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ORIGINAL ARTICLE

Physiological and proteomic analysis of young rice leaves grown under nitrogen-starvation conditions

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Abstract Rice grown in anaerobic waterlogged soil accumulates ammonium as a major source of nitrogen (N). We have compared the physiological symptoms of rice seedlings subjected to N-starvation stress with those receiving sufficient N, based on measurements of shoot/root length and weight and an analysis of protein expression patterns. N starvation marginally increased root growth but notably decreased shoot biomass. N uptake was reduced by >50% in the roots and shoots of N-starved seedlings. To better understand the mechanism of N starvation in rice, we performed a comparative proteome analysis of proteins isolated from rice leaves. Twenty-five differentially expressed proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrometry and electron spray ionization quadrupole TOF. Functional analysis of the N-starvation response proteins suggested their involvement in protein synthesis and fate, metabolism, and defense. These results indicate that these proteins may play important roles in regulating the plant's complex adaptation responses for N use during N starvation. The proteins may be useful for further characterization of protein function in plant N nutrition.

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Introduction

Nitrogen (N) is an essential inorganic macronutrient for plants and required in large quantities to achieve optimal growth and development, reflecting its role as a major constituent of proteins, nucleic acids, many cofactors, and primary or secondary metabolites (Marschner 1995). N-containing fertilizer is one of the major inputs to agricultural systems (Glass 2003). However, crop plants are able to utilize only 30–40% of fertilizer N (Raun and Johnson 1999), with the remaining N removed by denitrification, volatilization, soil erosion, microbial consumption, and leaching. These problems increase crop production costs and environmental perturbations. There is, therefore a need to improve the efficiency of N utilization in order to overcome these problems. Considerable efforts have been directed at verifying several genes involved in N transport and metabolism (Hirel et al. 2007). Nevertheless, little is known about the molecular mechanisms regulating the adaptability of plants to N starvation.

To cope with N starvation, plants have developed a variety of physiological and biochemical changes which include an increase in N uptake by high-affinity transporters, remobilization of N from older to younger leaves and reproductive parts, suppression of growth and photosynthesis, and increased accumulation of anthocyanin (Bongue-Bartelsman and Philips 1995; Ono et al. 1996; Chalker-Scott 1999; Ding et al. 2005; Peng et al. 2007). Nonetheless, the precise molecular mechanism(s) of the response of higher plants to N starvation remains unclear. The availability of an enlarged genomic database and

progressive improvements in proteomics technologies have led to the investigation of protein function and protein expression patterns in cereal plants.

In this study, we used an integrated physiology and two-dimensional gradient electrophoresis (2-DGE)-based proteomics approach to identify differentially expressed proteins responsive to N starvation. Identified proteins were broadly related to metabolism and the defense/stress response. The findings from this study may help researchers gain a better understanding of mechanism involved in the N-starvation response in plants and provide a basis for further characterization of the function and regulation of the proteins responsive to N starvation.

Materials and methods

Plant material and growth conditions

Mature rice seeds (cv. Jinheung) were obtained from the National Yeongnam Agricultural Experimentation Station. De-hulled seeds were sterilized with 70% ethanol for 10 min, rinsed in distilled water, and surface sterilized for 30 min using 3% sodium hypochlorite. The seeds were then rinsed extensively with sterile water and imbibed for 3 days at 4°C in sterile distilled water to break dormancy prior to being sown on a plastic supporting netting (mesh 1 mm^2) mounted in plastic containers at 28°C for 7 days. The emerging seedlings were then transferred to plastic containers containing complete or N-starved nutrient solution in which were positioned 12 mesh (1 cm^2) plastic panels. The complete nutrient solution contained 1.07 mM NH_4NO_3, 0.03 mM $NaH_2PO_4{\cdot}2H_2O$, 0.39 mM K_2SO_4, 0.39 mM KCl, 1.25 mM $CaCl_2{\cdot}2H_2O$, 0.82 mM $MgSO_4{\cdot}7H_2O$, 35.8 μM $FeSO_4{\cdot}7H_2O$, 9.1 μM $MnSO_4{\cdot}4H_2O$, 46.3 μM H_3BO_3, 3.1 μM $ZnSO_4{\cdot}7H_2O$, 0.16 μM $CuSO_4{\cdot}5H_2O$, and 0.05 μM $Na_2MoO_4{\cdot}2H_2O$. The N-deficient nutrient solution was the same as the complete nutrient solution, but without 1.07 mM NH_4NO_3. The pH of the solution was adjusted to 5.8, and the nutrient solution was continuously renewed. Plants were grown hydroponically at 28°C for 3 weeks in complete or N-starved nutrient solution prior to the experiments.

Analysis of physiological parameters of rice growth

Rice seedlings were harvested 1 or 3 weeks after culture in each nutrient solution. The length of the three longest roots and shoots were measured. Three plants were pooled for one replicate, and each experiment was conducted with three replicates (obtained from three different containers). The collected plants were dried in an oven at 105°C for 3 days and weighed. Dried samples were digested with sulfuric acid and hydrogen peroxide (Mizuno and Minami 1980).

Protein preparation and 2-DGE analysis

Rice leaves (2 g) grown in complete or N-starved nutrient solution for 3 weeks were sampled from three separate experiments. The leaves were ground in liquid N and homogenized in 10 mL of ice-cold Mg/NP-40 extraction buffer [0.5 M Tris–HCl, pH 8.3, 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% v/v β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% w/v polyvinylpolypyrrolidone (PVPP)] containing 0.7 M sucrose. The phenol extraction method was followed (Kim et al. 2001). Total leaf protein was extracted from three independent biological samples. The 2-DGE analysis was performed as described previously (Kim et al. 2008). A total of 250 μg of protein was loaded on 18-cm immobilized pH gradient (IPG) strips (pH 4–7) that were equilibrated with rehydration buffer [8 M (w/v) urea, 2% (w/v) CHAPS, 0.002% (w/v) Bromophenol G, 20 mM dithiothreitol (DTT), 0.5% (v/v) pharmalyte (pH 5–8)] for 12 h and focused at 50 V for 8 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, 8000 V for 5 h, 8000 V for 3 h, and 20 V for 2 h using an IPGphore3 platform (GE Healthcare, Buckinghamshire, UK). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in the second dimension as described by Laemmli (1970), using 12% polyacrylamide gels. The gels were stained with colloidal Coomassie Brilliant Blue G-250 (CBB) as described previously (Kim et al. 2008), and 2-DGE image analysis was performed using ImageMaster 2D Platinum ver. 6 software (Amersham Biosciences AB, Uppsala, Sweden).

In-gel digestion and mass spectrometry

Protein spots were carefully excised from CBB-stained gels and digested according to the method of Kim et al. (2004). Gel fragments were reduced in 20 mL of 10 mM DTT with 0.1 MNH_4HCO_3 for 45 min at 55°C, following which the DTT solution was substituted with 55 mM iodoacetamide in 0.1 M NH_4HCO_3. After washing, the dried gel pieces were re-swollen in 10 mL digestion buffer [25 mM NH_4HCO_3 and 12.5 ng/mL trypsin (sequencing grade; Promega, Madison, WI] and incubated at 37°C overnight. Tryptic digested peptides were extracted. The digestion mixture was redissolved in a solution containing water, acetonitrile, and trifluoroacetic acid (TFA) (93:5:2). The sample was bath-sonicated for 5 min and centrifuged for 2 min. The matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in acetone (40 mg/mL) and nitrocellulose in acetone (20 mg/mL) (Kim et al. 2004). This solution, the nitrocellulose solution, and isopropanol were mixed 100:50:50, and 2 μL of

the mixture was added to 2 μL of the peptide sample solution. A 1-μL sample of this final solution was spotted immediately onto a matrix-assisted laser desorption/ionization (MALDI) plate and left for 5 min. The MALDI plate was then washed with 0.1% (v/v) TFA. The gel spots were analyzed using a Voyager-DE STR MALDI time-of-flight (TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA). Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.010%, and a delay time of 150 ns. Des-Arg1-bradykinin (*m/z* 904.4681) and angiotensin 1 (*m/z* 1296.6853) were used as a two-point internal standard for calibration. Peptides were selected in the mass range of 500–3,000 Da. For data processing, the software package PerSeptive-Grams was used. Database searches were performed using Protein Prospector (http://prospector.ucsf.edu) and Mascot (http://www.matrixscience.com) websites. The electron spray ionization quadrupole (ESI-Q)–TOF procedure was conducted according to a previously described method (Kim et al. 2009).

Statistical analysis

Statistical analysis was performed by analysis of variance methodology. Differences between shoot or root growth means were assessed using Tukey tests ($P < 0.05$). SAS software (SAS Institute, Cary, NC) was used for all statistical analyses.

Results and discussion

Plant growth under N-starvation conditions

Rice seedlings subjected to N starvation were first grown under normal conditions for up to 7 days and then transferred to the N-starved nutrient condition for 1 or 3 weeks. Controls were grown in complete nutrient solution. The shoot and root growth of N-starved seedlings at 1 or 3 weeks of treatment were comparable with those of control plants grown in the complete nutrient. The dry weight of the leaves and roots was also measured at each time point. In the N-starved plants at 1 and 3 weeks of treatment, the shoot height decreased up to 21 and 38%, and the dry weight decreased up to 15 and 27%, respectively, relative to the control plants. However, root growth in the absence of N was approximately 12% higher than that of the control plants at both time points, resulting in large differences in the root-to-shoot ratios between each time point (Table 1). These data are consistent with typical *Arabidopsis* and rice growth caused by N depletion (Scheible et al. 2004; Li et al. 2006). In contrast to the increase in root growth at both time points, root dry weight was slightly decreased after 3 weeks (Table 1). This difference was within standard deviation errors. However, it may be possible that the lateral root was lost when the roots were dried, and N-starved increased lateral roots as well as primary roots capture nutrients necessary for continued leaf expansion.

Protein separation by 2-DGE analysis

Nitrogen starvation for 1 week resulted in no significant physiological changes compared to plants grown in N-starved (PK) conditions for 3 weeks (Table 1). However, the growth of rice leaves was highly retarded during the 3-week N-starvation period. These latter rice leaves were examined by 2-DGE. To compare proteins that were expressed in complete (NPK) and PK conditions, we extracted proteins from rice leaves grown in pairs (NPK vs. PK) for 3 weeks using phenol extraction with neutral IPG strips (pH 4–7) to obtain the best resolution of proteins on 2-DGE gels. CBB-stained gels were repeated three times

Table 1 Shoot and root tissue length and weight and shoot/root ratio of 1- and 3-week-old plants, respectively

Treatments (1 week)	Height (mm)			Weight (mg)		
	Shoot (S)	Root (R)	R/S	Shoot	Root	R/S
1-Week-old plants						
NPK	137.4 a	57.4 c	0.42 c	10.4 a,b	5.6	0.56 b
PK	109.1 b	64.7 c,b	0.59 a,b	8.9 b	7.0	0.82 a
F test	***	***	***	**	ns	**
3-Week-old plants						
NPK	213.6 a	97.9 b	0.46 c	19.5 a	7.7 a	0.39 a,b
PK	133.0 b	111.5 a	0.84 a	14.4 b	6.8 a	0.47 a
F test	***	***	***	***	***	***

NPK Complete nutrient solution, *PK* N-deficient nutrient solution, *ns* not significant

Each value represents the average of three individual identical experiments. Values followed by different lowercase letters indicate that the means differ significantly ($P < 0.05$) within a row

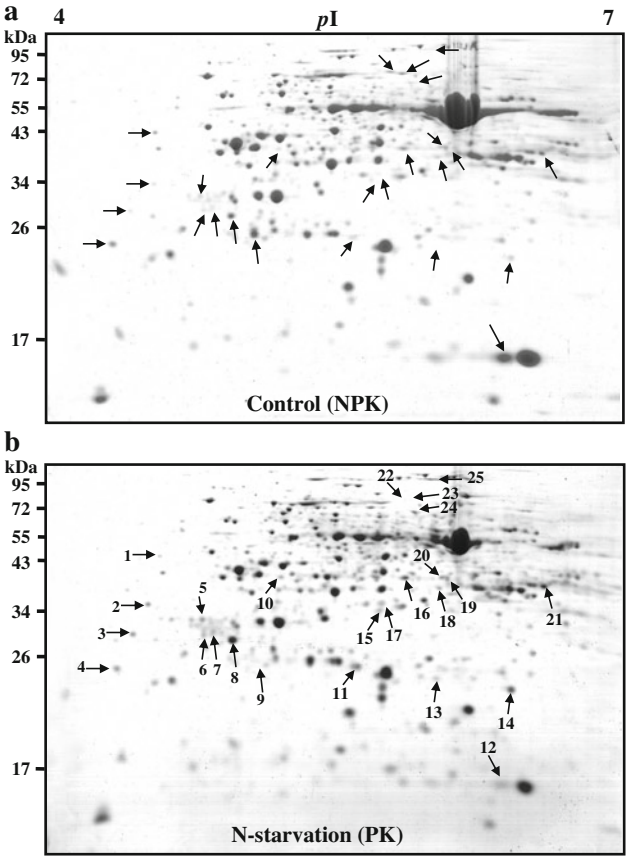

Fig. 1 Two-dimensional gradient electrophoretic (2-DGE) analysis of differentially expressed proteins in rice leaves in response to N starvation. Seedlings were grown for 3 weeks in complete (*NPK*) or N-deficient (*PK*; N starvation) nutrient solution. Total leaf protein was separated by 2-DE [pH 4–7, 18-cm immobilized pH gradient (IPG) strip] and gels were stained with colloidal Coomassie Brilliant Blue G-250 (CBB). **a** 2-DGE profile of proteins extracted from NPK-grown leaves, **b** 2-DE profile of proteins extracted from PK-grown leaves. Differentially regulated leaf proteins by N-starvation are indicated (*arrows*) and *numbered*. See Table 2 for identification of spot number

with independent protein samples (Fig. 1). More than 250 proteins were detected by 2-DGE. Proteins that were clearly induced or decreased following N starvation relative to the NPK condition were analyzed (Fig. 1a, b). Twenty-five proteins were differentially expressed as a result of N starvation (Fig. 2a, b). Among these proteins, 19 spots were markedly increased under the PK condition, whereas spots 1, 4, 9, 12, 22, and 23 were decreased (Fig. 2b). These results suggest that N starvation caused the up- or down-regulation of a few proteins in rice leaves, perhaps in response to plant adaptations to N depletion.

Identification of proteins involved in rice leaves responding to N-starvation

To better understand the mechanism of N starvation and the alteration of N homeostasis in plants, we analyzed a selection of differentially expressed proteins by MALDI–TOF and ESI-Q–TOF and identified by database searches with Protein Prospector and Mascot. The identified proteins were classified based on functional categories established by Bevan et al. (1998). These proteins which responded to N starvation were found to be involved in diverse biological processes, covering RNA synthesis (spot 3), signal transduction (spots 5–7), defense and stress (spots 11, 14, 15, 17), protein synthesis and fate (spots 1, 2, 8, 22, and 23), and metabolism (spots 4, 9, 10, 12, 13, 16, 18–21, 24, and 25) (Fig. 2; Table 2).

N-starvation can affect photosynthesis, respiration, and carbohydrate metabolism (Rufty et al. 1988; Wang and Tillberg 1996; Zhao et al. 2005). The proteins identified in our study included enzymes involved in photosynthesis and energy; among these, four proteins were probable components of the electron transport chain: one enzyme belonging to the family of oxidoreductases (spot 18), putative H(+)-transporting ATP synthase (spot 4), putative iron/ascorbate-dependent oxidoreductase (spot 10), and putative quinone-oxidoreductase QR2 (spot 13). Four enzymes involved in carbohydrate metabolism were also identified, namely, 2-carboxyarabinitol-1,5-bisphosphate (spot 12), glyceraldehyde-3-phosphate dehydrogenase (spot 21), NADP-dependent malic enzyme (spot 24), and aconitate hydratase (spot 25). The differential regulation of these proteins implies that N starvation results in a fundamental metabolic alteration.

The second major group in response to N starvation was attributed to protein synthesis and fate. Three of the proteins identified were involved in protein fate (spots 8, 22, and 23), and two proteins were factors of protein synthesis (spots 1 and 2). These proteins have been widely studied and are known to bind to folding and translocation intermediates and misfolded proteins and to be are involved in new protein synthesis under normal or stress conditions (Blond-Elguindi et al. 1993; Dai et al. 2006; Gething 1999). These results suggest that rice plants can recognize N deficiency and regulate changes of metabolism and protein states in order to maintain relative N homeostasis.

The third major group in response to defense or reactive oxygen species (ROS) stress comprised four proteins, which were linked to antioxidant/detoxifying reactions. Glutathione S-transferase (spot 11) is an enzyme catalyzing the conjugation of glutathione to a variety of toxic substrates arising from oxidative stress (Marrs 1996; Roxas et al. 2000). The other three proteins were peroxidases (spots 14, 15, and 17), which catalyze oxidoreduction between hydrogen peroxide and several different reductants. These results suggest that detoxification is a required response to N starvation, with these proteins playing an important role in plant ROS homeostasis.

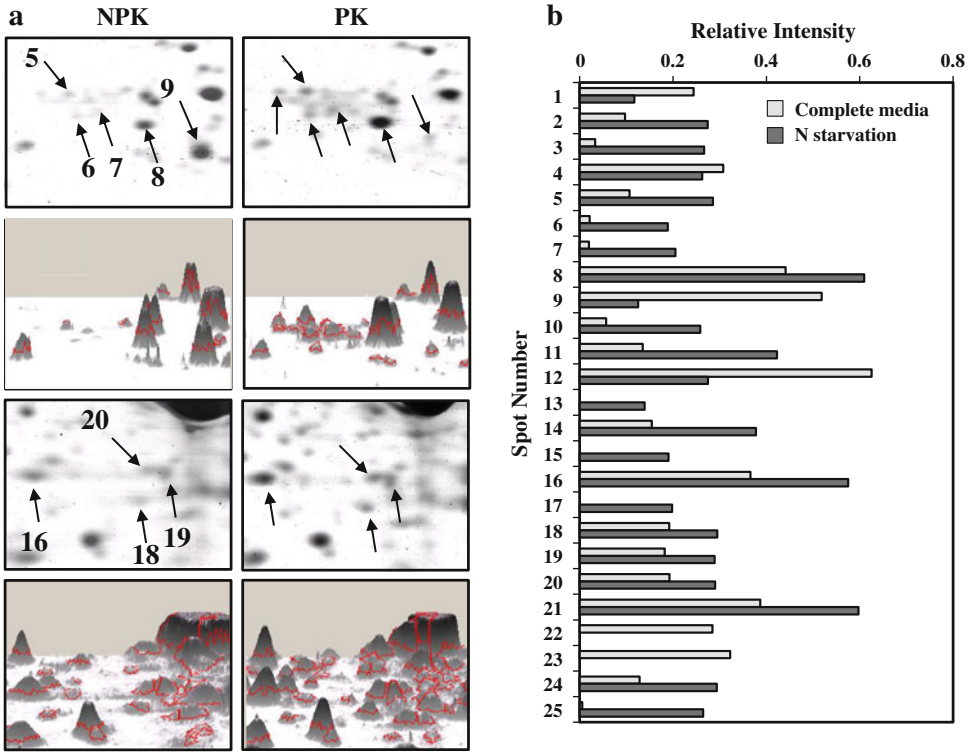

Fig. 2 Representative enlarged images of differentially regulated protein spots in response to N starvation. Three-dimensional image (**a**) and quantitative analysis (**b**) of differential protein spots on 2-D gels generated by ImageMaster software. See Table 2 for identification of spot number

Table 2 Identification of proteins (spots) differentially regulated by N starvation in rice shoots using MALDI–TOF and ESI-Q–TOF

Protein spot no.	Protein name	AC	Score	MP	SC	Ex Mr	Th Mr	Ex pl	Th pI	Up/down[a]
Metabolism										
4	Putative H(+)-transporting ATP synthase	gil115448701	75	5	21	25.14	26.2	3.84	4.98	↓
9	RNase S-like protein	gil115480399	69	6	26	26.53	28.38	4.5	5.25	↓
10	Putative iron/ascorbate-dependent oxidoreductase	gil115466724	101	8	29	42.48	40.09	4.7	5.3	↑
12	2-Carboxyarabinitol-1,5-bisphosphate	gil56966763	87	7	35	14.58	14.92	6.3	5.89	↓
13	Putative quinone-oxidoreductase QR2	gil115440369	76	5	37	25.75	21.69	5.8	6.06	↑
16	Reversibly glycosylated polypeptide	gil4158221	90	8	23	39.25	41.34	5.6	5.82	↑
18	Aldo/keto reductase (Os04g0338000)	gil115457788	163	12	32	38.58	38.21	6.1	6.03	↑
19	Cinnamyl alcohol dehydrogenase	125563652	2.51E + 09 (P)	12	34.9	39.12	38.08	6.2	6	↑
20	Alpha-1,4-glucan-protein synthase	115454033	111840 (P)	6	23.6	41.12	41.35	6.1	5.8	↑
21	Glyceraldehyde-3-phosphate dehydrogenase	gil115474601	72	6	24	39.24	36.39	6.8	6.61	↑
24	NADP dependent malic enzyme	gil54606800	86	8	20	68.59	65.36	6	5.79	↑
25	Aconitate hydratase	gil115450595	73	8	11	104.58	106.23	6.1	6.45	↑
Protein synthesis and fate										
1	30S ribosomal protein S1	gil115452675	114	9	25	43.24	41.89	4.28	4.57	↓
2	ACT domain-containing protein	gil125560701	85	7	25	33.18	35.49	4.17	6.67	↑
8	Luminal binding protein 5 precursor	gil125597253	68	6	25	28.47	37.19	4.2	6.2	↑
22	Endosperm lumenal binding protein	gil2267006	157	17	27	74.15	73.49	5.9	5.3	↓
23	Endosperm lumenal binding protein	gil2267006	157	17	27	75.19	73.49	6	5.3	↓
Defense and stress										
11	Glutathione S-transferase	gil115479659	86	8	33	25.75	25.17	5.7	5.5	↑
14	Peroxidase	2429286	5636 (P)	4	22.24	23.85	32.86	6.5	5.8	↑
15	Peroxidase	gil20286	65	5	22	34.73	32.85	5.5	5.77	↑
17	Peroxidase	gil20286	65	5	22	35.74	32.85	5.6	5.77	↑

Table 2 continued

Protein spot no.	Protein name	AC	Score	MP	SC	Ex Mr	Th Mr	Ex pl	Th pI	Up/down[a]
Signal transduction										
5[b]	Putative DREPP2 protein	Q6K4S7	42	–	–	33.18	24.04	4.3	4.73	↑
6	GF14-c protein	gil115476520	101	7	38	28.54	28.8	4.3	4.78	↑
7	GF14-c protein	gil115476520	77	6	30	28.5	28.8	4.4	4.78	↑
RNA synthesis										
3	Nucleoside-triphosphatase activity	125538986	827204 (P)	8	8.7	39.57	101.65	4	6.2	↑

MP Matched peptide, *Ex Mr/Ex pI* experimental molecular weight and pI, respectively *Th Mr/Th pI* theological molecular weight and pI, respectively, *P* database searches were performed using Protein Prospector, *ESI-Q–TOF* electron spray ionization quadrupole time-of-flight, *MALDI–TOF* matrix-assisted laser desorption/ionization–TOF, *AC* accession number, *SC* sequence coverage

[a] ↑, up-regulated; ↓, down-regulated

[b] Identified by ESI Q–TOF

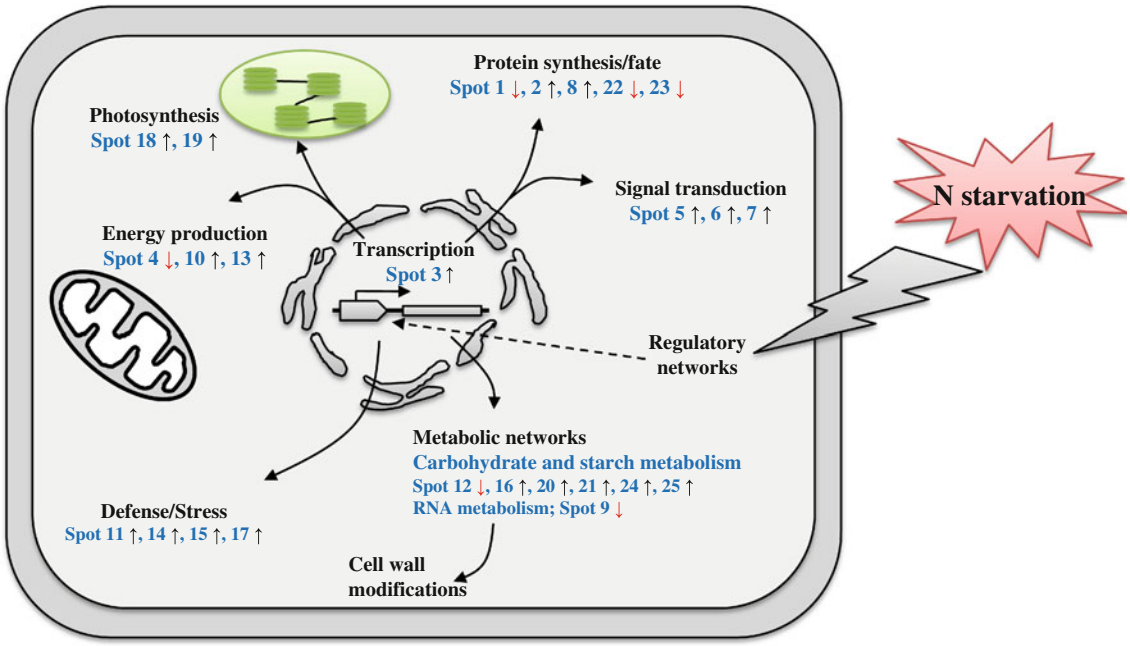

Fig. 3 An integrated view of cellular events responsive to N starvation in rice roots. N starvation affects cellular processes and responses at various levels

Conclusion

The 2-DGE-based proteomics approach used in our study resulted in the identification and cataloging of N-starvation responsive proteins found in rice leaves. These proteins were mainly involved in metabolism, protein synthesis/fate, and defense/stress responses, and they correlated well with rice leaf physiology and biochemical changes. These data provide a greater understanding of the cellular events regulated by N starvation in rice leaves, which involves changes in protein abundance of multiple cellular processes. Some of the major adaptive changes and differentially expressed rice leaf proteins discovered by our proteomic approaches in response to N-limited conditions are shown in Fig. 3. These findings provide advanced insight into rice leaf biology. The information reported here is only one step forward in our understanding of the underlying adaptation mechanism of rice leaves to N starvation. Further advances will require the use of multiple complementary proteomics approaches in order to develop a complete list of leaf proteins that respond to N starvation.

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