Research Article

Gel-based proteomics approach for detecting low nitrogen-responsive proteins in cultivated rice species

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ABSTRACT

Nitrogen fertilization is essential for increasing rice production to meet the food demands of increasing world's population. We established an *in vivo* hydroponic rice seedling culture system to investigate physio-biochemical/molecular responses of various rice japonica and indica cultivars to low nitrogen (N). Three-week-old seedlings grown in Yoshida's nutrient solution manifested stable and reproducible symptoms, such as reduced shoot growth and length under low N. Out of 12 genetically selected cultivars, 11 cultivars showed varied degrees of growth reduction response to applied N (4 and 40 ppm N for treatment and control, respectively), whereas one cultivar (no. 12) showed similar growth as the control though its leaf width was smaller than control. Leaves of a representative low N-responsive cultivar (BG90-2) were sampled for revealing protein profiles between low and normal (control) N application by two-dimensional gel electrophoresis (2-DGE). Forty-one proteins were identified with MALDI-TOF-MS and nESI-LC-MS/MS. Assignment of proteins into major (energy metabolism, photosynthesis and oxidative stress) and minor functional categories, revealed many novel low N-responsive proteins, including those having energy/photosynthesis- and defense/stress- and iron homeostasis-related functions. Results suggest the usefulness of proteomics in identifying novel N-responsive proteins and may provide potential markers for rice response to low N. **[Physiol. Mol. Biol. Plants 2009; 15(1) : 31-41]** *E-mail : rakwal-68@aist.go.jp*

Key words : *Rice, Two-dimensional gel electrophoresis, Mass spectrometry, Low nitrogen*

Abbreviations : *APX, Ascorbate peroxidase, CHAPS, 3-[(3-cholamidopropyl) dimethylaminol]-1-propanesulfonate, DTT, dithiothreitol, LSU, Large subunit, SOD, Superoxide dismutase, SSU, Small subunit*

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food crop for more than one-third of the world's population, accounting for 20 % of total calories consumed globally in 2000 [Word rice statistics from International Rice Research Institute, http://www.irri.org/science/ricestat/index.asp]. Moreover, it's a crop that has immense socio-economic impact on the people that grow it in South-East Asia; it has shaped the cultures and the lives of millions of people on our planet. As the rice plant is cultivated widely, it is also the most likely to endure a variety of stresses in the environment, which result in damage to the plant and ultimately affects yield, a critical point in rice cultivation.

In crop production, nitrogen (N) is one of the most essential elements for plant growth and high yield. It is usual practice for most farmers to use high nitrogen levels in order to obtain better yields. N fertilization is also a key ingredient in increasing rice production. With the introduction of high-yielding rice varieties (with high N fertilization) it has been possible to increase crop yields; however it has resulted in large increases in N

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use. However, in rice cultivation, the environment for plant growth is not very conducive for uptake of N fertilizers, resulting in reduced efficiency and N losses; combined with fertilizer imbalances and other macronutrient deficiencies, rice can be classified as a low N-use efficiency crop (Von Uexkull, 1993). To overcome this problem, rice cultivars have been subject to N-management strategies, including genetic manipulation. It should be emphasized that the acceptance and adoption of N-management strategies, often associated with high labor requirements, has not been very encouraging. There is always the risk in over-fertilization that it is not a very practical or economical approach to address the problems of mineral nutrition in general. Thus, there is an immediate and pressing need for identifying/developing N-efficient genotypes (Singh *et al.*, 1995, Broadbent *et al.*, 1987). Although not perfect, a genomic approach via genetic selection and plant breeding techniques is helping to develop rice varieties resistant to plant pathogens/pests, and abnormal environmental conditions, including nutrient deficiencies, drought and salinity. Recently, the first genetic selection approach for improving N-use efficiency in rice has been published (*Oh et al.*, 2005).

As N losses vary greatly between upland and flooded rice growth conditions, there is a great potential for increasing N uptake efficiency in rice. However, with the genetic selection procedures, time is a big constraint. Although we get a fair idea of how a cultivar behaves under a particular growth condition (N levels in our study), it is almost impossible to identify the underlying molecular components that help the rice plant to adapt or survive the stress/limiting factors. We believe that using high-throughput complementary genetic and proteomics approaches, the identification of such molecular components will serve in assigning potential markers in rice breeding/genetic selection programs. Today, proteomics is one of the leading "omic" technologies being used to study globally expressed proteins in a given tissue (Park, 2004; Agrawal *et al.*, 2005a,b,c; Agrawal and Rakwal, 2006).

Our proteomics-based approach for rice N-use efficiency uses a system – a hydroponic culture technique (Kim *et al.*, 2005) to analyse rice cultivar responses to low N application – in order to examine the molecular (protein) components affected therein. This is the first such study in rice and among plants in general. Our results discuss the correlation between identified proteins and low N-conditioned growth of rice seedlings, especially in the leaf tissues that were investigated in the present study.

MATERIALS AND METHODS

Plant materials

Rice (*Oryza sativa* L. cultivars japonica and indica) cultivars used were as follows: 1-Sobee, 2-Iipum, 3-Hwaseong, 4-Sampyeong, 5-Yangjo, 6-Palgong, 7-Samgiyeon3, 8-BG90-2 (indica), 9-Milyang182, 10-Ilpume(MNU), 11-SR19663, 12-SR19616. The cultivar numbers mentioned here are the same as shown in Figures 1-3. The seeds were stored in the cold room (4 ºC) till planting.

Hydroponic culture

Rice seeds were surface sterilized in 10 % H_2O_2 for 10 min and 70 % ethanol for 5 min, followed by washing in distilled water. The seeds were soaked in distilled water and germinated at 30 ºC in an incubator for 48 h. Uniformly germinated seeds were transferred to a stainless steel net stretched over a 6l plastic pot containing distilled water, and placed in an environmentally controlled growth chamber with a photoperiod of 12 h light/dark regime at 25/20 ºC, light intensity of 180 mE $m^{-2}s^{-1}$, and 60 % relative humidity. The seedlings were hydroponically-cultured for one week in distilled water, followed by replacement of distilled water with Yoshida's nutrient solution (YS), pH 5.8 (Hoai *et al.,* 2003; Agrawal *et al.*, 2002). The nutrient solution per dm^3, contains 40 mg each of N (nitrogen), K (potassium), Ca (calcium), and Mg (magnesium), 10 mg of P (phosphorus), 0.5 mg of Mn (manganese), 0.05 mg of Mo (molybdenum), 0.2 mg of B (boron), and 0.01 mg each of Zn (zinc) and Cu (copper), as NH_4NO_3, K_2SO_4, $CaCl_2$, $MgSO_4$, NaH_2PO_4, $MnCl_2$, $(NH_4)_6Mo_7O_{24}$, H_3BO_3, $ZnSO_4$. The nutrient solution contained 4 ppm N for low nitrogen treatment, while the control contained 40 ppm N (standard level in this study). For sampling, the fully expanded 3^{rd} /4^{th} leaves were harvested at 21 days from 5-6 randomly selected seedlings in two replications, immediately frozen in liquid nitrogen, and stored at -80 ºC until required for analysis.

Growth parameters

Rice seedlings were harvested randomly to measure growth parameters, such as the shoot fresh weight and lengths of shoot (the whole seedling above the seed). Values are presented as means from ten replications. Statistical analysis was performed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Extraction of total protein

Extraction of total protein was performed using trichloroacetic acid/acetone extraction protocol followed by solubilization in a modified lysis buffer (Agrawal and Rakwal, 2006). Leaf samples pooled from individual seedlings in two replications were placed in liquid nitrogen and ground thoroughly to a fine powder with a mortar and pestle (pre-cooled). The tissue powder (ca. 100 mg) was transferred to sterile tubes containing trichloroacetic acid acetone extraction buffer [TCAAEB, acetone containing 10 % (w/v) TCA, and 0.07 % 2-mercaptoethanol (2-ME)], and the proteins were precipitated for 1 h at -20 ºC, followed by centrifugation at 15,000 rpm for 15 min at 4 ºC. The supernatant was decanted, and the pellet was washed twice with wash buffer (acetone containing 0.07 % 2-ME, 2 mM EDTA, and EDTA-free proteinase inhibitor cocktail tablets (Roche) in a final volume of 100 ml buffer), followed by removal of all the acetone; the pellet was kept at –80 ºC for at least 24 h. The pellet was subsequently solubilized in lysis buffer (LB)-TT [7M urea, 2M thiourea, 4 % (w/v) 3-[(3-cholamidopropyl) dimethylaminol]-1-propanesulfonate, 18 mM Tris-HCl (pH 8.0), 14 mM trizma base, two EDTA-free proteinase inhibitor cocktail tablets in a final volume of 100 ml buffer, 0.2 % (v/v) Triton X-100 (R), containing 50 mM dithiothreitol (DTT)], and incubated for 20 min at 4 ºC with occasional vortexing, and centrifuged at 15,000 rpm for 15 min at 10 ºC. The supernatant was used for protein determination using a Coomassie Plus™ (PIERCE, Rockford, IL, USA) protein assay kit with bovine serum albumin as the standard, and stored in aliquots at -80 ºC till analyzed by 2-DGE.

Two-dimensional gel electrophoresis

2-DGE was carried out using pre-cast IPG strip gels (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on an IPGphor unit (GE Healthcare Bio-Sciences AB) followed by the second dimension using hand-cast (SDS-PAGE) gels on a Nihon Eido (Tokyo, Japan) vertical electrophoresis unit. The extract containing 100 µg total soluble protein was mixed with LB-TT containing 0.5% (v/v) pH 4-7 IPG buffer (GE Healthcare) to bring to a final volume of 250 µl. A trace of bromophenol blue (BPB) was added and the whole mixture was kept at room temperature (RT) for 5 min, vortexed, and centrifuged at 15,000 rpm for 15 min at 10 ºC followed by pipetting into a 13 cm strip holder tray. IPG strips (pH 4-7; 13 cm) were carefully placed onto the protein samples, covered with a lid, and placed into the IPGphor unit. The IPG strips were placed gel-face down onto the protein samples avoiding air bubbles and allowed to passively rehydrate with the protein samples for 1 h 30 min, followed by overlaying the IPG strips with 700 µl cover fluid, and this was directly linked to a five-step active rehydration and focusing protocol (13 cm strip). The whole procedure was carried out at 20 ºC, and a total of 60500 Vh was used for the 13 cm strip. Following IEF, the IPG strips were removed from the strip holder and blotted on kimwipe to remove the cover fluid. The IPG strips were immediately used for the second dimension or stored at -20 ºC.

The strip gels were incubated in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS) containing 2 % (w/v) DTT for 10 min (x 2 times) with gentle agitation, followed by incubation in the same equilibration buffer supplemented with 2.5 % (w/v) iodoacetamide for the same time periods as above at RT. Preceding the second dimension separation, IPG strips were then rinsed with cathode running buffer [0.025 M Tris, 0.192 M glycine and 0.2 % (w/v) SDS] and placed onto polyacrylamide gels and overlaid with overlay agarose solution [60 mM Tris-HCl, pH 6.8, 60 mM SDS, 0.5 % (w/v) agarose, 0.01 % (w/v) BPB]. The lower anode buffer contained 0.05 M diethanolamine and 0.05 M acetic acid. SDS-PAGE (4 % T, 2.6 % C stacking gels, pH 6.8 and 12.5 % T, 2.6 % C separating gels, pH 8.8) as the second dimension was carried out at constant current of 30 mA/ per gel for ca. 2.5 h. The stacking and separating gel buffer concentrations were 0.125 M Tris-HCl, pH 6.8, and 0.375 M Tris-HCl, pH 8.8, respectively. Molecular masses were determined by running standard protein markers (2.0 µl) (DualColor PrecisionPlus Protein™ Standard; Bio-Rad). For each sample, duplicate IPG strips and polyacrylamide gels were run under the same conditions.

Protein visualization and image analysis

The protein spots were visualized by staining the polyacrylamide gels with silver nitrate using a PlusOne Silver staining kit purchased from GE Healthcare Bio-Sciences AB. Protein patterns in the gels were recorded as digitalized images using a digital scanner (Canon CanoScan 8000F, resolution 300 dpi) and saved as TIFF files. The gels were quantitated in profile mode as instructed in the operating manual of the ImageMaster 2D Platinum software (GE Healthcare Bio-Sciences AB). The spots were excised from the 2-D gels, using a gel picker (One Touch Spot Picker, P2D1.5 and 3.0, The Gel Company, San Francisco, CA, USA), transferred to sterilized eppendorf tubes and stored at -30 ºC.

Mass spectrometry

The excised gel spots were destained with 100 µl of destain solution [30 mM potassium ferricyanide (Sigma), in 100 mM sodium thiosulfate (Merck)], with shaking for 5 min. After the solution was removed, the gel spots were incubated with 200 mM ammonium bicarbonate (Sigma) for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min and then rehydrated with 20 µl of 50 mM ammonium bicarbonate containing 0.2 µg modified trypsin (Promega, Madison, WI, USA) for 45 min on ice. After removal of solution, 30 µl of 50 mM ammonium bicarbonate was added and the digestion was performed overnight at 37 ºC. The peptides were desalted and concentrated using C18 nanoscale (porus C18) column (home made). For the analysis of MALDI-TOF MS by peptide-mass fingerprinting (PMF) method, the peptides were eluted by 0.8 µl of matrix solution [70 % acetonitrile (Merck), 0.1 % TFA (Merck), 10 mg/ml alpha-cyano-4-hydroxycinamic acid (Sigma)]. The eluted peptides were spotted onto a stainless steel target plate. Masses of peptides were determined using MALDI-TOF MS (Model M@LDI-R; Micromass, Manchester, UK). Calibration was performed using internal mass of trypsin auto digestion product (m/z 2211.105). To identify the protein, peptide masses from MALDI-TOF MS were matched with the theoretical molecular weight of peptides for proteins in the NCBI database using MASCOT software (www.matrixscience.com).

For analyses by MS/MS, 15 µl of the peptide solutions from the digestion supernatant was diluted with 30 µl in 5 % formic acid, loaded onto the column, and washed with 30 µl of 5 % formic acid. Peptides were eluted with 2.0 µl methanol/water/formic acid (50/49/1, v/v/v) directly into a precoated borosilicate nanoelectrospray needles (EconoTip™, New Objective, USA). MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The source temperature was 80 ºC. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles in the ion source combined with a nitrogen back-pressure of 0-5 psi to produce a stable flow rate (10–30 nl/min). The mass spectrometer operated in an automatic data-dependent MS/MS to collect ion signals from the eluted peptides. In this mode, the most abundant peptide ion peak with doubly or triply charged ion in a full scan mass spectrum (m/z 400–1500) was selected as the precursor ion. Finally, an MS/MS spectrum was recorded to confirm the sequence of the precursor ion using collision-induced dissociation (CID) with a relative collision energy dependant on molecular weight. The cone voltage was 40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of 6~7 x 10^{-5} mbar and the collision energy was 20–30 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter. The data were processed using a Mass Lynx Windows NT PC system.

To identify the protein, peptide masses from MALDI-TOF MS were matched with the theoretical molecular weight of peptides for proteins in the NCBI database using MASCOT software. Also, all MS/MS spectra recorded on tryptic peptides derived from spot were searched against protein sequences from NCBInr and EST databases using the MASCOT search program (www.matrixscience.com).

Immunoblotting

Electrotransfer of proteins on gel to a polyvinyldifluoride (PVDF) membrane (NT-31, 0.45 mM pore size; Nihon Eido) was carried out at 1mA/cm^2 using a semi-dry blotter (Nihon Eido) exactly as described before (Agrawal *et al.*, 2002; Rakwal *et al.*, 2003). Immunostaining was performed using spinach anti-APX [AP8, Mouse monoclonal, a kind gift from Dr. Akihiro Kubo and Dr. Hikaru Saji, National Institute for Environmental Studies (NIES), Tsukuba, Japan] and anti-superoxide dismutase (SOD; Rabbit polyclonal, Abcam Ltd.) antibodies. The ECL+plus Western Blotting Detection System protocol was used for blocking, primary and secondary antibody incubation, and stripping the antibody, followed exactly as described (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Immunoassayed proteins were visualized on an X-ray film (X-OMAT AR, Kodak, Tokyo, Japan) using an enhanced chemiluminescence protocol according to the manufacturer's directions (Amersham).

RESULTS AND DISCUSSION

Hydroponic culture and physiological changes in rice seedlings

The suitability of hydroponic culture system in studying the low N response was evaluated. The low N-triggered physiological/morphological changes to rice growth, such as reduction in seedling growth, in 12 selected rice cultivars (Oh *et al.*, 2005) were checked. Hydroponically culturing seedlings has a major advantage over experiments that use rice grown in the soil: Yoshida's nutrient solution (YS) nutrient solution will have actual

N concentration with roots in direct contact with N, and the system can be easily maintained in controlled conditions for better reproducibility. Figure 1 shows the effect of 4 ppm N (low N; treatment) on 21-day-old rice seedling growth; the shoot fresh weight was markedly reduced (A) along with shortening of the shoot length (B) in cultivars 1-11, whereas cultivar 12 showed a certain degree of adaptation to low N with almost similar growth parameters. It should be noted that the leaf width was considerably reduced (ca. 30 % of the control; 40 ppm N; standard level) in all the 12 cultivars, and the shoots were of a slight pale green color compared to the dark green color of the shoots in the control. These morphological data correlate well with the effect of low N in rice plants. Interestingly, the cultivar 8 (indica BG90-2) which was chosen as a representative cultivar for the subsequent proteomic analysis based on its reduced fresh weight and shoot length, has been adapted

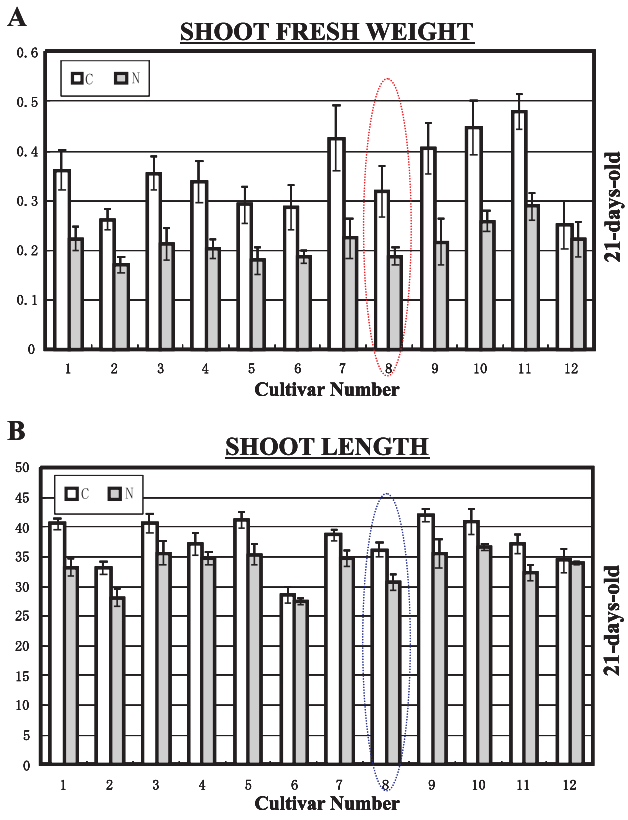

Fig. 1. Low nitrogen (N)-triggered changes in physiological parameters of rice seedlings. The shoot fresh weight and length of each cultivar (numbered 1-12) was measured as described in materials and methods. White bars – control (40 ppm N), and black bars – low N (4 ppm N). Values are means from ten replications. Error bars indicate standard deviations.

to low N conditions, was shown to produce high grain yield even under low N, and revealed high total dry matter contents (Oh *et al.*, 2005). The cultivar 12, which was almost similar in growth to the control, was also found to be an N-efficient genotype that produced high grain yield at both low and standard levels of N (Oh *et al.*, 2005). Therefore using the current evaluation system presented here, we can distinguish very early in the life cycle, between the cultivars selected for N-use efficiency by genetic selection in the field, and subsequently investigate molecular differences therein.

Low nitrogen-responsive proteome

The main aim of the study – to develop a proteomics approach for low N response in rice seedling – was fulfilled by using the leaf tissues from a representative cultivar 8 (BG90-2) as the source material for generating 2-D gel protein profiles (Figure 2). To investigate the temporal changes of protein profiles, 2-DGE analysis of total proteins from two biological replicates was performed. The representative silver nitrate-stained 2-D gels are shown in Figure 2. Quantitative image analysis using the ImageMaster software revealed, that a total of 50 silver nitrate-stained protein spots changed their intensities (equal to or greater than 1.5-fold over the WT spot) significantly. These changed spots were verified by visually checking the gel images under high-resolution. A low N-responsive cultivar 7 (japonica, Samgiyeon 3) showed a similar protein profile as cultivar 8, suggesting that varietal differences in at least two species of indica- and japonica-type do not significantly alter the low N response proteins profiles (data not shown). In cultivar 8, a total of 46 and 4 protein spots were induced and suppressed, respectively, when compared to the untreated control. The excised protein spots were analyzed by mass spectrometry (MS using MALDI-TOF or Q-TOF) resulting in the identification of 41 proteins (Table 1). These proteins were classified into various functional categories and as discussed below:

Major functional protein categories

Three major functional categories were assigned based on the changes in proteins in leaf under low N conditions:

Energy metabolism

This is by far the largest category changed in response to low N, which is logical as the plants show reduced growth, and there is a need for generating more energy for compensating reduced growth and leaf area. In the

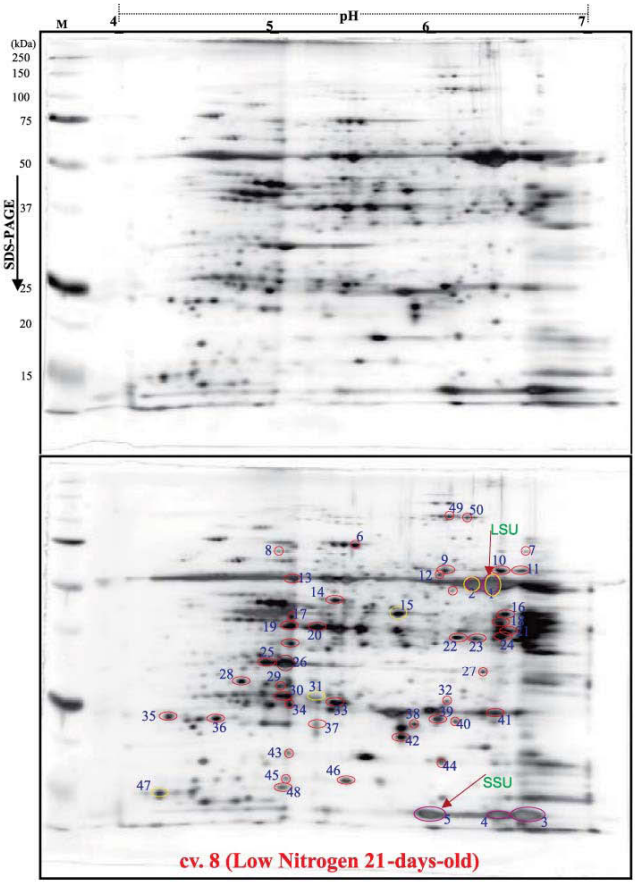

Fig. 2. Representative silver-stained 2-DGE protein profiles of leaf total protein extract from cultivar 8 reveals differentially expressed proteins in rice seedlings with low N. The control and low N gels are marked in blue and red respectively. 2-DGE was carried out as described in materials and methods. Numbered spots (1-50) show proteins selected for mass spectrometry; these spots (red: increased and yellow: decreased, over the control) were selected based on their induction/suppression in two independent gel replications. The arrows mark the RuBisCO large subunit (LSU, spot no. 1) and small subunit (SSU, spot no. 5), which also serve landmark proteins. All the RuBisCO proteins (spots 1-5) are also marked in pink for reference.

category, we could identify a total of 16 protein spots, belonging to various subcategories mentioned below.

ATP synthesis: Spots 9, 12, 13 and 45 are components of the ATP synthase complex, which plays a central role in energy transduction in bacteria, chloroplasts and mitochondria.

Carbon fixation: Spot 6 is a putative transketolase 1 (EC 2.2.1.1), involved in the pentose phosphate pathway responsible for the production of NADPH and several sugar phosphate intermediates. As a part of the carbohydrate transport and metabolism, this enzyme is critical to carbon dioxide (CO_2) fixation in photosynthetic organisms. Spot 20 is a fructose-bisphosphate aldolase class-I enzyme, which catalyzes the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

Citrate cycle (TCA cycle): Spot 10 is a putative dihydrolipoamide dehydrogenase precursor of pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes. Spot 22 and 23 are malate dehydrogenases, members of the NAD-dependent 2-hydroxycarboxylate dehydrogenases family, and are one of the key enzymes in the citric acid cycle, facilitating both the conversion of malate to oxaloacetate and replenishing levels of oxalacetate by reductive carboxylation of pyruvate.

Glycolysis and gluconeogenesis: Spot 15, 18 and 21 are similar to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a tetrameric NAD-binding enzyme. Spot 30 and 33 are putative triosephosphate isomerases, catalyzing a vital step in glycolysis converting D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate; this enzyme also plays an important role in fatty acid biosynthesis and the pentose shunt.

Nitrogen metabolism: Spot 41 is a carbonic anhydrase (carbonate dehydratase), a family of zinc-containing enzymes that catalyze the rapid interconversion of CO_2 and water into carbonic acid, protons, and bicarbonate ions.

Sulfur metabolism: The protein spot 19 is a putative plastidic cysteine synthase 1 of the pyridoxal-phosphate dependent enzyme family (EC 4.2.99.8). This enzyme is responsible for the final step in cysteine biosynthesis, a key limiting step in the production of glutathione, which is a thiol implicated in resistance to various environmental stresses (May *et al.*, 1998).

Photosynthesis

The second largest category is related to photosynthesis due to pale green color of leaves and associated energy requirements of the plants under low N. Protein spots 1-5 (ribulose-1,5-bisphosphate carboxylase/oxygenase, large and small subunits, RuBisCO LSU and SSU), 17, 25, 26 and 42 belong to the category of photosynthesis. RuBisCO is the major leaf protein that plays bifunctional roles as a carboxylase for mediating photosynthetic CO_2 assimilation, and as an oxygenase for catalyzing the

Table 1. Differentially expressed low nitrogen-responsive proteins and their identification by MS.

Spot No	MW (kDa)/p*I*	Protein Name	MW (da)/p*I*	Method	Accession	Functional Category
1	52/6.4	*Ribulose-1,5-bisphosphate carboxylase/ oxygenase,* LSU	49462/6.60	MALDI-TOF	AAU05134	P
2	52/6.2	*Ribulose-1,5-bisphosphate carboxylase/oxygenase,* LSU	49462/6.60	MALDI-TOF	AAU05134	P
3	12/6.5	Chain W, crystal structure of activated rice RuBisCO	15091/5.89	MALDI-TOF	1WDD_W	P
4	12/6.3	Ribulose bisphosphate carboxylase, SSU	20025/8.10	MALDI-TOF	ABA97181	P
5	12/5.9	Ribulose bisphosphate carboxylase, SSU	20025/8.10	MALDI-TOF	ABA97181	P
6	69/5.4	Putative transketolase 1	69407/5.43	MALDI-TOF	XP_550612	EM
7	66/6.5	No signal	–	–	–	–
8	67/4.9	Putative FtsH-like protein Pftf precursor	72607/5.54	MALDI-TOF	BAD45446	PTM/PT/C
9	59/6.0	ATPase alpha subunit	55202/5.95	MALDI-TOF	NP_922436	EM
10	58/6.4	Putative dihydrolipoamide dehydrogenase precursor	53009/7.21	MALDI-TOF	NP_908725	EM
11	58/6.5	Catalase (EC 1.11.1.6) catA	57066/6.75	MALDI-TOF	CSRZ	OS
12	57/6.0	ATP synthase F0 subunit 1	55624/5.85	MALDI-TOF	BAC19899	EM
13	55/5.0	Putative ATP synthase beta chain	59012/5.95	MALDI-TOF	XP_475868	EM
14	48/5.3	Translational elongation factor Tu	50610/6.19	MALDI-TOF	XP_466527	T/RSB
15	44/5.7	EST C74302(E30840)	47537/6.22	MALDI-TOF	XP_493811	EM
16	44/6.4	OSJNBa0053K19.11	44340/8.53	MALDI-TOF	XP_473945	AATM
17	43/5.0	Chloroplast inner envelope protein	47305/5.68	MALDI-TOF	ABB47817	P
18	42/6.3	Cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPDH	23480/7.88	Q-TOF	AAN59792	EM
19	41/5.0	Putative plastidic cysteine synthase 1	42104/6.28	MALDI-TOF	BAD87047	EM
20	40/5.2	Fructose-bisphosphate aldolase class-I	39556/6.85	MALDI-TOF	AAX95073	EM
21	39/6.4	OSJNBa0036B21.24	43031/7.62	MALDI-TOF	XP_472744	EM
22	37/6.1	Putative malate dehydrogenase	35667/8.74	MALDI-TOF	NP_917241	EM
23	37/6.2	Putative malate dehydrogenase	35642/8.22	Q-TOF	XP_475913	EM
24	38/6.4	RNA binding protein, putative	44815/9.06	MALDI-TOF	ABA97622	UA

Table 1. Continued .....

Spot No	MW (kDa)/p*I*	Protein Name	MW (da)/p*I*	Method	Accession	Functional Category
25	32/4.8	Oxygen-evolving complex protein 1	26603/5.13	MALDI-TOF	2002393A	P
26	32/5.0	Putative 33 kDa oxygen evolving protein of photosystem II	35068/6.10	MALDI-TOF	NP_918587	P
27	31/6.2	Jacalin homolog	33220/6.13	MALDI-TOF	ABA97248	D/S
28	30/4.7	No signal	–	–	–	–
29	29/4.9	Ascorbate peroxidase	27215/5.21	MALDI-TOF	XP_479627	OS
30	27/4.9	Putative Triosephosphate isomerase	32715/6.96	MALDI-TOF	BAD33340	EM
31	27/5.2	Ferritin	27970/5.47	MALDI-TOF	AAM74942	IH
32	26/5.0	No signal	–	–	–	–
33	26/5.3	Putative triosephosphate isomerase	27274/5.38	MALDI-TOF	XP_462797	EM
34	26/5.0	No signal	–	–	–	–
35	24/4.2	No signal	–	–	–	–
36	23/4.5	2-Cys peroxiredoxin	28307/5.67	MALDI-TOF	CAJ01693	OS
37	22/5.2	Germin-like protein 1	22017/6.01	Q-TOF	BAA74702	D/S
38	22/5.8	No signal	–	–	–	–
39	23/6.0	Dehydroascorbate reductase	23726/5.81	MALDI-TOF	AAV44199	OS
40	23/6.1	No signal	–	–	–	–
41	24/6.3	Carbonic anhydrase	29498/8.41	MALDI-TOF	NP_917149	EM
42	21/5.7	Unknown protein	25552/5.85	MALDI-TOF	XP_483228	P
43	18/5.0	Putative polyprotein	220543/9.30	MALDI-TOF	AAU44282	UA
44	17/6.0	Rieske Fe-S precursor protein	24211/8.54	MALDI-TOF	BAD30907	P
45	16/5.0	ATP synthetase epsilon subunit	15221/5.20	Q-TOF	BAA00335	EM
46	16/5.4	Putative superoxide dismutase [Cu-Zn]	20633/5.79	Q-TOF	XP_483791	OS
47	14/4.1	No signal	–	–	–	–
48	15/4.9	No signal	–	–	–	–
49	105/6.0	Putative glycine dehydrogenase	112382/6.51	MALDI-TOF	NP_916596	P
50	101/6.1	Putative glycine dehydrogenase	112382/6.51	MALDI-TOF	NP_916596	P

AATM, amino acid transport and metabolism; D/S, defense/stress; EM, energy metabolism; IH, iron homeostasis; LSU, large subunit; OS, oxidative stress; P, photosynthesis; PTM/PT/C, post-translational modifications/protein turnover/chaperones; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit; T/RSB, translation/ribosome structure and biogenesis; UA, unassigned. Italics denotes supressed protein spots.

first step of the photorespiratory pathway in plants. Decrease in the LSU of RuBisCO correlated well with the pale green leaf color. However, we did not find any significant change in the RuBisCO SSU under low N. On the other hand, spots 17, 25, 26 and 42 showed increased accumulations under low N. Spot 17 is a novel chloroplast inner envelope protein identified for the first time in rice. Spots 25 and 26 are the oxygen (O_2) evolving proteins (OEPs) of photosystem II (PSII) and OEP is involved in O_2 evolution and PSII stability (Sugihara *et al.*, 2000). These proteins have been previously shown to be induced by salt stress (Kim *et al.*, 2005) but reduced by ozone and sulfur dioxide fumigation (Agrawal *et al.*, 2002; Rakwal *et al.*, 2003). Spot 42 is also a novel protein possessing a domain region related to PsbP. This family consists of the 23 kDa subunit of O_2 evolving system of PSII or PsbP from various plants (where it is encoded by the nuclear genome) and cyanobacteria. The 23 kDa PsbP protein is required for PSII to be fully operational *in vivo*, it increases the affinity of the water oxidation site for Cl^- and provides the conditions required for high affinity binding of Ca^{2+}. These results suggest their potential role in enhanced photosynthesis to counteract the reductions in RuBisCO LSU or reduced photosynthesis in general. Spot 49/50 is a putative glycine dehydrogenase, is involved in photorespiration, and which has been suggested to be important for maintaining electron flow to prevent photoinhibition under stress condition (Wingler *et al.*, 2000). It has been previously reported that rice plants will become sensitive to chilling stress if glycine dehydrogenase is degraded that causes inhibition of photorespiration. Another novel protein is the Rieske Fe-S precursor protein (spot 44) of the cytochrome b_6/f complex is an indispensable component of the photosynthetic electron transport chain in chloroplasts. This protein was suggested to represent an ancient or intermediate state in the evolution of transport pathways at the thylakoid membrane in chloroplasts (Molik *et al.*, 2001). Induction of these last two proteins in cultivar 8 under low N suggests their use by cells in overcoming the effect of photoinhibition.

Oxidative stress

Reactive oxygen species (ROS), highly toxic byproducts of O_2 metabolism, are carefully regulated metabolites capable of signaling and communicating key information to the genetic machinery of the cell, and are also provoked by a variety of natural and stress stimuli that can seriously disrupt normal metabolism through oxidative damage to cellular components (Scandalios, 2002). A total of 5 oxidative stress-related proteins were identified in this study. Spot 11 is a catalase (EC 1.11.1.6; catA). Catalase uses hydrogen peroxide (H_2O_2) to oxidize toxins including phenols, formic acid, formaldehyde and alcohols. In plants, peroxisomes, which are involved in photorespiration, act quickly to remove H_2O_2 for preventing damage to cellular machinery. Its importance in normal cellular functioning cannot be underestimated. Another antioxidant enzyme SOD (EC 1.15.1.1) is a very necessary part of the antioxidant defense system in cells. Spot 46 is a putative Cu-Zn SOD, a ubiquitous metalloprotein that catalyzes the dismutation of superoxide radicals to O_2 and H_2O_2. Interestingly, 2-DGE coupled with immunoblotting resulted in the detection of increased amounts of SOD proteins cross-reacting with an anti-SOD antibody (Figure 3). Spot 29 is an ascorbate peroxidase (EC 1.11.1.11; APX), which converts H_2O_2 to water and O_2, and a key enzyme of H_2O_2-detoxification system in chloroplasts and cytosol, constituting an important component of the ascorbate-glutathione cycle. However, as compared to the observed induction of SOD proteins, no such increase could be seen for APX proteins, indicating that the SOD is a major player in the antioxidant defense in leaf of rice seedlings grown under low N. Thus, we have a potential maker protein (in Cu-Zn SOD) from the antioxidant family for low N response. We also identified for the first time from rice, another antioxidant enzyme, namely 2-Cys peroxiredoxin (spot 36). This family contains proteins

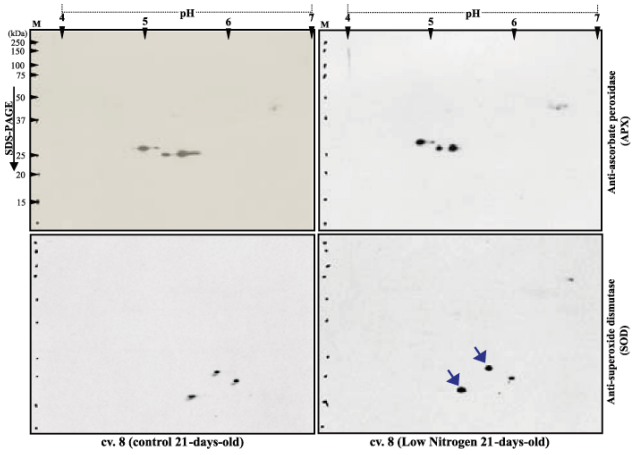

Fig. 3. Strong induction of cross-reacting superoxide dismutase (SOD), but not ascorbate peroxidase (APX) proteins in the low N-responsive cultivar 8. 2-DGE immunostaining with anti-APX and anti-SOD antibodies was carried out using the same PVDF membranes to comparatively detect the signals of cross-reacting proteins from rice leaf. The blue arrows mark the increased low molecular weight SOD cross-reacting protein spots in the low N treatment.

related to alkyl hydroperoxide reductase and thiol specific antioxidant. Spot 39 is a dehydroascorbate reductase and has glutathione dehydrogenase (ascorbate) activity/ antioxidant activity that also works to detoxify H_2O_2 in cells. Identification of most of the antioxidant enzymes strongly induced under low N conditions suggests their importance in sensing and counteracting the generated oxidative stress via the increased photorespiration and energy generation mechanisms mentioned above.

Minor functional protein categories

The remainder of the changed proteins spots are grouped under 6 categories as given below.

Amino acid transport and metabolism: Spot 16 is a glycine cleavage T-protein protein (aminomethyl transferase) belonging to a family of glycine cleavage T-proteins, part of the glycine cleavage multienzyme complex (GCV) found in bacteria and the mitochondria of eukaryotes; GCV catalyses the catabolism of glycine in eukaryotes.

Defense/Stress: Two novel proteins were identified here, including spot 27 as a jacalin homolog, which has a dirigent-like protein region, and this family contains a number of proteins which are induced during disease response in plants, including involvement in lignification. Spot 37 is a germin-like protein 1; germins and germine-like-proteins (GLPs) are known to function in pathogen resistance, but their involvement in defense against insect herbivores is poorly understood. It is quite interesting to see their induction under low N response in leaves, which suggests a potential link between N and plant vigor and ability to fight disease.

Iron homeostatis: Another novel finding was the spot 31, a ferritin, "iron storage protein", having the Eukaryotic Ferritin (Euk_Ferritin) domain. Ferritins are the primary iron storage proteins of most living organisms and members of a broad superfamily of ferritin-like diiron-carboxylate proteins. Previously, it has been shown that by introducing a soybean ferritin gene into rice, transgenic rice seeds stored three times more iron than the normal seeds (Goto *et al.*, 1999). Surprisingly it is one of the few proteins shown to be strongly decreased under low N conditions, suggesting its regulation by the availability of N in the system, and therefore may serve as an important marker protein for selection of N-efficient rice cultivars.

Posttranslational modifications, protein turnover, chaperones: Spot 6 a putative FtsH-like protein Pftf precursor. FtsH is the only membrane-bound ATP-dependent protease universally conserved in prokaryotes, and is part of the fundamental prokaryotic self-protection mechanism that checks if proteins are correctly folded. It also possesses regions associated with the AAA-superfamily of ATPases, having a wide variety of cellular activities, including membrane fusion, proteolysis and DNA replication, and the peptidase family M41. Its role remains undefined in the low N response, but it may be part of a protein synthesis and quality control in stressed leaf as discussed previously (Cui *et al.*, 2005).

Translation, ribosomal structure and biogenesis: Spot 14 is a translational elongation factor Tu (EF-Tu) belonging to the GTP-binding elongation factor family. These proteins promote the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis, and are predicted to be localized to the chloroplast.

Unassigned: Spot 24 is an RNA-binding protein, possessing putative NAD dependent epimerase/ dehydratase family and 3-beta hydroxysteroid dehydrogenase/isomerase family signatures. Spot 43 is retrotransposon gag protein. Their role in rice plant remains to be clarified.

CONCLUSIONS

N is a critical nutrient required for rice production. The study presented here is a novel approach for coupling the genetic-based selection of N-related genotypes with proteomics to unravel the molecular differences in rice plants grown under low N. As a preliminary study, high-throughput 2-DGE has resulted in the identification of numerous proteins from rice. Future studies at constructing 2-D gel reference map for use in comparative proteomics among cultivars for low N response will be necessary for precise identification of potential molecular protein markers to assist the breeders for screening N-efficient genotypes and help in understanding how rice adapts to low N availability.

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