JOURNAL OF INTEGRATED OMICS
A METHODOLOGICAL JOURNAL
HTTP://WWW.JIOMICS.COM

ORIGINAL ARTICLE | DOI: 10.5584/jiomics.v1i1.22

Proteomic analysis of nitrogen stress-responsive proteins in two rice cultivars differing in N utilization efficiency

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Received: 15 June 2010 **Accepted:** 9 September 2010 **Available Online**: 13 September 2010

ABSTRACT

Plant nitrogen utilization efficiency (NUE) has become critical important in modern agriculture, not only for crop growth and yield but also for reducing production cost. Moreover, one of the major negative environmental impacts of agricultural activities is associated with excessive nitrogen application. Improving NUE will ensure lower level of N fertilizer usage thus reduce environmental contamination. In order to understand the NUE mechanism of rice, the largest food crop in the world, a systematic proteomic study of investigating the nitrogen stress-responsive proteins in two rice cultivars differing in NUE is conducted. Four leaf-old seedlings were treated with normal nutrition solution and N-free solution for 12 h, 3 d and 7 d. Total proteins of leaves were extracted and separated by two-dimensional gel electrophoresis. Although more than 1000 protein spots were reproducibly detected, only a very small proportion of spots showed differential expression, including 10 and 24 up-regulated, 2 and 12 down-regulated in the two cultivars Chunyou 58 and Yongyou 6, respectively. This indicates that relatively simply biochemical pathways maybe involved with NUE thus the NUE as a trait maybe efficiently manipulated. Mass spectrometry based peptide mass fingerprinting (PMF) procedure identified 31 protein spots. Six stress-induced proteins were found, including DegP2, harpin binding proteins, Heat shock-related proteins, glutathione S-transferase GSTF14, Fibrillin-like protein and Glyceraldehyde-3-phosphate dehydrogenase. Apart from the stress related proteins, the other differential proteins identified were mainly these involved in the regulation of the main leaf biological function, photosynthesis metabolism, such as Rubisco activase, RuBisCo large subunit, etc. The study also detected two novel proteins, harpin binding protein and oryzains gamma precursor. The current study reveals new insights into N stress response and theoretical bases for improving NUE of rice crop.

Keywords: Mass spectrometry, rice (*Oryza sativa* L.), nitrogen utilization efficiency, Two-dimensional gel electrophoresis.

1. Introduction

A key element in modern agriculture is the application of nitrogen fertilizer, which has dramatically increased the crop yield [1]. In order to meet the food demand of the increased world population, application of nitrogen fertilizer in the world has been increased by 10 folds in the last half century, It was predicted that the increase trend will continue in this century, from 87 million tones in 2000 to 236 million tones in 2050 [2]. Meanwhile, most of the high yield varieties of the major crops developed in the last several decades had high nitrogen demand for the realization of yield potential [3]. On the other hand, less than half of the N fertilizers applied to the field was absorbed and utilized by crops; the majority of them was lost to the atmosphere or leached into groundwater, lakes and rivers, causing increasingly severe pollutions to the environments [4]. Therefore, development of crop cultivars with high N utilization is essential for agricultural sustainability and environmental protection.

The improvement of N fertilizer utilization could be realized by enhancing the ability of N uptake and/or increasing N utilization efficiency (NUE). For the former, a crop or a special cultivar has high ability of N uptake from the soils with low N concentration, which is referred as high uptake effi-

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ciency. For the latter, a crop or a cultivar may make the best use of N nutrient that the plant absorbed from soil for producing biomass or harvest organs, which is evaluated by grain yield or biomass production per unit nitrogen amount[1], expressed as NUEY (N utilization efficiency of yield) and NUEB (N utilization efficiency of biomass), respectively. Although NUEY is affected by many physiological processes, including nitrogen contribution to spikelet production during early panicle formation stage, and contribution to sink size by decreasing the number of degenerated spikelets and increasing hull size during the late panicle formation stage [5], the fact that higher rice yield is achieved mainly due to greater biomass production [6] provided the possibility to explore the relationship between NUEP and NUEY.

As an essential plant macronutrient, nitrogen is required for a variety of physiological processes. It comprises 1.5–2% of plant dry matter and approximately 16% of total plant protein [4]. For rice, the leaf N, about 75% of total plant N, is associated with chloroplasts, which are physiologically important in dry matter production through photosynthesis [7]. It is also an important constituent of many important compounds, including amino acids, proteins (enzymes), nucleic acids, chlorophyll and several plant hormones.

NUE is considered as the function of N in carbohydrate production, which is closely related to the C/N balance. For plants, N and C metabolism is tightly linked in most biochemical pathways, which involve in carbon fixation, nitrogen transfer and utilization etc. Although roots play a dominant role of nitrogen uptake, leaf is the major organ for carbon and nitrogen metabolism. N drives plant dry matter production through the control of both leaf area index (LAI) and leaf photosynthesis [8]. Moreover, the photosynthetic NUE (PNUE), which is dependent on the level of CO_2 saturation of Rubisco, is another important factor to consider when NUE is compared among different genotypes. At low N level, greater PNUE and NUE were found in C3 plants relative to C4 plants, whereas at high N level, the opposite is true [9]. Consequently, identification of the regulatory elements controlling the balance between N available to maintain photosynthesis and the reallocation of the remobilized N to sink organs such as developing young leaves is of major importance, particularly when N supply is restricted. Therefore, the complex regulators of N related to primary CO_2 assimilation, the photo-respiratory processes, and as storage pool need further investigation for optimizing NUE under low N level [10-12]. In addition, the recent finding that synthesis, turnover, and degradation of Rubisco are subjected to a complex interplay of regulations renews the concept of the importance of N use and recycling by the plants [13]. Attempts have also been made to identify some of the components responsible for the physiological control of the 'stay-green' phenotype particularly in relation to NUE. For example, in both sorghum and maize, delayed leaf senescence allowed photosynthetic activity to be prolonged, which had a positive effect on N uptake capacity of the plants [14-16].

In general, a low or zero nitrogen application causes nutritional imbalance. Plants can perceive the stress signals and transmit them to the cellular machinery to activate adaptive responses. The adaptation is generally completed by regulating gene expressions. Proteome dynamics under the stress conditions reflects the regulatory gene expressions. In the current study, in order to understand the NUE mechanism of rice, the largest food crop of the world, we adopted a systematic proteomic approach to investigate the nitrogen stress-responsive proteins in two rice cultivars differing in NUE.

2. Material and methods

2.1 *Plant materials and stress treatments*

Seeds of two rice cultivars, Chunyou 58 (high NUE) and Yongyou 6 (Low NUE), were germinated and grown hydroponically in nutrient solution containing 2.9 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 1.0 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.7 mM $MgSO_4 \cdot 7H_2O$, 9.1 μM $MnCl_2 \cdot 4H_2O$, 0.52 μM $(NH_4)_6Mo7O_{24} \cdot 4H_2O$, 18 μM H_3BO_3, 0.15 μM $ZnSO_4 \cdot 7H_2O$, 0.16 μM $CuSO_4 \cdot 5H_2O$, 36 μM $FeCl_3 \cdot 6H_2O$. The pH value of the solution was adjusted to 5.5 using 1 M HCl or NaOH solution as required [17]. Half concentration of the nutrient solution was applied for the first 3 days and then changed to full nutrient solution. At the emergence of the fourth leaf, the seedlings were transferred into either a nutrient solution without N supply as stress treatment or a nutrient solution with the normal N concentration as control. Nutrient solutions were renewed every four days. The upper expanded leaves were harvested after 12 hours, 3 days, and 7 days after the treatment, and kept frozen in liquid nitrogen and kept at -80 ℃.

2.2 *Sample preparation*

Leaf proteins were extracted by phenol extraction coupled with ammonium acetate precipitation [18]. Three separate extractions were conducted from three leaf samples of each treatment. Briefly, 1) 0.4 g frozen plant tissue with 30 mg PVPP was grinded into a fine powder using cold pestle and mortar. 2) Suspend the powder in cold phenol extraction buffer (0.7 M sucrose; 0.1 M KCl; 0.5 MTris-HCl, pH 7.5 and 50 mM EDTA, 1% w/v DTT, pH 7.5; complete protease inhibitor cocktail (Roche Applied Science)), then add an equal volume of phenol saturated with Tris-HCl, pH 7.5, and after 30 min shake the mixture at 4℃. 3) Centrifuge at 5,000 g for 30 min at 4℃, then collect the upper phenolic phase; discard the lower aqueous phase. 4) Add extraction buffer to the collected phenolic phase; repeat steps 5-6 and then repeat Step 5 again. 5) Add 5 volumes of cold 0.1 M ammonium acetate in methanol to the collected phenol phase; stored at –20℃ overnight. 6) Centrifuge the sample for 30 min at 5000 g at 4℃ and carefully remove the supernatant with a pipette and discard. 7) Add 2 volumes (based on the volume of the last collected phenolic phase) of ice-cold methanol to wash

the pellet, centrifuge the sample for 10 min at 5,000 g at 4℃; repeat step 7 two more times to remove ammonium acetate and phenol, lipids and pigments and repeat step 9 twice again using acetone instead of methanol to replace. 8) Dry the pellet gently in a fume hood, and store the clear supernatants in aliquots at -80℃ until analyzed. The protein concentrations were measured by a Bradford assay using bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA).

2.3 Two-dimensional electrophoresis

Two-DE was performed according to the manual obtained from GE Healthcare Life Sciences (Little Chalfont, United Kingdom). Extracellular protein preparation containing 150 μg protein was separated by 2-DE using 24 cm immobilized pH gradient strips pH4-7 (GE Healthcare, Milwaukee, WI, USA). Briefly, sample was diluted with rehydration solution (8 M Urea, 2 M Thiourea, 4% w/v CHAPS, 20 mM w/v DTT, 0.5% v/v IPG buffer pH 4–7, 0.002% w/v bromophenol blue) to 0.5-1 mg protein per 100 μl. Immobiline DryStrip gels (pH 4-7, 24 cm; GE Healthcare Life Sciences) were then rehydrated with 450 mL of mixture solutions in 17 cm strip holders and electrofocused with the GE Healthcare Life Sciences IPGphor. Initially, all protein extracts were subjected to 2-DE. Among the three biological replicates of each leaf-treatment sample, the one with best 2-DE quality were chosen to run two times more 2-DE. About 100 mg of protein were loaded using in-gel rehydration. The focusing protocol was as follows: 50 mA per strip at 20 ℃; (i) rehydration with 30 V for 12 h;(ii) 500 V for 1 h (step and hold);(iii) 1000 V for 1 h (step and hold); and (iv) 8000 V for 10 h (step and hold) was applied until the total Vh reached 100 kVh. After IEF, the strips were equilibrated twice with gentle shaking for 15 min in SDS equilibration buffer.

The first step was performed in a equilibration solution containing 6 M urea, 30% w/v glycerol, 2% w/v SDS, 1% w/v DTT, 50 mM Tris-HCl buffer, pH 8.8 and 0.002% w/v bromophenol blue. The second step was performed in a solution modified by the replacement of DTT with 2.5% w/v iodoacetamide. When the equilibration was finished, the strips were loaded onto vertical SDS PAGE (12.5% T constant). The second dimension SDS electrophoresis was run using an Ettan DALTsix electrophoresis Unit (Amersham Biosciences). A denaturing solution (0.5% Agarose in running buffer) was loaded onto the gel strips and electrophoresis was performed in a Laemmli running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS). The gels were run at 2-2.5 W per gel for the first 40 min and followed by 17 W per gel for 6 h until the dye front reached the bottom of the gel. For quantitative analysis of protein abundance profiles, gels were stained by silver-staining according to the manufacturer's instructions (GE Healthcare, Milwaukee, WI, USA). The stained gels were scanned in an ImageScanner (PowerLook1100 scanner, UMAX) and were analyzed with ImageMaster 2D Elite software. The three technical replicates of each biological sample were pooled and averaged.

2.4 In-gel digestion and MALDI-TOF MS analysis

Spot detection was realized without spot editing. The spots were quantified using the % volume criterion. Only those with significant and reproducible changes were considered to be differentially accumulated proteins. Protein spots were excised from the Silver-stained gels and transferred into 0.2 mL Eppendorf tubes. Each spot was washed twice in milli-Q water (Millipore), destained by washing with 50% MeOH/ 50mM NH_4HCO_3 for 30 min. The gels were then washed twice in milli-Q water, dehydrated by addition of ACN (acetonitrile, Fisher A/0626/17), and dried in a SpeedVac (Thermo Savant, Holbrook, NY, USA) for 30 min. Subsequently, the gel in each tube was rehydrated in 3 μl of proteomics grade trypsin (Sigma) solution (20 ng/mL 40 mM NH_4HCO_3 in 9% ACN) and incubated at 37℃ for 16 h. Peptides were extracted twice by adding 40 μl of solution containing 50% can and 5% TFA (trifluoroacetic acid, GE HealthCare). The extracted solutions were concentrated to 5-10 μl in a lyophilizer (Virtis, Gardiner, NY, USA). Peptide mixtures were mixed with an equal volume of 10 mg/mL CHCA (Sigma) saturated with 50% ACN in 0.1% TFA (Sigma) and analyzed with a Voyager-DE STR MALDI-TOF-TOF mass spectrometer (ABI4700 System, USA) using a delayed ion extraction and ion mirror reflector (Applied Biosystems, Foster city, CA, USA). MS analysis was conducted with a MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Data were analyzed using GPS Explorer software (Applied Biosystem) and MASCOT software (Matrix Science, London, UK). Parameters were set to Variable Modification - Oxidation, 1 Allowed Missed Cleavage. NCBInr and *Oryza sativa* (rice) was selected as the database and taxonomy, respectively.

3. Results and Discussion

Physiological response to nitrogen stress

Four-leaf age seedlings were exposed to N-free nutrient solution and sampled at different times. Obvious nitrogen stress symptoms were observed in the seedlings, such as yellow leaves and impaired tillers. Highly significant difference in nitrogen content and accumulation were also found between the N stress treatment and the control (Table 1). In normal condition with sufficient N supply, cultivar Yongyou 6 had higher dry weight than Chunyou 58, which was consistent with the difference in nitrogen accumulation between the two cultivars. However, when the seedlings were exposed to N stress, Yongyou 6 showed higher loss of dry weight than Chunyou 58. The two cultivars both showed significant decline of nitrogen content and accumulation under N stress relative to the normal condition.

2-DE analysis of leaf proteins in nitrogen stressed rice

Total proteins in the fully-expanded leaves were extracted and separated by 2-DE using pH 4–7 IPG strips in IEF. More than 1,000 protein spots were reproducibly detected on gels by ImageMaster 2D Elite software. Spots with biological

Table 1. Shoot dry weigh and N content and accumulation of the rice cultivars under the different N treatments.

Cultivar	Treatment	Shoot dry weight (g/pot)			N content (%)			Shoot N accumulation(mg/pot)		
		12h	3d	7d	12h	3d	7d	12h	3d	7d
Chunyou 58	0-N	0.14a	0.21a	0.42b	6.24a	4.22b	2.04b	8.7a	8.9a	8.6b
	Control	0.15a	0.22a	0.53a	6.34a	5.37a	4.54a	9.2a	11.8a	24.0a
Yongyou 6	0-N	0.28a	0.44b	0.58b	6.46a	4.21b	3.27b	18.1a	18.5b	18.9b
	Control	0.30a	0.65a	0.82a	6.37a	5.14a	4.00a	19.1a	33.4a	32.8a

significance (ratio > 1.3) between the two treatments are showed in Figure 1. In order to investigate changes in protein accumulation profiles between the control and N-stressed rice plants, the ratio of differentially accumulated proteins between N stress and the normal treatments was calculated, and the proteins with the ratio of over 1.3 were further examined (Fig. 2). Apparently, there were more proteins, which showed significant and reproducible changes in Yongyou 6 than in Chunyou 58. In addition, the two cultivars differed greatly in the number of differential proteins (up or down regulation) over the time of treatment. Chunyou 58 reached the maximum differential proteins in 3 d after the treatment of N stress, while Yongyou 6 did not show the obvious difference over the time of treatment.

There were 31 protein spots in the two cultivars that showed reproducible changes during the treatment, and were selected for MALDI-TOF MS analysis. Among them, 2 and 11 were down-regulated spots for Chunyou 58 (C-D1, C-D2) (Fig. 2A) and Yongyou 6 (Y-D1-Y-D10) (Fig. 2B), respectively; and 8 and 16 were up-regulated spots for Chunyou 58 (C-U1–C-U6) (Fig. 2A) and Yongyou 6 (Y-U1-Y-U11) (Fig. 2B), respectively. The abundance ratios, i.e. the percentage volumes in treated samples over the percentage volumes in control samples, in different stages are shown in Figure 3. Qualitative changes of spots have been found. For example, Y-D6 was visible in all stages of the N stress treatment but invisible in control samples, suggesting that it was induced under N stress treatment. Some differentially accumulated proteins showed quantitative changes in a time-dependent manner. For instance, C-U5, Y-U5, Y-U4 and Y-U16 showed smaller difference between the treated and control samples in early stages of N stress, such as at 12 h. Their abundance ratios were greater at 3 d or 7 d (Fig. 3), indicating that the syntheses of the proteins in the treated sample were enhanced. In addition, spot Y-U4 was observed with a dramatic increase in the abundance in the treated sample at 3 d, while spot Y-D7 decreased dramatically and almost disappeared in the treated sample at 7 d.

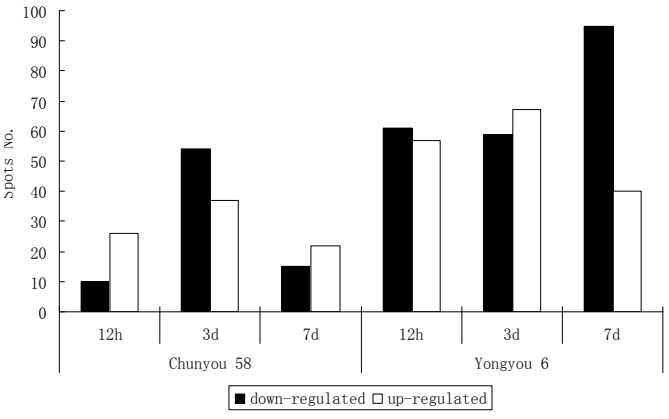

Figure 1. Number of spots whose abundance ratio of the differentially accumulated proteins were over 1.3 after N stress treatment. The percentage volume was considered as the abundance of each spot. The abundance ratio of each spot was calculated by percentage volume in treated samples/ percentage volume in control samples as up-regulated spots (□), while the ones was calculated by percentage volume in control sample/ percentage volume in treated sample as down-regulated spots (▪).

N stress responsive proteins identified by MS

A total of 37 differentially accumulated protein spots were analyzed and identified by MALDI-TOF/ TOF MS with high probability (Table 2). "Spots view" of 15 protein spots of time-dependent changes was shown in Figure 4 as examples. Four identified proteins were found in both varieties in all times during the stress treatment (Table 2). Spots C-U1 and Y-U6 were identified as the same protein, ribulose-1, 5-bisphosphate carboxylase/oxygenase activase. However, they were located at different positions on the gels, with different Mr and pI (Fig. 2 A and B), indicating that they might be isoforms of ribulose-1, 5-bisphosphate carboxylase/ oxygenase activase. It can be assumed that the enzyme is up-regulated under stress since its expression is enhanced with decreased RuBisCo abundance which will reduce photosynthesis. Spots C-D1 and Y-D10 were identified as rubisco large subunit with similar Mr and pI. Spots C-U8 and Y-U15 were identified as H protein subunit of glycine decarboxylase 3'-partial. Spots C-D2 and Y-D5 were identified as putative transposase.

Five proteins were involved in photosynthetic metabolism, including ribulose-1,5-bisphosphate carboxylase/oxygenase activase (C-U1 and Y-U6), type II light-harvesting chlorophyll a/b-binding protein (C-U4), carbonic anhydrases (C-U5), rubisco large subunit (C-D1 and Y-D10), 23kDa polypeptide of photosystem II (Y-U9), dTDP-glucose 4-6-dehydratase-like protein (C-U7) and H protein subunit of glycine decarboxylase 3'-partia (C-U8 and Y-U15). Six proteins were the stressor response to N stress i.e. DegP2(Y-D6), harpin binding proteins(Y-D11), Heat shock-related proteins

(Y-U2), glutathione S-transferase GSTF14(Y-U4), Fibrillin-like protein(Y-U6) in Yongyou 6, and Glyceraldehyde-3-phosphate dehydrogenase (C-U3) in Chunyou 58.

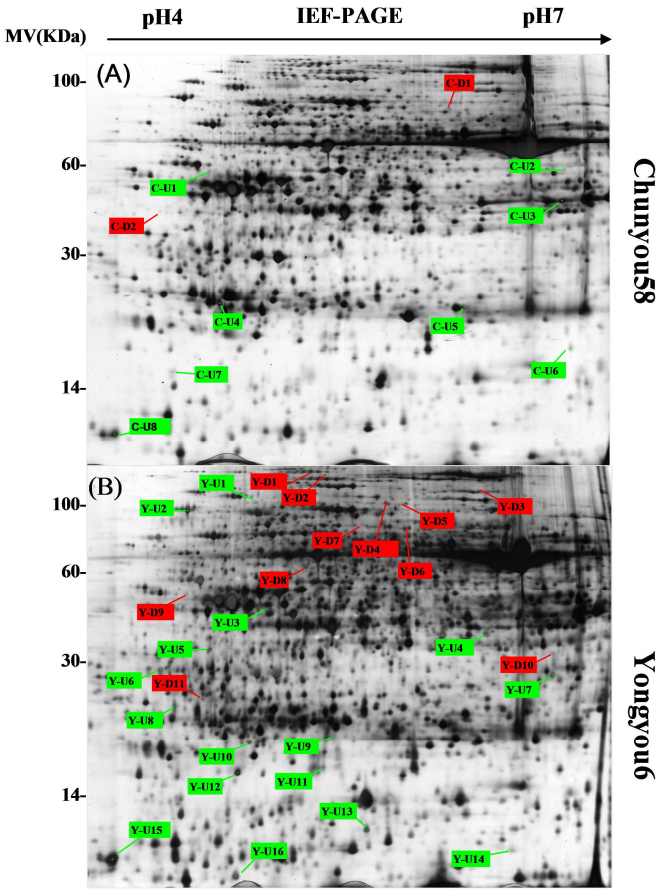

Figure 2. Representative 2-DE maps of rice leaf proteins. Differentially accumulated protein spots (Ratio>1.3) which appeared in all time are selected for MALDI-TOF MS analysis and indicated by label in the map. Two and twelve down-regulated spots for Chunyou58 (C-D1, C-D2) and Yongyou6 (Y-D1-Y-D10) are indicated by red marker. Ten and twenty four up-regulated spots for Chunyou58 (C-U1–C-U6) and Yongyou6 (Y-U1-Y-U11) are indicated on the map by green marker

Photosynthesis and photorespiration

Rubisco activase (RCA, spots C-U1 and Y-U6) is the key enzyme for the rapid formation of the critical carbamate in the active site of RuBisCo. It is modulated either by reaction with CO_2 and Mg^{2+} to carbanylate a lysine residue in the catalytic site, or by the binding of inhibitors within the catalytic site [19]. A variable number of RCA genes have been reported in different plant species. In monocotyledonous plants, two genes have been detected [20]. Two mature RCA polypeptides, with molecular mass ranging between 41 kDa and 47 kDa are present in most plants [21]. Increased 43 Kda/41 KDa was found in low light intensity whereas the decreased one was found in water-stress [20]. Our result showed that RCA (C-U1 and Y-U6) was strongly up-regulated by N stress in most expanded leaves of both rice cultivars (Table 2). N stress can reduce the photosynthesis either by impairing activation state of RuBisCo, which is often attributed to the loss of RCA activity or by reducing the abundance of RuBisCo proteins. The reduced RuBisCo was proved by a dramatic down- regulation of RuBisCo large subunit (RLS) (C-D1 and Y-D10) in N stress samples (Table 2). These results suggest that the RCA was over-expressed as a feedback mechanism for decrease of RCA activity or RuBisCo content in both rice cultivars. However, different additional protective strategy was found between Chunyou 58 and Yongyou 6. Type II light-harvesting chlorophyll a/b-binding protein (LHCP) (C-U4) and carbonic anhydrases (CA) (C-U5) were found in the N treated Chunyou 58 and 23 kDa polypeptide of photosystem II (PsbP-PSII) (Y-U9) was strongly up-regulated in treated samples of Yongyou 6. LHCP is an approximately 25,000-D thylakoid membrane protein, which captures and transmits the energy from the sunlight into biomass [22]. The increased LHCP was found under N stress in this experiment. With the reduced photosynthetic efficiency resulting from N stress, the up-regulated LHCP in N stressed sample might be a compensation for the low photosynthetic efficiency in order to capture and transform more energy to produce the carbohydrate for plant growth. AC forms a family of enzymes, which catalyze rapid conversion of carbon dioxide to bicarbonate and protons. In plants, AC may increase CO_2 concentration within chloroplasts in order to enhance carboxylation rate of RuBisCO [23]. Rengel (1995) found that higher photosynthetic rate under Zn deficiency was related to higher CO_2 availability due to higher CA activity in some wheat genotypes [24]. It may be assumed that the less reduction of biomass in N-stressed Chunyou 58 comparing to Yongyou 6 might be attributed to higher CA activity. PsbP-PSII is one of subunits of the oxygen-evolving complex (OEC) of PSII. The 23-kDa subunit allows PSII to evolve oxygen under both Ca^{2+} and Cl^{2-} limiting conditions, suggesting that it acts as a concentrator of these ions [10]. The dramatic up-regulation of PsbP-PSII was found in N-stressed samples of Yongyou 6, which may act as a compensation for the decreased photosynthesis induced by N stress. The difference in regulated metabolisms between the two rice cultivars may be one of the major causes that lead to more biomass reduction for Yongyou 6 than for Chunyou 58.

A marked increase in H protein subunit of glycine decarboxylase 3'-partial was found in N-stressed rice plants (H-GDC) (Chunyou 58, C-U8; Yongyou 6, Y-U15). H-protein is the lipoyl-protein component of the glycine decarboxylase complex (GDC), which oxidizes glycine to support photorespiration [25]. GDC consists of four proteins, including H-protein that helps to receive the released CO_2. It was reported that the expression of H-protein gene in leaf was stimulated by light [26]. In this experiment, more H-protein was found in N-stressed samples, indicating that photorespiration might be enhanced when the plants are exposed to N stress.

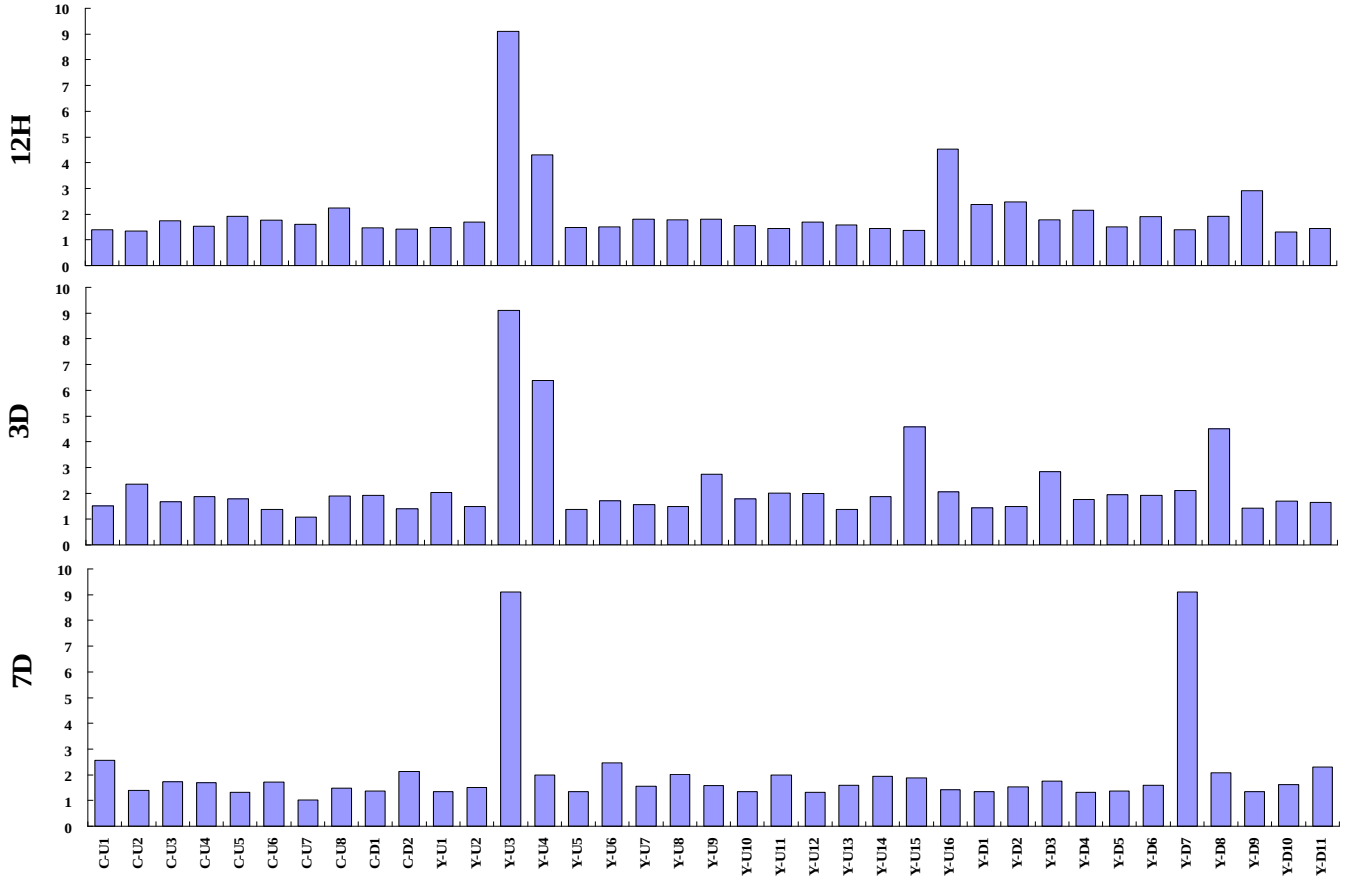

Figure 3. Abundance ratios of the differentially accumulated proteins after 12 h, 3 d and 7 d of N stress treatment. The percentage volume was considered as the abundance of each spot. The abundance ratio of each spot was calculated by percentage volume in treated samples / percentage volume in control samples. The up-regulated proteins include C-U1 to C-U6 (A, D, G), and Y-U1 to Y-U11 (B, E, H); the down-regulated proteins are C-D1 to C-D2 (A, D, G), and Y-D1 to Y-D10 (C, F, I). Spots with * means either the abundance ratio of differentially accumulated protein was over 10,000 or the protein was absent in the treated or control sample.

N stress- induced proteins

Many N stress-related proteins were identified in this study. In general, Yongyou 6 was more sensitive to N stress than Chunyou 58. There were 5 N stress-induced proteins in Yongyou 6, including two down-regulated ones: DegP2(Y-D6) and harpin binding proteins(Y-D11), and three up-regulated ones: heat shock-related proteins(Y-U2), glutathione S-transferase GSTF14(Y-U4), and Fibrillin-like protein(Y-U6). On the contrast, only one stress-related protein was found in Chunyou 58, i.e. glyceraldehyde-3-phosphate dehydrogenase (C-U3). DegP2 is a member of a large family of related Deg/Htr serine proteases found in most organisms, including bacteria [27], humans [28] and plants [29]. Bacterial DegP/HtrA protease has been implicated in tolerance to various stresses, including oxidation, salinity, pH and heat [28]. The current results showed that this protein was reduced under N stress (Table 2), suggesting that the effect of DegP/HtrA protease on enhancing plant tolerance under the stress condition relies on the nitrogen nutrition. Fibrillin-like protein is a glycoprotein, which is essential for the formation of elastic fibers [30]. As lipid-binding proteins of plastids, fibrillin are induced under abiotic stress conditions. Yang (2006) reported that ABA treatment increased fibrillin accumulation, thus enhancing the tolerance of photosystem II to light stress-triggered photo-inhibition in *Arabidopsis* [31]. In this study, fibrillin–like protein were increased in N stressed plants of Yongyou 6, indicating that as a feedback mechanism for N deficiency, the efficiency of photosynthesis was improved by inducing more fibrillin proteins to protect the photosystem II. The harpin protein group, which is first found and identified by Wei et al. (1992) in *Erwinia amylovora* [32], may elicit multiple plant responses, causing beneficial effects on crop improvement [33]. The current results indicated the possible defense mechanism of rice plants in response to N stress by inducing harpin proteins, thus enhancing photosynthesis and nitrogen uptake. Heat shock-related proteins (HSP) are a class of functionally related proteins, whose expression is increased when cells are exposed to elevated temperatures or other stresses [34]. The function of glutathione S-transferase GSTF14 protects cells from injury by a wide range of stresses in plants [35]. A significant up-regulation of heat shock related proteins and GSTF14 was found in N stressed Yongyou 6 (Table 2).

Table 2. Differentially accumulated proteins identified by MS.

MS SPOT	Rank Protein Name	Accession No.	Protein Score	Protein MW	Protein PI	Ratio		
						12h	3d	7d
C-U1	ribulose-1,5-bisphosphate carboxylase/oxygenase activase [Oryza sativa]	gi|115486823	218	48127.9	5.85	1.40	1.52	2.55
C-U2	putative gypsy-type retrotransposon [Oryza sativa (japonica cultivar-group)]	gi|18071410	39	165471.2	9.53	1.36	2.36	1.40
C-U3	glyceraldehyde-3-phosphate dehydrogenase [Oryza sativa]	gi|2331137	173	36707.0	9.55	1.74	1.66	1.75
C-U4	type II light-harvesting chlorophyll a/b-binding protein [Oryza sativa Japonica Group]	gi|218174	54	28566.4	5.61	1.54	1.88	1.68
C-U5	carbonic anhydrase 3 [Oryza sativa]	gi|5917783	134	29585.0	8.41	1.92	1.79	1.34
C-U6	putative metalloproteinase [Oryza sativa (japonica cultivar-group)]	gi|14165330	42	32250.9	6.36	1.75	1.38	1.72
C-U7	dTDP-glucose 4-6-dehydratase-like protein [Oryza sativa]	gi|18201659	40	26091.4	7.1	1.60	1.08	1.03
C-U8	H protein subunit of glycine decarboxylase 3'-partial [Oryza sativa (japonica cultivar-group)]	gi|10257441	67	7156.6	8.98	22.25	11.89	1.50
C-D1	rubisco large subunit	gi|476752	79	45614.8	8.43	1.46	1.91	1.38
C-D2	putative transposase [Oryza sativa (japonica cultivar-group)]	gi|34015353	49		6.56	1.42	1.39	2.13
Y-U1	ribulose-1,5-bisphosphate carboxylase activase [Oryza sativa]	gi|13569643	273	21737.8	4.78	1.49	2.04	1.35
Y-U2	heat shock-related protein [Oryza sativa (japonica cultivar-group)]	gi|29367425	404	45014.5	5.02	1.69	1.48	1.52
Y-U3	Os06g0176700 [Oryza sativa (japonica cultivar-group)]	gi|115466716	203	40022.5	5.16	1000000	1000000	1000000
Y-U4	glutathione S-transferase GSTF14 [Oryza sativa(japonica cultivar-group)]	gi|46276327	514	30766.5	7.77	4.52	6.38	2.00
Y-U5	putative protein kinase ADK1 [Oryza sativa Japonica Group]	gi|52077492	41	26201.6	9.55	1.49538	1.37301	1.34866
Y-U6	fibrillin-like protein [Oryza sativa (japonica cultivar-group)]	gi|29367475	510	33923.7	5.04	1.51	1.72	2.47
Y-U7	Putative wall-associated protein kinase [Oryza sativa (japonica cultivar-group)]	gi|14029040	40	53505.1	6.01	1.8159	1.55712	1.55934
Y-U8	oryzain gamma precursor [Oryza sativa Japonica Group]	gi|218185	51	39692.5	7.07	1.79	1.48	2.01
Y-U9	23kDa polypeptide of photosystem II [Oryza sativa]	gi|2570499	271	27173.9	9.06	1.82	2.74	t
Y-U10	Os08g0455800 [Oryza sativa (japonica cultivar-group)]	gi|115476734	240	21683.7	5.15	1.56	1.79	1.34
Y-U11	hypothetical protein [Oryza sativa Japonica Group]	gi|42407348	54	7766.8	10.96	1.44	2.02	2.00
Y-U12	Os08g0478200 [Oryza sativa (japonica cultivar-group)]	gi|115476908	371	19712.9	5.19	1.70	1.98	1.33
Y-U13	Os10g0471300 [Oryza sativa (japonica cultivar-group)]	gi|115482468	161	18653.4	5.61	1.57	1.38	1.61
Y-U14	hypothetical protein LOC_Os03g43310 [Oryza sativa (japonica cultivar-group)]	gi|53370666	39	20432.4	10.86	1.45	1.87	1.94
Y-U15	H protein subunit of glycine decarboxylase 3'-partial [Oryza sativa (japonica cultivar-	gi|10257441	60	7156.6	8.98	1.37	4.58	1.88

	group)]							
Y-U16	Os06g0705100 [Oryza sativa (japonica cultivar-group)]	gi|115469830	298	24997.9	8.74	1000000	2.06	1.42
Y-D1	putative chloroplast inner envelope protein [Oryza sativa (japonica cultivar-group)]	gi|10140720	812	108209.8	5.37	2.38	1.43	1.35
Y-D2	putative SecA [Oryza sativa Japonica Group]	gi|52075758	410	114899.0	5.78	2.47	1.49	1.54
Y-D3	Os03g0401300 [Oryza sativa (japonica cultivar-group)]	gi|115453437	72	93362.3	5.94	1.79	2.84	1.77
Y-D4	Os02g0285800 [Oryza sativa (japonica cultivar-group)]	gi|115445587	360	74035.2	7.08	2.16	1.76	1.32
Y-D5	putative transposase [Oryza sativa (japonica cultivar-group)]	gi|34015353	45	66527.4	6.56	1.51	1.94	1.37
Y-D6	putative DegP2 protease [Oryza sativa (japonica cultivar-group)]	gi|51038169	37	65771.4	5.73	1.94	1.93	1.61
Y-D7	Os06g0562600 [Oryza sativa (japonica cultivar-group)]	gi|115468554	268	59720.8	5.71	1.39	2.11	1000000
Y-D8	eukaryotic initiation factor 4A [Oryza sativa Japonica Group]	gi|303844	207	47393.1	5.43	1.91	4.52	2.08
Y-D9	hypothetical protein [Oryza sativa (japonica cultivar-group)]	gi|13236651	142	45168.2	5.27	2.90	1.43	1.35
Y-D10	rubisco large subunit	gi|476752	157	45614.8	8.43	1.31	1.70	1.63
Y-D11	harpin binding protein 1 [Oryza sativa (indica cultivar-group)]	gi|38679325	78	28457.0	8.92	1.43	1.65	2.30

Glyceraldehyde 3-phosphate dehydrogenase (C-U3) (GAPDH, EC 1.2.1.1) was up-regulated in N stressed Chunyou 58. GAPDH plays important roles in various cellular processes. It is a central glycolytic protein with pivotal role in energy production, and is also an abundant and crucial enzyme in glycolysis and gluconeogenesis in most plants [36]. Moreover, GAPDH is a protein with multi-function, involving in the translational control of gene expression [37]. For the last decade, there were many reports that GAPDH works as a stressor associated with oxidative stress in cells that undergo apoptosis [38]. It may be suggested that over-expression of GAPDH in N stressed Chunyou 58 acts not only as an oxidative signal to N deficiency, but also an energy production through its glycolytic function.

Membrane transporter

Putative chloroplast inner envelop protein (Y-D1) and SecA protein (Y-D2) were down-regulated in N stressed Yongyou 6. Chloroplast inner envelop is highly specialized with transport proteins, which involved in the movement of ions, small molecules, or macromolecules. SecA proteins were found in the thylakoid membrane as well as the cytoplasmic membrane, and they involved in protein translocation across the thylakoid membrane [39]. The current results showed that the translocation across the chloroplast or thylakoid membrane was inhibited in Yongyu 6 under N stress.

Different protective strategies under N stress between two cultivars

Resolving the cause and effect relationship in plants subject to a nitrogen limitation is difficult because nitrogen stress initiates a series of complex physiological responses varied over the time and the stress degree. Many of the physiological metabolisms were directly or indirectly involved with the stress effect. For example, the net photosynthesis rate in most extended leaves acclimate to N stress with highly up-regulated RCA in two cultivars as well as LHCP / CA and PsbP-PSII in Chunyou 58 and Yongyou 6, respectively. The similar results were also reported by De Groot et al. (2003) that photosynthetic light-harvesting and electron-transport activity acclimate to nitrogen stress so that the internal relationships between electron transport by photosystems I and II do not change; the linear relationship between PSII, and PSI was not affected [40]. In protein profile, RCA could play a role of a chaperone, either in helping target the thylakoid membrane or in protection of translation machinery related to thylakoid against abiotic stress [41]. Thus the up-regulated RCA could protect the photosynthesis machinery under N stress. Despite of the similar protection from the RCA, different protective strategies were drown out from cultivars differing in NUE. LHCP / CA were up regulated in Chunyou 58 resulting in the slightly biomass decrease subjected to a nitrogen limitation. Only PsbP-PSII was consistently up regulated for carbon production metabolism over the stress period in Yongyou 6. This may be considered as the major cause for the biomass differentiation between the cultivars.

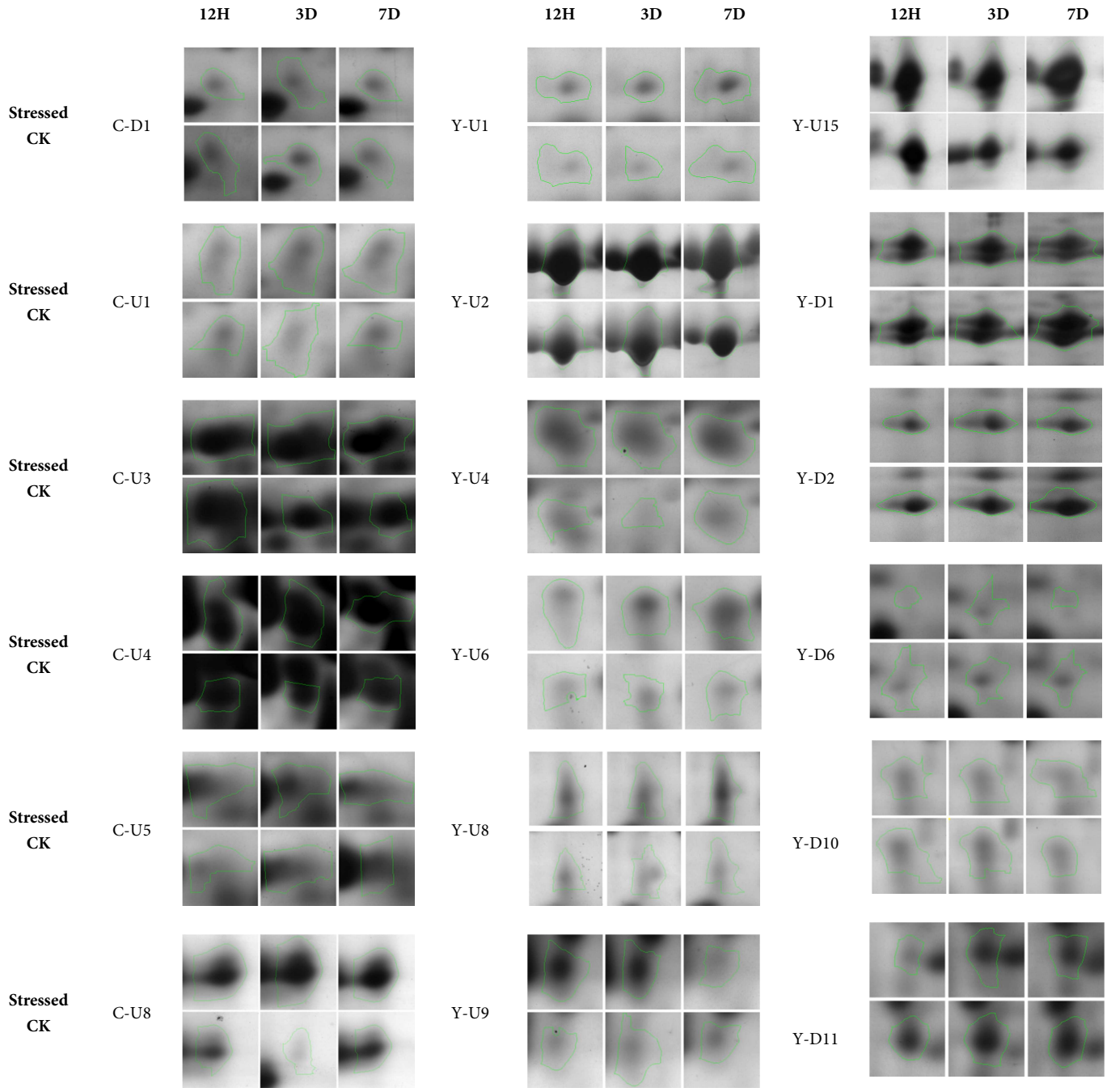

Figure 4. Time-dependent changes of the 15 of 48 differentially accumulated proteins. Proteins in leaves were extracted from both control and stressed samples after 12h, 3d and 7d treatment and separated by 2-DE.

4. Concluding remarks

A systematic proteomic analysis of the leaf proteins in N stressed rice was carried out in this study. Of the six protein spots involved in photosynthesis and photorespiration, three were identified in the two rice cultivars at all times during the treatment. These are: Rubisco activase, RuBisCo large subunit, and H protein subunit of glycine decarboxylase 3'-partial. Six stress-induced proteins were identified, including DegP2, harpin binding proteins, heat shock-related proteins, glutathione S-transferase GSTF14, fibrillin-like protein and glyceraldehyde-3-phosphate dehydrogenase. Two proteins, i.e. putative chloroplast inner envelop protein and SecA protein are related to membrane translocation, Moreover, two novel proteins, harpin binding protein and oryzains gamma precursor, were found in the rice leaves under N stress. These results provide useful information for further investigation of their functions using genetic or genomic approaches.

Acknowledgements

We thank Bureau of Science and Technology of Zhejiang, China for its financial support to this research project and Dr. Younan Ouyang of Chinese Rice Research Institute for his kind help in nitrogen measurement.

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