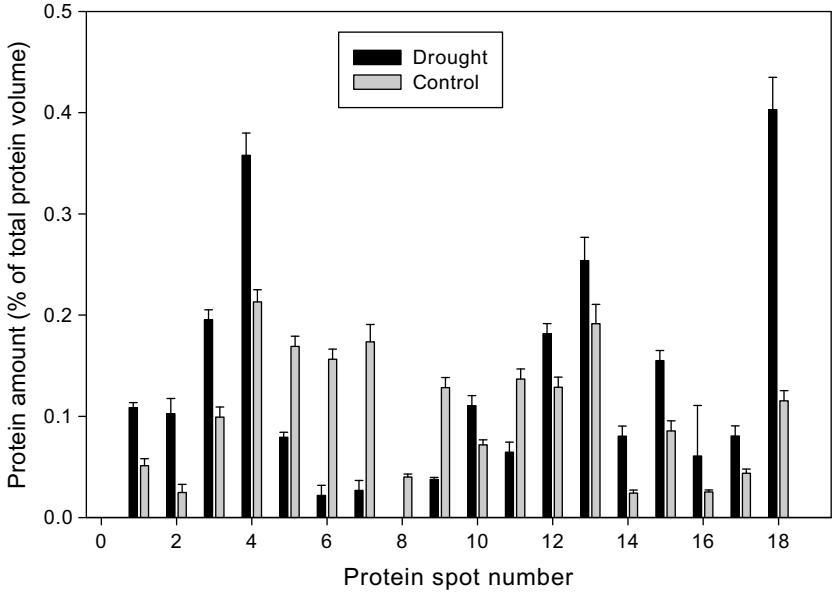
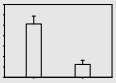
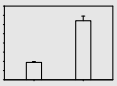
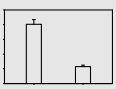


Fig. 2. The abundance ratios of 18 rice leaf proteins in drought-stressed plants and control plants, compared with the average abundances. Abundance is expressed as spot quantity as a percentage of the total spot quantity over the 2D gel. The protein spot numbers are the same as in Fig. 1.

Table 1
Identification of the differentially expressed proteins shown in Fig. 1 by MS + MS/MS data.

Spot No. [a]	Change[b] a b	Protein Accession	Protein description	MW/p*I*[c]	CI %[d]	No of peptide[e]
2		trm|Q40741	LEA-like protein	20.5/5.8	99.97	16
11		trm|Q9ZSU7	Rieske Fe–S precursor protein	23.9/8.5	100	12
18		spt|P93407	Superoxide dismutase [Cu–Zn], chloroplast precursor	21.3/5.9	100	9

[a] The protein spot number was given in Fig. 1.
[b] Change: average in abundance from three independent treatments ± SD. Column a, drought stress; column b, control.
[c] Molecular mass and isoelectric point (p*I*) are theoretical values.
[d] CI (confidence interval) % was generated by the GPS Explorer software associated with the MALDI TOF/TOF mass spectrometer (Applied Biosystems).
[e] The number of peptides that matched with the identified protein in mass analyses.

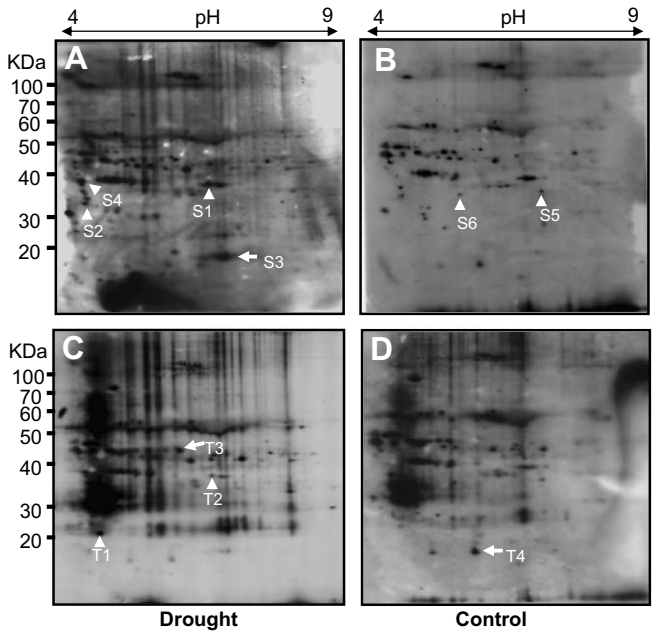


Fig. 3. The distinct different phosphoproteins from the drought-stressed rice leaves for 4 days. Leaf proteins were separated by two-dimensional gel electrophoresis and transferred onto membranes. Proteins were probed by anti-phosphoserine (A,B) or anti-phosphothreonine antibodies (C,D). Phosphoserine- and phosphothreonine-containing proteins were labeled with the letters S and T, respectively. Only proteins identified by mass spectrometry are labeled.

drought-induced S-like ribonuclease (spot T1, Table 2) were detected. Failure to detect other phosphopeptides in the sample proteins separated by two-dimensional electrophoresis has been reported by other investigators [16,23–25].

To determine the possible phosphorylation sites in the other identified phosphoproteins, two computer algorithms, NetPhos [26] and PHOSIDA [27], were used to analyze these proteins. NetPhos or PHOSIDA predicted that all the identified proteins contained at least one serine or threonine phosphorylation site (Table 2).

Gene ontology (GO) analysis

To understand the biological processes and cellular components involved in drought stress response in rice, gene ontology analyses were performed using the differentially regulated proteins, including both differentially up- or down-regulated proteins and phosphoproteins. As shown in Table 3 (Supplementary data), one of the differentially expressed proteins was involved in response to abiotic stress, one was involved in photosynthesis, and the other was involved in oxidation/reduction. When analyzed based on biological processes, 3 of the 10 variable phosphoproteins were involved in metabolic processes, two functioned in signal transduction, one was involved in protein translation, and the others remained unknown (Table 3, Supplementary data).

Discussion

Drought-responsive proteins

LEA-like protein and chloroplast Cu–Zn SOD were up-regulated by drought stress, whereas Rieske Fe–S precursor protein was down-regulated. Compared with the results of Salekdeh et al. [3], only the down-regulation of Rieske Fe–S precursor protein and the up-regulation of chloroplast Cu–Zn SOD were revealed in both studies. The lack of commonality in most of the identified drought-responsive proteins between our study and theirs [3] may be due to the different sampling times (2-week-old rice versus 3-week-old rice) and different times of drought treatment (4 days versus 23 days).

LEA-like protein probably functions in abiotic stress tolerance by minimizing the negative effects of oxidation [28]. Mowla et al. [29] identified an *Arabidopsis* LEA-like protein, AtLEA5, that increased the tolerance of Deltayap1 cells to oxidants, such as H_2O_2, diamide, menadione, and *tert*-butyl hydroperoxide. In response to drought stress, the abundance of the chloroplast Cu–Zn SOD was reported to increase in rice and wheat [3,30]. However, the down-regulation of Rieske Fe–S precursor protein may induce the decrease of photosynthesis. This could be a mechanism to optimize the rice plant's water use during restricted availability. When the rice roots are submitted to stress, they will produce abscisic acid (ABA), which is transported to the aerial parts of the plant; ABA in turn causes drastic changes in the major photosynthetic protein (i.e., Rieske Fe–S precursor protein) and accumulation of certain defense/stress-related proteins (i.e., LEA-like protein and chloroplast Cu–Zn SOD) [31], which then induce stomatal closure to prevent evaporation.

Drought-responsive phosphoproteins

In total, 10 proteins exhibited altered phosphorylation in response to drought stress and were identified by tandem mass spectrometry.

Table 2
The list of phosphoproteins identified by tandem mass spectrometry.

Spot No.[a]	Regulation by drought[b]	Protein Accession	Protein description	MW/pI[c]	CI %[d]	No of peptide[e]	S[f]	T[g]
S1	Up	trm|Q941V4	NAD-malate dehydrogenase	42.7/7.01	99.9	8	+	
S2	Up	trm|Q7X7H3	OSJNBa0084K20.14 protein	40.2/6.75	100	20	+	
S3	Up	trm|O49149	Abscisic acid- and stress- inducible protein	15.5/6.2	100	9	+	
S4	Up	gb|AAO37485.1	Ribosomal protein	34.9/4.97	100	16	+	
S5	Down	trm|Q9FTY4	r40c1 protein	30.1/6.27	100	12	+	
S6	Down	trm|Q7XMK5	OSJNBb0039L24.13 protein	22.4/5.47	100	18	+	
T1	Up	trm|Q8RYA7	Drought-induced S-like ribonuclease	28.3/5.25	100	10		+
T2	Up	trm|Q8W3D0	Ethylene-inducible protein	33.1/6.19	100	11		+
T3	Up	spt|P49027	Guanine nucleotide-binding protein beta subunit-like protein	36.2/5.97	100	14		+
T4	Down	trm|O49001	Germin-like protein 1	21.8/6.01	100	3		+

[a] The protein spot number was given in Fig. 1.
[b] Up, increased phosphorylation by drought; down, decreased phosphorylation by drought.
[c] Molecular mass and isoelectric point (p*I*) are theoretical values.
[d] CI (confidence interval) % was generated by the GPS Explorer software associated with the MALDI TOF/TOF mass spectrometer (Applied Biosystems).
[e] The number of peptides that matched with the identified protein in mass analyses.
[f] A plus sign indicates that the protein contains at least one predicted phosphoserine site by NetPhos.
[g] A plus sign indicates that the protein contains at least one predicted phosphothreonine site by Phosida.

Three of these proteins—NAD-malate dehydrogenase, ribosomal protein and G protein beta subunit-like protein—were already known to be phosphorylated under various conditions of drought and/or by exogenous ABA in different species. NAD-malate dehydrogenase (EC 1.1.1.37) is an enzyme that catalyzes the formation of oxaloacetate from malate and NAD. Malate dehydrogenase in maize root plasma membrane was down-regulated by treatment with humic substances [32] and was reported to be phosphorylated in rice [33–35]. Meanwhile, the ribosomal protein (spot S4) has been identified as a phosphoprotein in rice and *Arabidopsis* [36–39]. G protein beta subunit-like protein was phosphorylated in rice in response to exogenous ABA treatment [16]. It is a member of the WD-repeat protein family, which is implicated in a variety of processes, ranging from signal transduction and transcription regulation to cell cycle control and apoptosis.

Four of these proteins—abscisic acid- and stress-inducible protein, ethylene-inducible protein, drought-induced S-like ribonuclease, and OSJNBa0084K20.14—are involved in stress responses or metabolism (Table 3, Supplementary data), but the function of phosphorylation of these proteins is not clear. Abscisic acid- and stress-inducible protein was induced by water stress in *Solanum chacoense* [40], loblolly pine [41], maize [10], and *Elymus elongatum host* [42]. In addition, drought-induced S-like ribonuclease, an S-like RNase homolog, was up-regulated in rice during drought stress [11].

Three of these proteins—OSJNBb0039L24.13 protein, germin-like protein (GLP) 1 and putative r40c1 protein—were found to be dramatically dephosphorylated during drought stress. The OSJNBb0039L24.13 protein functions in signal transduction. GLPs appear to be involved in defense mechanisms in some plants [43,44], and the function of the putative r40c1 protein was still unknown (Table 3, Supplementary data). The functional importance of their dephosphorylation remains to be determined. In the future, it will be of interest to experimentally determine the kinases and phosphatases that act on the ten drought-regulated phosphoproteins.

Acknowledgments

This work was supported by the Natural Science Foundation of Fujian (No. 2009J0052), the National Natural Science Foundation of China (No. 30020170; 30871494) and the US National Science Foundation (Grant MCB0505458).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.067.

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