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Cold-induced changes of protein and phosphoprotein expression patterns from rice roots as revealed by multiplex proteomic analysis

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Abstract

Cold stress is a critical abiotic stress that reduces crop yield and quality. The response of the rice proteome to cold stress has been documented, and differential proteomic analysis has provided valuable information on the mechanisms by which rice adapts to cold stress. A global analysis of the change in protein phosphorylation status in response to cold stress remains to be explored, however. Here, we performed a phosphoproteomic analysis of rice roots following exposure to cold stress using a two-dimensional gel electrophoresis–based multiplex proteomic approach. Differentially expressed proteins and phosphoproteins were detected and identified by matrix-assisted laser desorption ionization time of flight/time of flight mass spectrometry combined with querying rice protein databases. Nineteen protein gel spots (stained with silver) showed a twofold difference in abundance of protein spots from gels with and without cold stress; these proteins were identified to be involved in redox homeostasis, signal transduction, and metabolism. Twelve of the thirteen phosphoprotein gel spots (stained with Pro-Q Diamond) that showed a twofold abundance difference were identified, including the following nine proteins: enolase, glyceraldehyde-3-phosphate dehydrogenase, nucleoside diphosphate kinase, ascorbate peroxidase, adenosine kinase, CPK1 adapter protein 2, ATP synthase subunit alpha, methionine synthase 1, and tubulin. Phosphorylation site predictors were used to confirm that the identified proteins had putative phosphorylation sites. These results suggest that phosphorylation of some proteins in rice roots is regulated in response to cold stress.

Keywords: Cold stress; Multiplex proteomics; Phosphoprotein; Rice roots.
Abbreviations: 2DGE, two-dimensional gel electrophoresis; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ ionization-time of flight/time of flight mass spectrometry; NCBInr protein database, National Center for Biotechnology Information nonredundant protein database.

Introduction

Low temperature is one of the most serious stressors for plants, causing reduced plant growth and rolled and withered leaves (Allen and Ort, 2001). Rice, a cold-sensitive plant, is an important food crop. Because its growth and yield are significantly affected by cold stress, the molecular mechanism of cold adaptation/resistance has been of great interest. Proteomics were widely used to analyzed the changes of protein expression profiles responding to various stress conditions (Kamal et al., 2010; Shin et al., 2010). Considerable effort has been made in studying the expression of cold-responsive proteins that may be involved in cold tolerance (Yan et al., 2006; Neilson et al., 2011). The differential expression of proteins that are involved in the cold stress response and/or recovery in different rice tissues has also been reported (Yan et al., 2006; Neilson et al., 2011). These results provide useful information for understanding cold stress–responsive genes and/or proteins and their related metabolic pathways. In addition to monitoring steady-state levels of protein expression, co- and post-translational protein modifications can also be monitored using proteomic methods. In plants, protein phosphorylation appears to be the predominant post-translational modification in response to environmental stress, and it plays an important role in defense signaling. Given the importance of protein phosphorylation in the regulation of cellular processes, a major goal of proteomic research today is to identify and understand the function of phosphoproteins in higher organisms. For plants, this research is at an early stage, although phosphorylated proteins have been

identified on a large scale in rice treated with various hormones (Khan et al., 2005), under high-heat stress (Chen et al., 2011), and under drought stress (Ke et al., 2009). The phosphoprotein-specific fluorescent stain, Pro-Q Diamond, has been widely used to detect phosphoproteins following their separation by 2DGE (see for example, Knowles et al., 2003). Multiplex proteomic technology allows direct, in-gel detection of phosphate groups attached to Tyr, Ser or Thr residues and enables the quantitative changes of particular protein gel spots to be monitored in a time-dependent manner, *i.e.*, during a treatment course or during different developmental stages; this is an especially useful tool in revealing differential expression of phosphoproteins. Using this method, Chitteti and Peng (2007) investigated dynamic changes in the phosphoproteome of the Arabidopsis cotyledon during cell dedifferentiation, Chen et al. (2011) detected the changes in 11 phosphorylated proteins in response to high-heat stress in rice leaves, and Chinnusamy et al. (2006) analyzed the proteome and phosphoproteome differential expression profiles in rice roots under salinity stress. The roots of a plant form a functionally important organ: many critical metabolic and regulatory activities take place there that eventually control the absorption and transport of materials and nutrients. Root architecture is determined by a combination of the intrinsic developmental program and the external biotic and abiotic stimuli of a plant (Lynch 1995). To better understand the molecular programs induced by cold stress, we investigated the changes to the proteome and phosphoproteome in rice roots in response to cold injury. Multiplex staining of the protein and phosphorylated protein gel spots following high-resolution 2DGE allowed us to quantify changes in protein phosphorylation after rice roots were subjected to cold stress.

Results and discussion

Rice root proteome patterns revealed by Pro-Q Diamond phosphoprotein in-gel stain and silver stain

To examine the proteome and phosphoproteome of rice roots, proteins were isolated from 2-week-old rice roots, separated by 2DGE, and stained for phosphoproteins (using Pro-Q Diamond dye) and then for total proteins (using silver staining). The majority of protein spots that were heavily stained by Pro-Q Diamond had relatively low pIs (Fig. 1). In contrast, the silver-stained protein spots were more evenly distributed across the 2DGE gel (Fig. 2). Many protein spots that were heavily stained with silver were not stained, or were weakly stained, by Pro-Q Diamond. Conversely, many spots that were weakly stained by silver were heavily stained by Pro-Q Diamond, as illustrated by comparing the spots labeled in Figure 1A and 1B. Approximately 115 and 730 protein spots were consistently detected when stained with Pro-Q Diamond and silver, respectively, in all three biological replicas.

Rice root proteome and phosphoproteome following cold stress

To investigate the changes in protein expression in rice roots when subjected to cold stress, we performed 2DGE analysis of rice root protein extracts in three biological replicates and at two different temperatures (15 ºC and 6 ºC). Gels were run in (at least) triplicate for each sample and showed a high level of reproducibility. Replicate gels were stained in parallel with Pro-Q Diamond, and typical gels are shown in Figure 1B and 1C. More than 115 and 730 protein spots were reproducibly detected by PDQuest 8.0 software for the Pro-Q Diamond–stained and silver-stained gels, respectively (Figs. 1 and 2). Quantitative image analysis revealed 13 protein spots (Pro-Q Diamond staining) and 19 spots (silver staining) that had significantly changed intensities (*i.e.*, by more than twofold, and with $p < 0.05$) as a result of at least one of the cold stress treatments (Fig. 3). Of these, for the Pro-Q Diamond–stained gels, 12 spots indicated upregulation and 1 spot indicated downregulation; for the silver-stained gels, 14 spots indicated upregulation, and 5 spots indicated downregulation.

Analysis of differentially expressed Pro-Q Diamond–stained rice-root proteins under cold stress

We examined the response of rice roots to cold stress, at 15 ºC and 6 ºC, by monitoring changes in the relative expression levels of putative phosphoproteins (*i.e.*, those stained by Pro-Q Diamond). Thirteen Pro-Q Diamond–stained protein spots showed a greater than twofold upregulation or downregulation in all three biological replicas (Fig. 1 and Fig. 3B). These protein spots were excised from the gel, in-gel digested with trypsin, and analyzed by MALDI TOF/TOF MS. Twelve of the 13 putative phosphoproteins were identified with high confidence (Table S1) and are mainly involved in carbohydrate metabolism, signal transduction, and redox homeostasis. Among those involved in carbohydrate metabolism (spots 1, 2, 5 and 11), enolase and glyceraldehyde-3-phosphate dehydrogenase are typical plant stress-response proteins and are phosphorylated and strongly induced in response to salt, low or high temperature, and anaerobic stress in plant roots (Forsthoefel et al., 1995; Kosová et al., 2011). The identified phosphoproteins involved in signal transduction are nucleoside diphosphate kinase (spot 3), adenosine kinase (spot 6), and calcium-dependent protein kinase (CPK1) adapter protein 2 (spot 7). We observed that levels of nucleoside diphosphate kinase increased under cold stress; this is consistent with the report that this kinase showed high levels of autophosphorylation, which confers an enhanced tolerance to multiple environmental stresses in *Arabidopsis* that elicit accumulation of reactive oxygen species (Moon et al., 2003). Spot 6 was identified as adenosine kinase, which has been shown to be phosphorylated by sucrose nonfermenting-related kinase in ProQ Diamond–stained gels in *Arabidopsis* (Shin et al., 2007). Spot 7 was identified as CPK1 adapter protein 2, which has been found to participate in vesicular trafficking of proteins (Chehab et al., 2007). Spots 4 and 10 were both identified as the redox homeostasis protein, ascorbate peroxidase (APX); the two spots are presumably due to differing degrees of phosphorylation. Spot 8 was identified as ATP synthase subunit alpha, and its phosphorylation was induced under cold stress. It has been reported that the phosphorylation status of ATP synthase is induced under heat stress in rice leaves (Chen et al., 2011). Using four different phosphorylation site predictors, all 13 Pro-Q Diamond–stained protein spots were identified as phosphoproteins (data not shown). Because different stress response pathways often "cross talk" with each other (Wang et al., 2003; Chinnusamy et al., 2006), it is not surprising that these phosphorylated proteins may be also induced under cold stress conditions.

Analysis of differentially expressed silver-stained rice-root proteins under cold stress

We also examined the response of rice roots to cold stress, at 15 ºC and 6 ºC, by monitoring changes in the relative expression levels of all proteins (*i.e.*, using silver stain). Fourteen proteins displayed a greater than twofold upregulation, and five displayed greater than twofold downregulation in all three biological replicas (Fig. 2 and Fig. 3A). MALDI-TOF/TOF MS

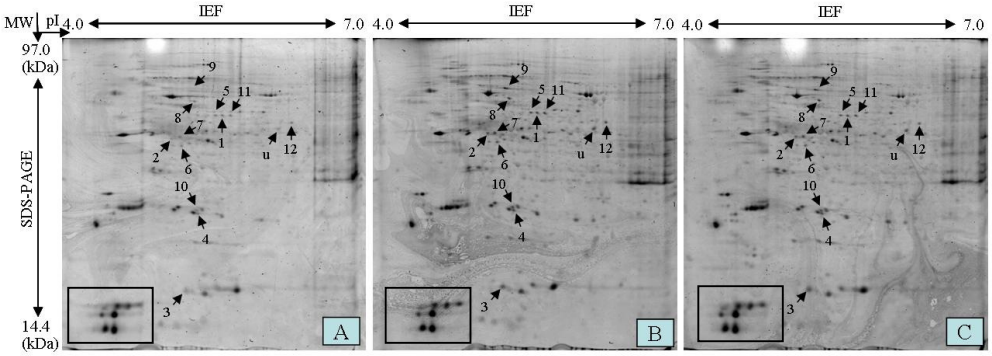

Fig 1. Images of the Pro-Q Diamond–stained 2DGE gels showing the phosphoproteins extracted from rice roots after exposure to cold stress conditions. The protein extracts were obtained from rice roots after exposure to: (A) normal temperature (control); (B) 15 ºC for 12 h; (C) 6 ºC for 12 h. Arrows identify the differential expression protein spots used in this study.

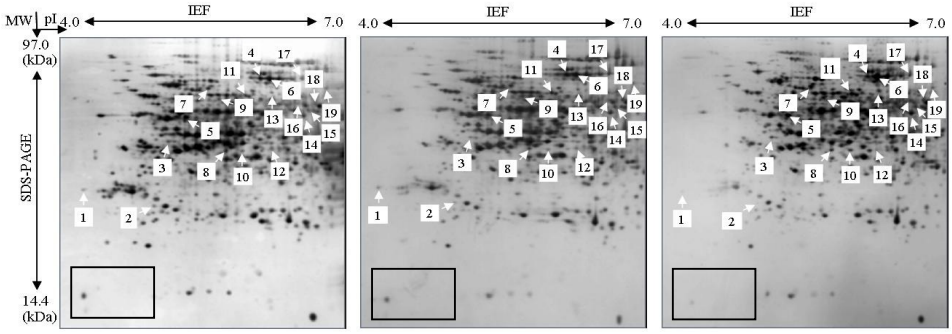

Fig 2. Images of the silver-stained 2DGE gels showing all proteins extracted from rice roots after exposure to cold stress conditions. The protein extracts were obtained from rice roots after exposure to: (A) normal temperature (control); (B) 15 ºC for 12 h; (C) 6 ºC for 12 h. Numbers in the figures show the protein spots expressed differentially in gels.

analysis identified all 19 proteins with high confidence (Table S2); these proteins were found to be involved in redox homeostasis, amino acid or carbohydrate metabolism, defense response, and protein metabolism. Of the 14 upregulated proteins, 6 have been reported to be differentially regulated under cold stress in prior rice proteome studies, including glyceraldehyde-3-phosphate dehydrogenase, catalase, pentatricopeptide repeat–containing protein, fructokinase, heat shock 70 protein, and phenylalanine ammonia-lyase (Cui et al., 2005; Yan et al., 2006; Hashimoto et al., 2007; Lee et al., 2009). In addition to these six proteins, the roles of ascorbate peroxidase and dehydroascorbate reductase in response to cold stress are well documented (Zhang et al., 2008). We also observed that four proteins were upregulated following cold stress: TPA: class III peroxidase 59 precursor, a putative TCP-1/cpn60 chaperonin family protein, 26S proteasome regulatory particle triple-A ATPase subunit3, and 26S protease regulatory subunit 7. Isoforms and/or homologs of these proteins have been reported to be upregulated by cold stress in rice (Cui et al., 2005; Yan et al., 2006; Hashimoto et al., 2007; Lee et al., 2009).

Materials and methods

Rice growth and cold stress treatment

The japonica "super" rice cultivar, Huaidao 9 (a cold tolerent cultivar, bred by the Rice Research Institute of Huaian Academy of Agricultural Sciences, China) was used as the experimental material. Rice seeds were surfaced-sterilized with 0.1% sodium hypochlorite and rinsed with distilled water. The sterilized seeds were soaked in distilled water and allowed to germinate in darkness at 28 ºC for 48 h. Rice seedlings were grown hydroponically in a growth chamber in Hoagland solution with a 14 h d^{-1} photoperiod of 400 µmol m^{-2} s^{-1} photosynthetic photon flux density and a 28 ºC/25 ºC light/dark cycle in 75% relative humidity. Second-week-old seedlings were subjected to air temperatures and Hoagland solution at 15 ºC and 6 ºC in the growth chamber for 12 h, respectively. The roots from the cold-treated and control plants were then collected to extract the proteins.

Protein extraction from rice roots

Proteins were extracted from the rice roots as described (Yang et al., 2010) with the following modifications. In brief, frozen roots were ground in liquid nitrogen, then mixed with a solution of 10% (v/v) trichloroacetic acid in acetone containing 0.07% (w/v) dithiothreitol (DTT) at –20 ºC for 1 h, to allow the proteins to precipitate. The mixture was centrifuged at 15,000 × *g* at 4 ºC for 30 min, and the precipitate was washed with ice-cold acetone containing 0.07% w/v DTT (to remove pigments and lipids) until the pellet was colorless. Pellets were dried by vacuum centrifugation, then resuspended by mixing with extraction buffer (recipe follows) at room temperature for 30 min, followed by five periods of sonication for 30 s each.

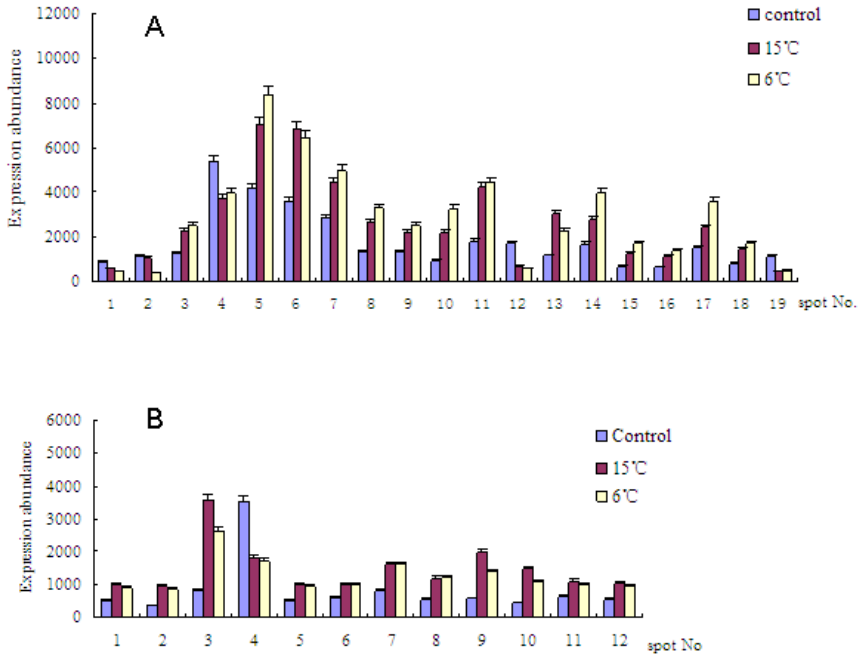

Fig 3. Plot of the 2DGE protein spot intensities, indicating expression levels, for proteins extracted from rice roots after exposure to cold stress conditions. The gels were treated with: (A) silver stain, and (B) Pro-Q Diamond stain.

The extraction buffer consisted of 7 M urea, 2 M thiourea, 4% (w/v)
3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate,
20 mM DTT, 0.2% (v/v) carrier ampholyte (pH 4.0–7.0), 1 µl per 30 mg plant tissue of Protease Inhibitor Cocktail for Plant Cells (Sigma-Aldrich, USA), and 10 µl per 1 ml of extraction buffer of Phosphatase Inhibitor Cocktail (Sigma-Aldrich). After extraction, the solution was centrifuged at 15,000 × *g* for 30 min, and the resulting supernatant stored at –80 ℃. Protein extract was quantified using a 2-D Quant kit (GE Healthcare, USA) according to the manufacturer's instructions.

2DGE experiments

2DGE was conducted as described (Yang et al., 2010) with the following modifications. In brief, a 450-µg sample of extracted proteins was separated by isoelectric focusing (IEF) using immobilized linear pH gradient strips (Immobiline DryStrip Gels, pH 4.0–7.0, 24 cm; GE Healthcare) in an IEF apparatus (Ettan IPGphor 3 IEF apparatus; GE Healthcare). The strips were passively rehydrated in rehydration solution for 13 h prior to use. The voltage/time profile used was: 250 V, 1 h; step to 500 V, 1 h; step to 2000 V, 1 h; step to 8000 V, 3 h; then 8000 V until 72,000 Vh was reached. Separated proteins on the IEF strips were then reduced and alkylated by first soaking for 15 min in 10 ml of equilibration solution (0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% (v/v) glycerol and 2% (w/v) SDS) with an added 2% (w/v) DTT and then for 15 min in 10 ml of the equilibration solution containing 2% (w/v) iodoacetamide. Separation along the second dimension was achieved by SDS-PAGE using 12.5% polyacrylamide gels and electrophoresing at 2 W/gel for 90 min, then 12 W/gel for 6 h, in a Multiphor II Electrophoresis System (GE Healthcare).

Multiplex fluorescent gel staining and image analysis

The 2DGE gels were fixed overnight in 40% methanol/10% acetic acid and first stained for phosphoproteins with Pro-Q Diamond phosphoprotein gel stain (Invitrogen, USA) according to manufacturer's instructions, and then for total proteins with silver stain to visualize all protein spots. To stain the phosphoproteins, the 2DGE gels were incubated for 3 h in ProQ Diamond, then destained with 20% (v/v) acetonitrile in 50 mM sodium acetate, pH 4.0, for 3 h. After destaining, the gels were scanned for phosphoprotein spots using a Typhoon Trio imager (GE Healthcare) with an excitation wavelength of 532 nm and a 610-nm band pass emission filter. After the scan images were acquired, the gels were stained with silver to visualize all proteins (including non-phosphorylated proteins), following the method of Shevchenko et al. (1996). For each temperature condition that the plant roots were exposed to (15 ℃, 6 ℃ and control) three replicate gels, stained for phosphoprotein and total protein, were prepared and compared. Quantitative and qualitative analysis was performed using the PDQuest (version 8.0) 2D Analysis Software package (Bio-Rad, Hercules, CA, USA). Spot intensities were normalized so that the total image density, summed over all spots, was equal for all gels in each analysis. Only spots that were present in all three replicate gels were considered. To quantify changes in protein levels between cold-stressed and control plant roots, a threshold of a twofold intensity change was set. Protein and phosphoprotein spots that were significantly different (*i.e.*, where $p < 0.01$ in the Student's *t*-test) were considered to be upregulated or downregulated.

MALDI-TOF/TOF MS analysis

Protein spots with differential expression patterns on gels were manually excised from gels, washed with Millipore pure water for three times. In-gel digestion of proteins, MALDI-TOF/TOF-MS analysis were performed as described (Chitteti and Peng, 2007). Mass spectra were collected using a MALDI TOF/TOF MS (ABI 4700 Proteomics Analyzer, Applied Biosystems, USA).

Database searching

All spectra of proteins were submitted to database searching using online MASCOT program (http://www.matrixscience.com) against NCBInr databases. The following parameters were used: digestion enzyme, trypsin with one missed cleavage; MS (precursor-ion) peak filtering, 600–4000 *m/z* interval, monoisotopic, signal-to-noise ratio (S/N) >10, mass tolerance, 150 ppm; MS/MS (fragmentation) peak filtering, monoisotopic, $M+H^+$, S/N > 3; MS/MS fragment tolerance, 0.2 Da; database, *Oryza* taxonomic subdatabase of NCBInr databases. During the initial MS scan, data were analyzed by peptide mass fingerprinting, and preliminary protein identification was made by searching the *Oryza* subdatabase using MASCOT (www.matrixscience.com). Proteins with high-confidence, *i.e.*, with a cross confidence interval > 95%, were automatically selected for *in silico* digestion, and the corresponding three most prevalent peptides/precursor ions present in the mass spectra were selected for further MS/MS analysis, to yield RDA_1 (the best match). Sample spots that did not yield high-confidence identification after preliminary peptide mass fingerprinting, or after RDA_1 identification, were subjected to further analysis: by selecting the 20 most intense precursor ions in the mass spectra for further MS/MS analysis, RDA_2 was generated. Data from the above peptide mass fingerprinting analysis, RDA_1, and RDA_2 MS/MS spectra were together subjected to a combined MASCOT search. Only proteins with a total protein score > 95% were considered positively identified.

Functional classifications of identified phosphoproteins

Protein functions were assigned to the identified proteins using the protein function database Pfam (http://pfam.sanger.ac.uk/; Bateman et al., 2002) or Inter-Pro (www.ebi.ac.uk/interpro/; Apweiler et al., 2001). Identified proteins were categorized by their functional annotation, such as that described for *Arabidopsis thaliana* (Bevan et al., 1998).

Prediction of phosphorylation sites of identified phosphoproteins

Putative phosphorylation sites of the proteins that had been experimentally identified as phosphorylated were predicted using NetPhos v2.0 (www.cbs.dtu.dk/services/NetPhos/) and the ScanProsite tool of the Prosite database, release [20.78] (www.expasy.org). Additional database searches using Phospho.ELM (www.phospho.elm.eu.org), BRENDA (www.brenda-enzymes.org), and PubMed (http://www.ncbi.nlm.nih.gov/PubMed/) were also carried out.

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