Transcriptome response to nitrogen starvation in rice

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Nitrogen is an essential mineral nutrient required for plant growth and development. Insufficient nitrogen (N) supply triggers extensive physiological and biochemical changes in plants. In this study, we used Affymetrix GeneChip rice genome arrays to analyse the dynamics of rice transcriptome under N starvation. N starvation induced or suppressed transcription of 3518 genes, representing 10.88% of the genome. These changes, mostly transient, affected various cellular metabolic pathways, including stress response, primary and secondary metabolism, molecular transport, regulatory process and organismal development. 462 or 13.1% transcripts for N starvation expressed similarly in root and shoot. Comparative analysis between rice and *Arabidopsis* identified 73 orthologous groups that responded to N starvation, demonstrated the existence of conserved N stress coupling mechanism among plants. Additional analysis of transcription profiles of microRNAs revealed differential expression of miR399 and miR530 under N starvation, suggesting their potential roles in plant nutrient homeostasis.

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1. Introduction

Nitrogen (N) is one of the major macronutrients required for plant growth and development. It is not only the constituent of key cell molecules such as amino acids, nucleic acids, chlorophyll, ATP and several plant hormones, but also the pivotal regulator involved in many biological processes, including carbon metabolism, amino acid metabolism and protein synthesis (Frink *et al*. 1999; Crawford and Forde 2002). Limitation of N causes both molecular and developmental adaptation in all organisms. In higher plants, N limitation leads to dramatic changes in plant growth and development, such as root branching, leaf chlorosis and fewer seed production (Stitt and Krapp 1999; Good *et al*. 2004). Plant growth and crop production requires abundant N, which is generally the most common limiting nutrient for growth and yield of crops worldwide. Large amounts of nitrogen fertilizers are applied to meet the high N requirement of crop plants. However, applications of large quantities of fertilizers to increase crop yield are not economically sustainable and also lead to environmental pollution. Crop plants use only less than half of the applied nitrogen fertilizers (Socolow 1999). The unused N are inevitably leached into the underground water system and lost to the atmosphere, leading to severe environmental pollution. Recent analysis showed that soil acidification in China resulted mainly from high-N fertilizer inputs (Guo *et al*. 2010). Therefore, efforts have been directed to understanding the

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molecular basis of plant responses to N deficiency and to identifying N responsive genes whose expression can be manipulated to enable plants to use N more efficiently.

The complex and diverse physiological and biochemical changes induced by N limitation suggested that numerous genes and various metabolic and regulatory pathways were required to develop plant adaptive responses to N limitation. Thus, genome-wide investigation of gene expression by microarray represented an effective approach for analysing gene regulatory networks in organisms with sequenced genomes (Zhu *et al*. 2001; Zhu 2003; Morley *et al*. 2004; Hubner *et al*. 2005; Tsai *et al*. 2006). Recently, this technology has been applied to identify pathways involved in light control of genome expression (Tepperman *et al*. 2001; Ma *et al*. 2002), circadian clock (Harmer *et al*. 2000) and in expression profiles of plant genes under environmental stresses, such as low N nutrient (Lian *et al*. 2006; Bi *et al*. 2007; Peng *et al*. 2007), low phosphorus (P) nutrition (Wang *et al*. 2002; Hammond *et al*. 2003; Uhde-Stone *et al*. 2003; Wasaki *et al*. 2003; Wu *et al*. 2003; Misson *et al*. 2005), pathogen infections (Maleck *et al*. 2000) as well as cold, salt and drought treatments (Kawasaki *et al*. 2001; Seki *et al*. 2001; 2002). Numerous studies of nitrate response reactions induced by resupply of nitrate after depletion have been reported, which identified large numbers of genes in *Arabidopsis*, including genes that are directly involved in nitrate transport, nitrate reduction and nitrite reduction, ammonium assimilation, and generation of NADPH through the oxidative pentose phosphate pathway (Wang *et al*. 2000; 2003; 2004; Palenchar *et al*. 2004; Price *et al*. 2004; Scheible *et al*. 2004). Many new nitrate responsive genes were also identified using cDNA arrays containing 5524 *Arabidopsis* genes/clones (Wang *et al*. 2000) and 1280 tomato genes (Wang *et al*. 2001), including two genes encoding enzymes of the nonoxidative pentose phosphate pathway, a calcium antiporter, an MYB transcription factor, two putative protein kinases, an Asn synthetase and non-symbiotic hemoglobin.

miRNAs are small (20–24 nt) non-translated RNAs found in plants and animals which are processed from the stem-loop region of single-stranded endogenous long-precursor transcripts (Bonnet *et al*. 2006; Mallory and Vaucheret 2006; Zhang *et al*. 2006b; Sunkar *et al*. 2007). They usually negatively regulate gene expression at the post-transcriptional level by partial base-pairing to their complementary mRNA (Lee *et al*. 1993; Reinhart *et al*. 2000; Carrington and Ambros 2003; Bartel 2004). miRNAs have recently been shown to play critical roles at each major stage of plant development (Jones-Rhoades *et al*. 2006), acting at the core of a gene regulatory network, targeting genes that are themselves regulators, such as those encoding transcription factors and F-box proteins that are involved in organ morphogenesis and plant development (Rhoades *et al*. 2002; Mallory *et al*. 2004; Vaucheret *et al*. 2004; Guo *et al*. 2005). In addition, some miRNAs have also been shown to be involved in the coordination of nutrient homeostasis. For example, miR395 was shown to be strongly induced by low sulphate concentration and repressed by P limitation, miR398 was shown to respond to copper deprivation and repressed by both N and P limitation, and miR399 increased drastically in low-phosphate media in *Arabidopsis* and other plant species (Jones-Rhoades and Bartel 2004; Fujii *et al*. 2005; Aung *et al*. 2006; Bari *et al*. 2006; Chiou *et al*. 2006; Sunkar *et al*. 2006; Chiou 2007; Yamasaki *et al*. 2007; Buhtz *et al*. 2008; Doerner 2008; Hsieh *et al*. 2009; Pant *et al*. 2009).

Previously, transient changes in gene expression have been reported in nitrate-starved *Arabidopsis* and tomato seedlings when nitrate is resupplied (Wang *et al*. 2000; 2001; 2003; Scheible *et al*. 2004), also reported in N-stressed *Arabidopsis* (Bi *et al*. 2007) and rice (Lian *et al*. 2006) seedlings by Gene Chip and cDNA array, respectively. In the present study, we applied the Affymetrix Rice Genome array to analyze transcriptomic response to N starvation time points in rice shoot and root, and also used plant miRNA array to identify differentially expressed miRNAs in rice plant at 7 days after N starvation. Genes and their transcription responses to N starvation identified from this study provided more useful information to better understanding the molecular mechanism of plant adaptation to N starvation compared with those from cDNA array reported (Lian *et al*. 2006), which gave us a better understanding of potential target genes for nitrogen-use efficiency improvement of rice crop.

2. Materials and methods

2.1 *Plant growth condition and N concentration measurement*

Seeds of the rice cultivar Hejiang 19 (*Oryza sativa* ssp. *japonica*) were germinated and grown hydroponically in nutrient solution containing 1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM $NaSiO_3$, 50 μm Fe-EDTA, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μMH_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$, 40.5 μM citric acid, pH 5.5 (Yoshida *et al*. 1976). The culture solution was refreshed every 3 days. At five-leaf stage, seedlings were transferred into a nutrient solution without N as N starvation treatment, and a nutrient solution with complete nutrients as the control.

Root and shoot materials were harvested separately at 1 h, 24 h and 7 days after N starvation treatment. Both root and shoot were sampled from three separate experiments for total RNA isolation used in microarray analysis and quantitative reverse transcriptase PCR (qRT-PCR) analysis. In addition, both root and shoot were sampled

from three separate experiments for N content measurement, which were analysed by flow injection analyser (FIA-3700, Flow Injection Analysis, Germany) according to the manufacturer's instructions.

2.2 *Microarray preparation*

The Affymetrix GeneChip Rice Genome Arrays were used in this study (*http://www.affymetrix.com/products/arrays/specific/rice.affx*). RNA isolation, purification and microarray hybridization were conducted by the CapitalBio Corporation (Beijing, China) according to the Affymetrix standard protocols (Affymetrix GeneChip Expression Analysis Technical Manual). The probe intensity files (.cel files) were generated using the GeneChip Operating System (GCOS, Affymetrix). The Affymetrix Rice Genome array contains 57,381 probe sets. Each probe set consists of 11 pairs of 25-mer perfect match (PM) and mismatch (MM) probes which differ only at the middle base.

2.3 *Measurement of expression levels*

The probe intensity files (.cel files) for expression profiling were read into R (*http://www.R-project.org*). Background correction, quantile normalization and summarization were performed using the GC Robust Multi-array Average (GCRMA) method in Bioconductor gcrma package (Wu *et al*. 2004). We used expression flags from Affymetrix MicroArray Suite 5.0 to indicate whether a gene was expressed and represented as present (P), marginal (M) and absent (A) calls. Only probe sets with 'Present' calls in at least two out of three replicates in at least one tissue of certain time point were selected for further analysis.

2.4 *Gene annotation and probe set–gene association*

The annotation of genes and gene families used in this study were downloaded from TIGR/MSU Rice Genome Pseudomolecules Release 5 (Ouyang *et al*. 2007). Due to the update of genome assemble and gene annotation, we revaluated the definition of probe sets in the microarray. All PM probes were mapped to the rice genome, probe sets with more than half of the total PM probes (greater than six for most of the probe sets) in a unique genomic location were considered as core probe sets. Each core probe set with at least four PM probes located in a TIGR/MSU transcriptional unit (TU) was considered to represent this TU. Based on above criterions, 40,525 core probe sets on the array were associated with 33,811 unique TUs (genes). Kinase genes from Rice Kinase Database (*http://rkd.ucdavis.edu/*) or genes under GO: 0016301 (kinase activity) were merged and defined as kinase-related genes. Transcription factor activity genes were selected based on Rice Transcription Factor Database (*http://ricetfdb.bio.uni-potsdam.de*) and genes under GO:0003700 (transcription factor activity). Transport activity genes were defined by merging genes with text 'transport' in TIGR/MSU annotation and genes under GO: 0005215 (transporter activity).

2.5 *Identification and cluster analyses of differentially expressed genes*

Differentially expressed genes between control and treatments at each time point were identified using an extended rank product method implemented in the Bioconductor package RankProd (Hong *et al*. 2006) with parameters of 100 permutations and false discovery rate (FDR) less than 0.05. Cluster analyses were performed for genes showing significant differential expression between control and treatments using logarithmic ratios of geometric mean of expression values between control and treatments on two ways. For illustrating the time-course responses to N starvation, expression ratios were clustered by descending sort in each time point along the order of 1 h, 24 h and 7 days, root first then shoot. To characterize the interactions of responses to N starvation, hierarchical clustering with the complete linkage and Pearson correlation coefficients was applied to the entire data set using R.

2.6 *Gene ontology enrichment analyses*

A combination of *classic* and a modified *weight* Fisher's exact test (FET) implemented in Bioconductor topGO package (Alexa *et al*. 2006) were used to provide a deeper view in biological function levels based on gene ontology enrichment analyses using biological processes data from TIGR/MSU version 5 Gene Ontology (GO) annotation (Ouyang *et al*. 2007). The two methods can generate complementary view of gene ontology enrichment: the *weight* method can reliably detect locally most significant GO terms but down-weight genes in less significant neighbours, comparing to the *classic* FET. Thus, we used the *weight* method to detect significant terms while used *P*-values produced by the *classic* FET to draw GO heatmap.

2.7 *Identification of homologous genes between rice and Arabidopsis*

We selected 56,278 unique rice proteins from TIGR/MSU Rice Annotation Release 5 and 27,029 *Arabidopsis* proteins from TAIR Arabidopsis Annotation Release 7. For gene locus with multiple proteins, the longest protein was selected. Putative homologous gene groups between rice and *Arabidopsis* were identified using Inparanoid (Remm *et al*. 2001; Berglund *et al*. 2008) based on reciprocal NCBI

TBLASTX (Altschul *et al*. 1990). The reciprocal best-match homologous gene pairs between rice and *Arabidopsis* were considered as putative orthologous gene pairs.

2.8 *Prediction of potential targets of miRNAs*

The rice plant sample at 7 days after N starvation was used to hybridize with plant miRNA chip and analysed using SAM, Significant differentially expressed miRNAs were identified based on their Q-value of less than 0.01 and up- or down-regulated greater than twofold. The mature sequences of miRNAs were downloaded from miRBase (Griffiths-Jones 2006). Then the potential targets of each miRNAs were predicted by using Plant microRNA Potential Target Finder (miRU; *http://bioinfo3.noble.org/miRNA/miRU.htm*) with mature sequence of miRNA, dataset of TIGR Rice Genome mRNA (OSA1 release 5) and default parameters (Zhang 2005).

2.9 *Additional microarray data*

In addition to the rice microarray data generated from this study, microarray data from published studies generated by Affymetrix ATH1 GeneChip array were also used for the analyses. Microarray data of the nitrate responses in *Arabidopsis* seedling, root and shoot (Wang *et al*. 2003; Scheible *et al*. 2004; Bi *et al*. 2007) were downloaded from the authors' supplemental materials.

2.10 *qRT-PCR analysis*

Total RNA was extracted with TriZol reagent (Invitrogen, Germany) according to the manufacturer's instructions. For qRT-PCR analysis, first-strand cDNAs were synthesized from DNaseI-treated total RNA using Superscript II Reverse Transcriptase (Invitrogen) and One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. qRT-PCR was performed in an optical 96-well plate with an ABI PRISM 7500 qRT-PCR system (Applied Biosystems, Foster City, CA, USA). Each reaction contained 12.5 μL of 2×SYBR Green Master Mix reagent (Applied Biosystems), 3.0 μL of cDNA samples, and 200 nM each of the gene-specific primers in a final volume of 25 μL. The thermal cycle used was as follows: 95°C for 3 min; 45 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s. All gene-specific primers for qRT-PCR are listed in supplementary table 6, and designed on the basis of the cDNA sequences. The specific primer for the rice *actin* gene (NM_197297) was used as an internal control. The primers were designed by Primer Express Software (Foster City, CA, USA), and were checked by BLAST program in the rice genomic sequence available in The Institute for Genomic Research (TIGR; *http://rice.plantbiology.msu.edu/*) database to ensure that the primers amplify a unique and desired cDNA segment. The specificity of the reactions was checked by melting curve analysis, and three replicates of each cDNA sample were used for qRT-PCR analysis.

3. Results

3.1 *General features of the N starvation responsive gene expression profile*

Measurements of *in vivo* N concentration revealed a continuous decline in both root and shoot from 24 h to 7 days after nutrient starvation: the N concentration reduced by 22% in root and 16% in shoot after 7 days of N starvation (figure 1A). And plants displayed the obvious phenotypes (less biomass, longer root, less chlorophyll content, etc.) at 7 days after N starvation compared with plants under normal nutrient condition (figure 1B). To study gene transcriptome changes in rice root and shoot after N starvation, root and shoot materials were harvested at 1 h, 24 h, and 7 days after N withdrawal and microarray experiments were conducted with Affymetrix Rice Genome Array. The data are highly reproducible among biological replicates (correlation coefficients of all biological replicates are all greater than 0.99). Six representative genes induced or repressed from the microarray analysis were selected for qRT-PCR analysis. The qRT-PCR results were quite consistent with the results from microarray data (figure 2), thus validating the microarray result in this paper.

A total of 32,341 probe sets were used to detected transcripts under different N conditions and time points (see Materials and methods for details). When contrasting to normal nutrient condition, 3518 (10.88%) genes altered their transcript levels in whole plant under N starvation condition, with the estimated percentage of false-positive predictions (PFP) <0.05 (supplementary figure 1C). The details of these differentially expressed genes are listed in supplementary table 1. There were total of 2214 transcripts significantly changed in root compared with 1766 transcripts in shoot, and 462 (20.9% and 26.2% of the root and shoot changed transcripts respectively) of those transcripts were shared between root and shoot (supplementary figure 1C). This is similar to a previous study in rice using cDNA array, which showed that genes in root responded to N stress much more quickly than in shoot (Lian *et al*. 2006). This result suggested that genes in different organs always served specific functions, and distinct strategies were used by those two organs in response to N starvation.

3.2 *Differential expression patterns in different time points/tissues responded to N starvation*

Details of numbers of up- or down-regulated transcripts in different time points/tissues responded to N starvation were presented in figure 3A. Across three time points examined,

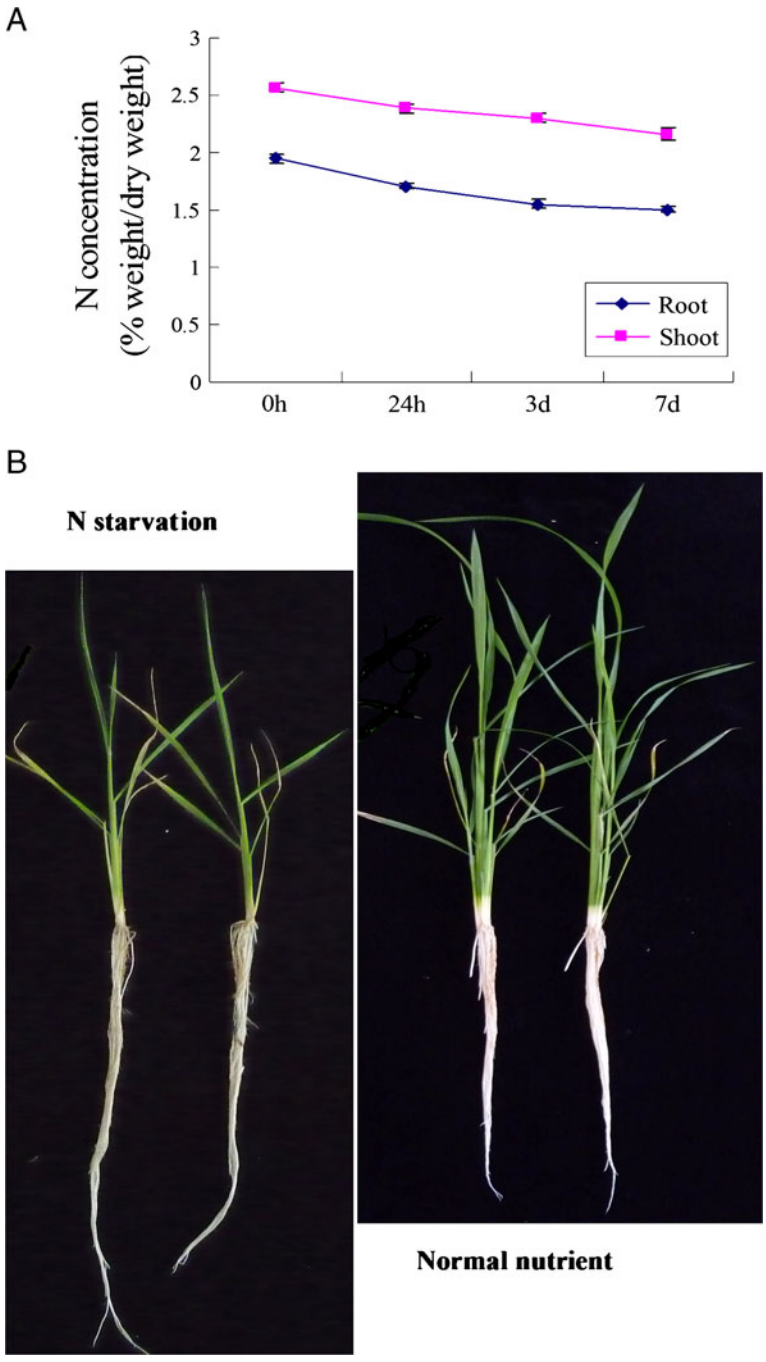


Figure 1. *In vivo* N concentration in root and shoot at 0 h, 24 h, 3 days, 7 days after N starvation treatment (**A**), and the phenotypes of rice plants under 7 days after N starvation treatment and normal nutrient conditions (**B**).

the differentially expressed genes progressively increased in shoot with the duration of N starvation, while in root, they decreased at 24 h and then increased to the maximum at 7 days (figure 3A). In addition, there were more genes differentially regulated in root than in shoot under N starvation, and unlike the situation in root, where the number of up-regulated genes was close to the number of down-regulated genes, the up-regulated genes were more than down-regulated genes in shoot (figure 3A).

Nevertheless, most of the significantly altered expression only transiently occurred at a specific time point in the time series examined, suggesting that plant respond to nutrient starvation by inducing or repressing different sets of genes at different time points. As shown in supplementary figure 1A

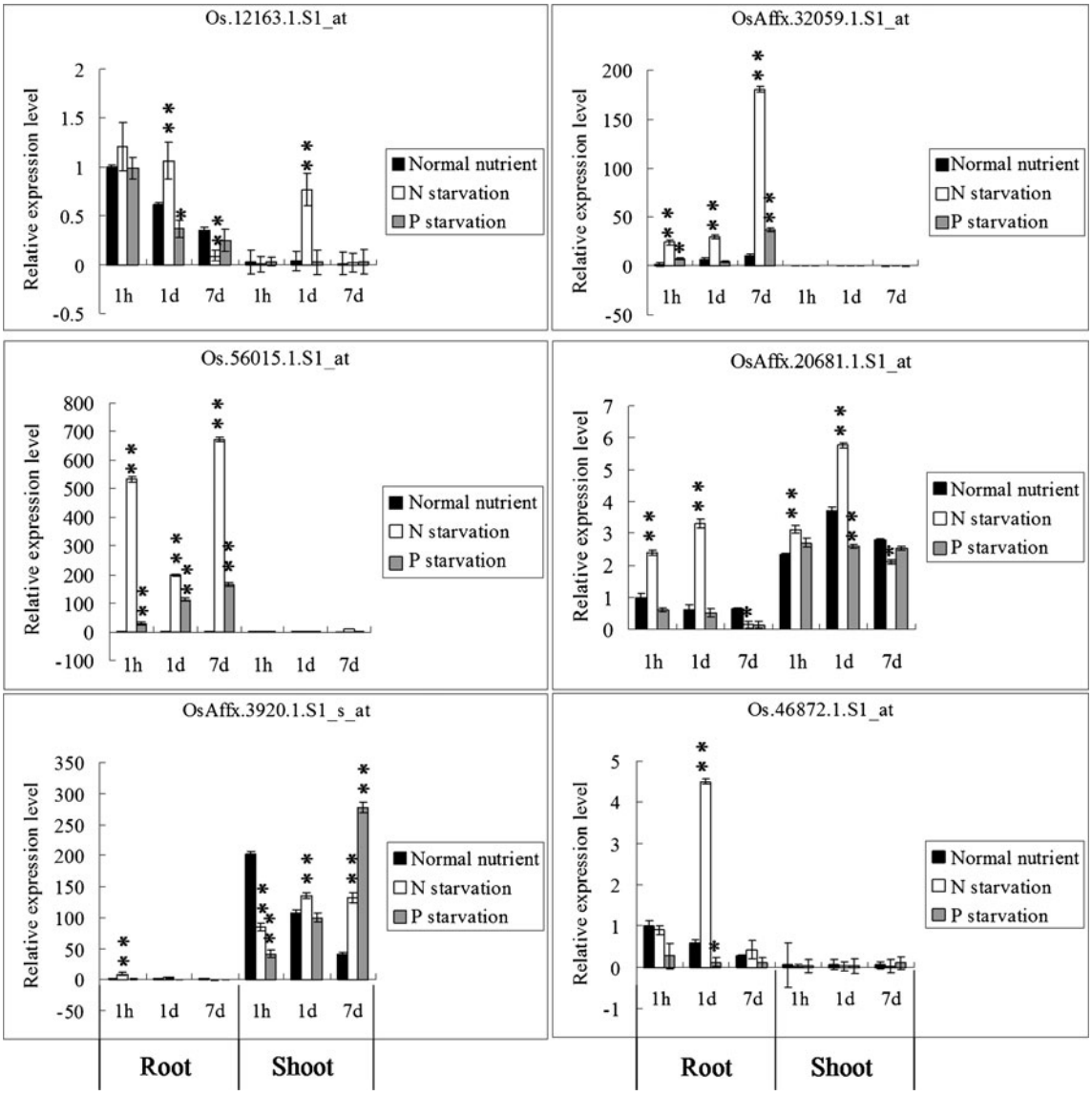
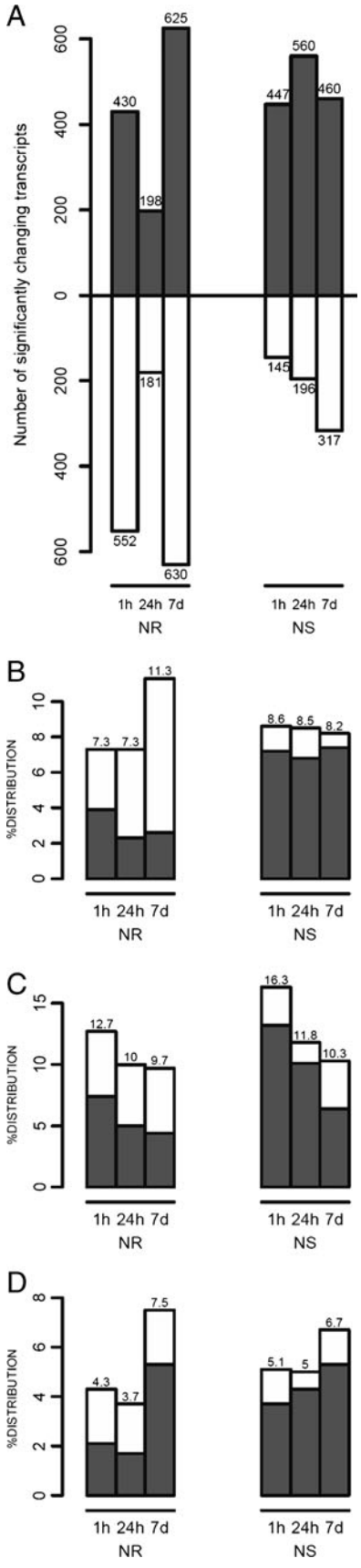


Figure 2. Comparison of the microarray data and qRT-PCR analysis for 6 selected genes in root and shoot at 1 h, 1 days, 7 days after N and P starvation treatment. Y-axis means the relative expression level of genes; values are mean ± SD from three independent biological replicates. *, **Significant differences at the levels of P=0.05 and P=0.01, respectively.

and B, no genes were constitutively up- or down-regulated in root and only 11 genes in shoot were up-regulated at all three time points after N starvation (supplementary figure 1A and B). This is similar to the results reported in rice by Lian *et al*. (2006) in which only 3 genes were changed at three time points (20 min, 1 h and 2 h). Such temporal transcriptional trends into N starvation can be also demonstrated both in root and in shoot by clustering analysis that different sets of genes were responsive to N starvation either in root or shoot at three time points (figure 4).

Genes encoding kinases, transcription factors and transporters that responded at the beginning of N deprivation were further analysed in figure 3. Among three time points after N starvation, more kinase-related genes were down-regulated in root, while more genes were up-regulated in shoot (figure 3B). Large numbers of transcription-factor-related genes were transiently activated after 1 h of N deprivation but few stay activated after 7 days (figure 3C). In contrast, genes encoding transporters seem activated along the N stress progression (figure 3D), suggesting an adaptation in the metabolic and physiological processes. Taking together, these results indicate a complex regulatory mechanism towards N deprivation stress in rice.



◀ **Figure 3.** Number of significantly changed transcripts detected from the microarray experiment. (**A**) The number of differentially expressed transcripts responded to N starvation stress in each tissue (NR: root; NS: shoot) and time points (1 h, 24 h, 7 days), *grey* for up-regulated genes and *white* for down-regulated genes. The percentage of kinase-relative genes (**B**), transcription factor related genes (**C**) and transporter activity genes (**D**) in each tissue (NR: root; NS: shoot) and time points (1 h, 24 h, 7 days) were also counted and presented.

3.3 *Functional classifications of the differentially expressed genes*

To gain a broad view of the intrinsic influence of N deprivation and the adaptation strategies thatplant used, a classic and a modified weight Fisher's exact test (FET) provided with Bioconductor topGO package (Alexa *et al*. 2006) were used to classify the functions of identified differentially expressed genes based on gene ontology enrichment analyses. Supplementary table 2A, B and C summarize the biological process, molecular function and cellular component with significant genes up- or down-regulated in root and shoot at three different time point (1 h, 24 h, 7 dyas) after N starvation respectively (weight <0.01). Results showed that the differentially expressed genes mainly belong to six biological process categories: stress response (such as endogenous stimulus, abiotic and biotic stimulus), primary metabolism (such as nitrate assimilation, amino acid and derivative metabolism, carbon utilization and fixation, photosynthesis, lipid metabolism, sulphate reduction and carbohydrate metabolic process), secondary metabolism (including antibiotic biosynthetic process), molecular transport (including electron, metal ion, multidrug, lipid, phosphate, citrate and sulphate transport), regulatory process (including transcriptional regulation, signal transduction, pollen–pistil interaction, cell communication, protein amino acid phosphorylation and dephosphorylation, DNA catabolic process) and organismal development (such as pollination, nodulation, ciliary or flagellar motility, embryonic and reproductive structure development).

Our results displayed that transcription of many genes responsive to both biotic and abiotic stimuli were significantly altered to couple with N starvation conditions. This observation is supported by early reports in *Arabidopsis* (Hammond *et al*. 2003; Wu *et al*. 2003; Misson *et al*. 2005; Bi *et al*. 2007).

In our study, genes involved in nitrate transportation were differentially expressed and the whole pathways of nitrate metabolism were also altered: nitrate reductase (NR, two out of two genes changed, expressed here as 2/2), nitrite reductase (NiR, 2/3), glutamine synthetase (GS, 2/4), glutamate synthase (GOGAT, 3/3) and glutamate dehydrogenase (GDH, 1/4) (supplementary table 1). These severe alterations in nitrate assimilation genes were accompanied by extensive changes in cellular transport, which is essential for plant cellular homeostasis and growth under various conditions.

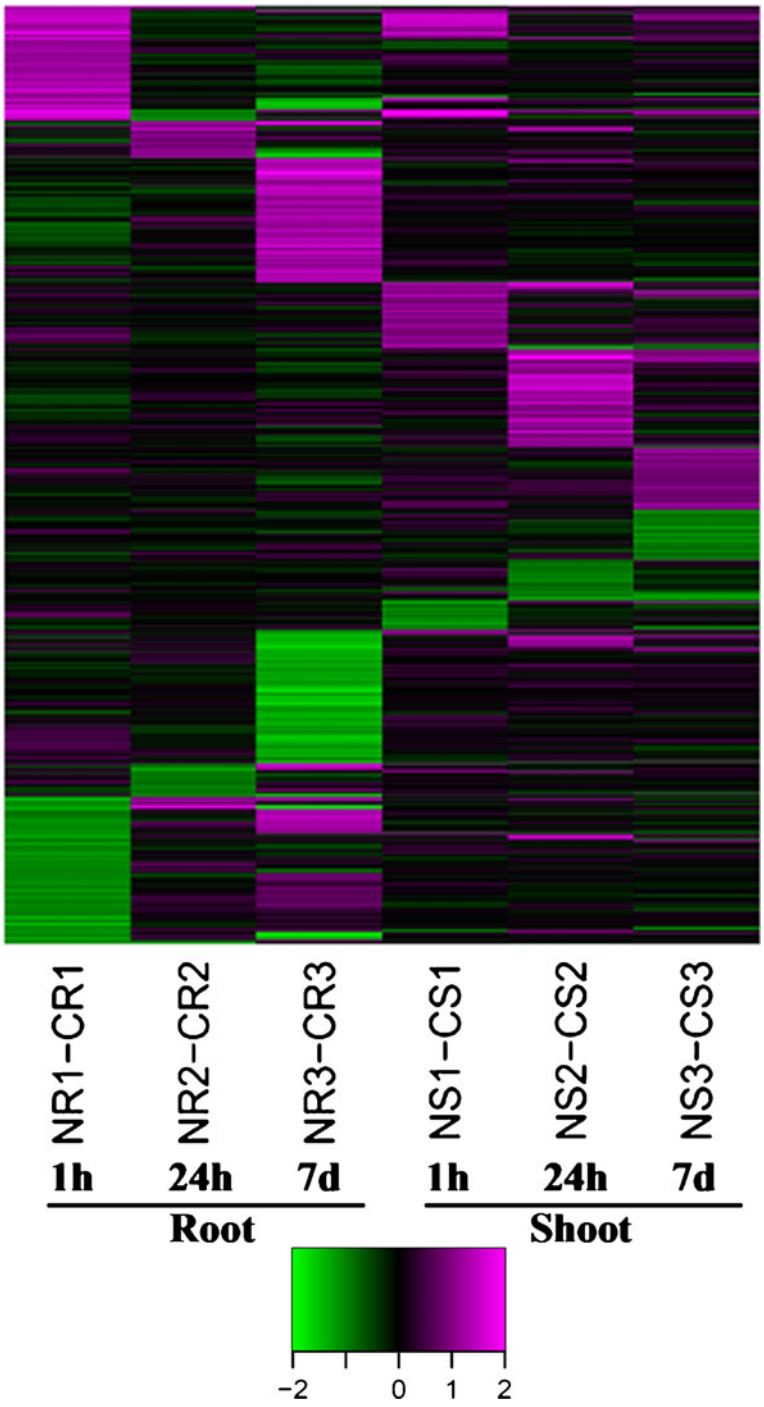


Figure 4. Clustering analysis of rice genes that exhibited differential expression in response to N starvation at three time points examined in either root or shoot. Expression patterns of differentially expressed genes at 1 h (NR1-CR1), 24 h (NR2-CR2), 7 days (NR3-CR3) in root and at 1 h (NS1-CS1), 24 h (NS2-CS2), 7 days (NS3-CS3) in shoot after N starvation. The color scale was shown at the bottom, and purple for up-regulated genes and green for down-regulated genes.

Many genes involved in electron, metal ion, multidrug, lipid, phosphate, citrate and sulphate transport were significantly changed after N starvation (supplementary table 2).

As yellow leaf is one of the most obvious phenotype when a plant is grown under N starvation condition, genes involved in photosynthesis would be expected to be down-regulated in

leaves. Although many genes involved in photosynthesis were reported to be down-regulated in *Arabidopsis* under N starvation (Bi *et al*. 2007; Peng *et al*. 2007), we did not observed significant number of genes involved in photosynthesis change their expression in shoot during all three time points. However, we found that a large number of such genes were down-regulated in root during N starvation (supplementary table 2). A similar phenomenon was also observed in gene expression profiling in *Arabidopsis* (Himanen *et al*. 2004) or rice (Lian *et al*. 2006) that the expressions of these photosynthesis genes were little affected in leaves.

Transcriptional factors are of special interest as they are capable of coordinating the expression of several or many downstream target genes, even entire metabolic and developmental pathways. In this study, a large number of genes involved in regulation of transcription (GO:0045449; GO:0006350; GO:0006355) and signal transduction (GO:0007165) were differentially expressed during N starvation (supplementary table 2).

In addition, a large number of genes involved in carbon (C) metabolic pathways were significantly altered during N starvation. This suggests a stringent relationship between the levels of C and N metabolism and a balanced C/N ratio in plants. Genes involved in pollination were also significantly changed during N starvation, suggesting an important role of N in rice pollination process

3.4 *Comparative analysis of differentially expressed genes between rice and Arabidopsis under N starvation condition*

In this study, we also compared the differentially expressed genes in rice identified in our microarray, with differentially expressed genes in *Arabidopsis* identified in earlier reports and available in the public domain, to determine the conserved genes responsive to N starvation condition (supplementary table S3). A total of 8796 putative orthologous gene pairs covered by microarrays of both species were identified, of which 337 gene pairs responded to N starvation or inducement in both species. To further dissect conservative gene networks, we identified putative homologous gene groups between rice and *Arabidopsis* using Inparanoid (Remm *et al*. 2001; Berglund *et al*. 2008), and 73 gene groups were found in common between rice and *Arabidopsis* responded to N starvation (supplementary table 4).

Table 1 shows that totally 7 groups of genes directly related to N metabolism which involved in nitrate and ammonium transport, nitrate and nitrite reduction, glutamine synthesis pathway were up-regulated in both rice and *Arabidopsis* during N starvation, except that *AMT1;1* gene (AT4G13510.1) was down-regulated in *Arabidopsis* (table 1). It is reasonable that N-metabolism-related genes especially those encoding transporters were strongly induced in order to uptake nitrate or ammonium more efficiently and to produce more N metabolites that maintained plants surviving under N starvation condition.

The pH dependency of the activity of the plant nitrate transporters located in plasma membrane is well known, as well as the involvement of the H^+-ATPase in the formation of a more favourable electrochemical gradient (Miller and Smith 1996; Wang and Crawford 1996). In the present study, two genes (LOC_Os03g17350.1 and LOC_Os07g12900.1) in rice and one gene (AT4G30110.1) in *Arabidopsis* encoding ATPases were up-regulated, along with genes encoding transporters including sulphate transporter 1;2, ABC transporter and ATPase, HPP family proteins in both rice and *Arabidopsis* (table 1). An increased activity of ATPases may suggest their important role in N transport regulation under N starvation condition, similar to the hypothetic role of plasma membrane ATPase in the regulation of nitrate and ammonium uptake (Martinoia *et al*. 2007; Pagliarani *et al*. 2008). ATP binding cassette (ABC) superfamily may use the energy of ATP hydrolysis to drive transporting a wide range of substrates including peptides, sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic acids and glutathione conjugates (Rea *et al*. 1998). HPP family proteins, which contain a conserved HPP motif, are integral membrane proteins with four transmembrane spanning helices and may function as transporters.

Cytochrome P450s are one of the largest superfamilies of enzymes and are ubiquitously distributed in all biological organisms; they catalyse the oxidation of a wide range of chemical reactions by activating dioxygen (Werck-Reichhart and Feyereisen 2000; Isin and Guengerich 2007), including phenolic and lipid metabolism, biosynthesis of isoprenoids, alkaloids and other amino-acid-derived compounds in plants (Morant *et al*. 2003; Schuler and Werck-Reichhart 2003). Plant cytochromes P450 are also major players in the detoxification and activation of environmental contaminants, including herbicides, pesticides and other industrial pollutants (Morant *et al*. 2003). To date, there is no detailed report about the function of cytochrome P450 involved in nitrogen metabolic pathway in plants, except one putative cytochrome P450 gene was up-regulated by nitrogen starvation (Karlsson *et al*. 2008). In contrast, we found four genes in rice (LOC_Os08g43390.1; LOC_Os02g30100.1; LOC_Os10g34480.1; LOC_Os07g33480.1) and siz genes in *Arabidopsis* (AT3G61880.1; AT4G37400.1; AT5G23190.1; AT5G08250.1; AT5G36110.1; AT5G36140.1) encoding cytochrome P450 were differentially expressed under N starvation condition (table 1), providing a new insight into the function of cytochrome P450 in plant.

In addition, a lot of genes encoding hormone responsive proteins, protein kinases and other domain/motif-containing proteins were also differentially expressed in both rice and *Arabidopsis* during N starvation (table 1).

Table 1. Differentially expressed genes that conserved in rice and *Arabidopsis* (Wang *et al.* 2003; Scheible *et al.* 2004; Bi *et al.* 2007) under N starvation condition

Gene locus	Signal Ratio (log 2)	Gene annotation
		N-related proteins
		N transport
AT1G12110.1	2.45	NRT1.1 (nitrate transporter 1.1); transporter
LOC_Os10g40600.1	1.11	Peptide transporter PTR2, putative, expressed
AT1G08090.1	4.1	ATNRT2:1 (high-affinity nitrate transporter 2.1)
AT1G08100.1	5.4	ATNRT2.2 (high-affinity nitrate transporter 2.2)
AT5G60770.1	2.35	ATNRT2.4 (high-affinity nitrate transporter 2.4)
LOC_Os02g02170.1	2.26	High-affinity nitrate transporter, putative, expressed
LOC_Os02g02190.1	2.28	High-affinity nitrate transporter, putative, expressed
AT5G50200.3	2.45	Putative component of high-affinity nitrate transporter
LOC_Os02g38230.1	2.25	Component of high-affinity nitrate transporter, putative, expressed
AT4G13510.1	−0.4	AMT1;1 (ammonium transport 1)
LOC_Os02g40710.1	2.65	ammonium transporter 1, member 1 precursor, putative, expressed
LOC_Os02g40730.1	1.5	Ammonium transporter 1, member 2, putative, expressed
		N reduction
AT1G77760.1	3.65	NIA1 (nitrate reductase 1)
AT1G37130.1	0.9	NIA2 (nitrate reductase 2)
LOC_Os02g53130.1	2.67	Nitrate reductase, putative, expressed
LOC_Os08g36480.1	2.1	Nitrate reductase 1, putative, expressed
AT2G15620.1	4.55	NIR1 (nitrite reductase)
LOC_Os01g25484.1	1.71	Ferredoxin–nitrite reductase, chloroplast precursor, putative, expressed
		N assimilation
AT5G35630.1	1	GS2 (glutamine synthetase 2); glutamate-ammonia ligase
LOC_Os04g56400.1	1.29	Glutamine synthetase, chloroplast precursor, putative, expressed
		Others
		Other transporters
AT1G78000.1	1.75	SULTR1;2 (sulphate transporter 1;2)
LOC_Os03g09980.1	1.35	Sulphate transporter 1.2, putative, expressed
AT3G55090.1	−0.45	ABC transporter family protein
AT2G37360.1	−0.55	ABC transporter family protein
LOC_Os03g17350.1	1.35	ATPase, coupled to transmembrane movement of substances, putative, expressed
AT4G30110.1	2.85	HMA2 (Heavy metal ATPase 2); cadmium-transporting ATPase
LOC_Os07g12900.1	1.12	Cadmium/zinc-transporting ATPase 2, putative
AT3G47980.1	2.95	Integral membrane HPP family protein
AT5G62720.1	4.1	Integral membrane HPP family protein
LOC_Os03g48030.1	1.65	HPP, putative, expressed
		Hormone responsive proteins
AT1G16510.1	-0.7	Auxin-responsive family protein
AT3G12830.1	0.8	Auxin-responsive family protein
LOC_Os04g52670.1	1.13	OsSAUR21 - Auxin-responsive SAUR gene family member, expressed
AT1G49660.1	−0.3	Similar to cell-death-associated protein-related (TAIR: AT1G49650.1)
LOC_Os09g28630.1	1.29	Gibberellin receptor GID1L2, putative, expressed
AT2G43820.1	−0.8	UDP-glucoronosyl/UDP-glucosyltransferase/UDP-glycosyltransferase family protein
LOC_Os04g12970.1	−2.29	Indole-3-acetate beta-glucosyltransferase, putative, expressed
AT1G22360.1	−0.55	UDP-glucoronosyl/UDP-glucosyl transferase family protein
LOC_Os02g51930.1	−1.17	Cytokinin-*O*-glucosyltransferase 2, putative, expressed

Table 1 (continued)

Gene locus	Signal Ratio (log 2)	Gene annotation
LOC_Os02g51910.1	1.14	Cytokinin-*O*-glucosyltransferase 2, putative, expressed
		cytochromes
AT3G61880.1	2.05	CYP78A9 (cytochrome P450 78A9); oxygen binding
LOC_Os08g43390.1	1.21	Cytochrome P450 78A3, putative, expressed
AT4G37400.1	0.55	CYP81F3 (cytochrome P450, family 81, subfamily F, polypeptide 3); oxygen binding
LOC_Os02g30100.1	−2.02	Cytochrome P450 81E1, putative, expressed
AT5G23190.1	0.65	CYP86B1 (cytochrome P450, family 86, subfamily B, polypeptide 1); oxygen binding
AT5G08250.1	1.4	Cytochrome P450 family protein
LOC_Os10g34480.1	1	Cytochrome P450 86A2, putative, expressed
AT5G36110.1	−0.4	CYP716A1 (cytochrome P450, family 716, subfamily A, polypeptide 1); oxygen binding
AT5G36140.1	−0.45	CYP716A2 (cytochrome P450, family 716, subfamily A, polypeptide 2); heme/iron ion binding/monooxygenase
LOC_Os07g33480.1	1.28	Taxane 10-beta-hydroxylase, putative, expressed
		Protein kinases
AT5G49760.1	0.65	Leucine-rich repeat family protein / protein kinase family protein
AT1G72300.1	1.2	Leucine-rich repeat transmembrane protein kinase, putative
LOC_Os11g14050.1	1.65	Receptor protein kinase-like, putative, expressed
LOC_Os02g05920.1	−1.15	Phytosulfokine receptor precursor, putative, expressed
AT2G37710.1	−0.35	RLK (receptor lectin kinase); kinase
AT4G02410.1	−0.7	Lectin protein kinase family protein
LOC_Os07g03780.1	−1.33	Lectin-like receptor kinase 7, putative
LOC_Os07g03870.1	−1.43	Lectin-like receptor kinase 7, putative, expressed
LOC_Os07g38810.1	−1.92	Lectin receptor-type protein kinase, putative, expressed
AT3G04810.1	−0.45	Protein kinase, putative
LOC_Os07g08000.1	1.48	Serine/threonine-protein kinase Nek4, putative, expressed
AT4G05200.1	−0.6	Protein kinase family protein
LOC_Os07g35290.1	−1.12	Receptor-like serine-threonine protein kinase, putative, expressed
LOC_Os07g35690.1	−1.42	CRK6, putative, expressed
LOC_Os07g35300.1	−1.09	CRK10, putative, expressed
AT2G17220.1	−0.3	Protein kinase, putative
LOC_Os04g47620.1	1.06	Protein kinase APK1B, chloroplast precursor, putative, expressed
		Other domain/motif-containing proteins
AT1G66160.1	−0.8	U-box domain-containing protein
LOC_Os03g13740.1	−1.27	Immediate-early fungal elicitor protein CMPG1, putative, expressed
AT1G80440.1	1.75	Kelch repeat-containing F-box family protein
LOC_Os06g39370.1	1.26	Protein kinase Kelch repeat: Kelch, putative, expressed
AT3G02550.1	−1.3	LOB domain protein 41/lateral organ boundaries domain protein 41 (LBD41)
LOC_Os01g32770.1	1.1	Seed specific protein Bn15D17A, putative, expressed
AT3G05200.1	0.6	Zinc finger (C3HC4-type RING finger) family protein (ATL6)
AT5G27420.1	−0.95	Zinc finger (C3HC4-type RING finger) family protein
LOC_Os08g37760.1	−1.07	Zinc finger, C3HC4 type family protein, expressed
AT4G27900.1	1.45	Similar to unknown protein (TAIR: AT5G53420.1); contains InterPro domain CCT;(InterPro: IPR010402)
LOC_Os05g51690.1	1.01	CCT motif family protein, expressed
AT2G23340.1	−0.6	AP2 domain-containing transcription factor, putative
LOC_Os04g55520.1	1.68	Dehydration responsive element binding protein, putative, expressed

3.5 *Up- or down-regulated miRNAs involved in N starvation condition*

In the past several years, an increasing number of miRNAs were discovered and deposited in the RNA Registry at *www.sanger.ac.uk/Software/Rfam/*, due to the improved method in identification of species-specific miRNAs (Zhang *et al*. 2006a). Among them, three miRNAs (miR395, miR398 and miR399) were identified as regulators of plant nutrient homeostasis. To examine their expression behaviour and to discover additional miRNAs that might involved in N starvation, the rice plant sample at 7 days after N starvation was used to hybridize with plant miRNA chip and analysed using SAM (supplementary table 5). Significant differentially expressed miRNAs were identified based on their Q-value of less than 0.01 and up- or down-regulated greater than twofold. Results showed that the expression level of osa-miR399i increased~threefold and osa-miR530 decreased~twofold by N starvation (supplementary table 5A), which were validated by qRT-PCR analysis (supplementary figure 2). The complete list of the predicted target genes of osa-miR399i and osa-miR530 can be found in supplementary table 5B and C. These potential targets of miRNAs were predicted using miRU (Plant microRNA Potential Target Finder; *http://bioinfo3.noble.org/miRNA/miRU.htm*).

The predicted target genes of osa-miR399i included *OsPHO2* (LOC_Os05g48390), which encodes an ubiquitin conjugating (UBC) enzyme. *OsPHO2* is the orthologue of *AtPHO2* (At2g33770) in *Arabidopsis* which is a conservative target of miR399 (Sunkar and Zhu 2004), involved in the targeted protein degradation pathway (Bachmair *et al*. 2001; Fujii *et al*. 2005; Kraft *et al*. 2005; Chiou *et al*. 2006; Chiou 2007), and regulates several Pi transport processes including Pi loading, redistribution and recycling (Fang *et al*. 2009). Our qRT-PCR analysis (primer sequences were presented in supplementary table 6) results showed that the expression level of *OsPHO2* (LOC_Os05g48390) was significantly decreased in root at 7 days after N starvation ($P<0.01$; figure 5). In addition, another predicted targeting gene (TNNI3K, LOC_Os01g54480) of osa-miR399i which encodes putative expressed serine/threonine-protein kinase was also significantly decreased in root at 7 days after N starvation ($P<0.05$; figure 5). This is in accordance with the induced expression level of osa-miR399i but in contrast to the previous reports in which up-regulation of miR399 was considered as a specific response to phosphorus deficiency but not other nutrients such as nitrogen, potassium, sulfate or carbohydrates (Fujii *et al*. 2005; Bari *et al*. 2006).

Under N starvation, osa-miR530 was also decreased to 45%, but no targets for miR530 have been identified till date (Liu *et al*. 2005). However, in this study, several genes were predicted to be targets of osa-miR530 in rice (supplementary table 5C), and most of them were significantly increased at 7 days after N starvation from our qRT-PCR analysis ($P<0.05$; figure 5). For example, one of the targets of osa-miR530, an expressed protein (LOC_Os08g02180), was up-regulated both in root and shoot at 7 days after N starvation, and six predicted targeting genes (including LOC_Os01g70430, LOC_Os02g52390, LOC_Os04g02280, LOC_Os08g07830, LOC_Os08g34140 and LOC_Os09g36000) were up-regulated in shoot at 7 days after N starvation (figure 5). These results indicated an important role of miR530 in N nutrient metabolic pathway in plants.

4. Discussions

4.1 *Distinct transcriptional response to N starvation in root and shoot*

In this study, we observed that different sets of genes were responsive to N starvation by altering their transcript levels at different time points in the same organ or in different organs at the same time points. The 'early' (at 1 h after N starvation) transcriptional response consisted of genes involved in the signal transduction pathways, encoding transcription factors and kinases. The 'late' (at 7 days after N starvation) responsive genes were more related to the metabolic processes, substance transportation and physiological pathways, correlating with the morphological changes at 7 days after nutrient starvation. Different sets of genes responsive to N starvation at different time points indicated that there were different strategies to adapt to the short-term N deprivation and long-term N deprivation.

We also observed that some of the genes with similar functions were up- or down-regulated at the same time point. For example, 38 genes response to stress (GO:0006950) were up-regulated in root at 1 h while other 49 stress responsive genes were down-regulated; also, 26 genes responsive to stress were up-regulated in root at 24 h while other 22 stress responsive genes were down-regulated. These results suggested there was an existence of a compensatory mechanism within these biological processes: different members in the same gene family may express differentially to balance the gene expression and metabolic products during nutrient starvation.

4.2 *Cross-talk between N and C/P/S in response to nutrient starvation in plants*

It is well known that the deficiency of one mineral leads to imbalances of nutrients and influences the uptake of other nutrients (Schachtman and Shin 2007). It was also known that cross-talks in signal transduction pathways occurred between nitrogen and carbon metabolism (Muller *et al*. 2007; Yuan and Liu 2008). It is generally agreed that

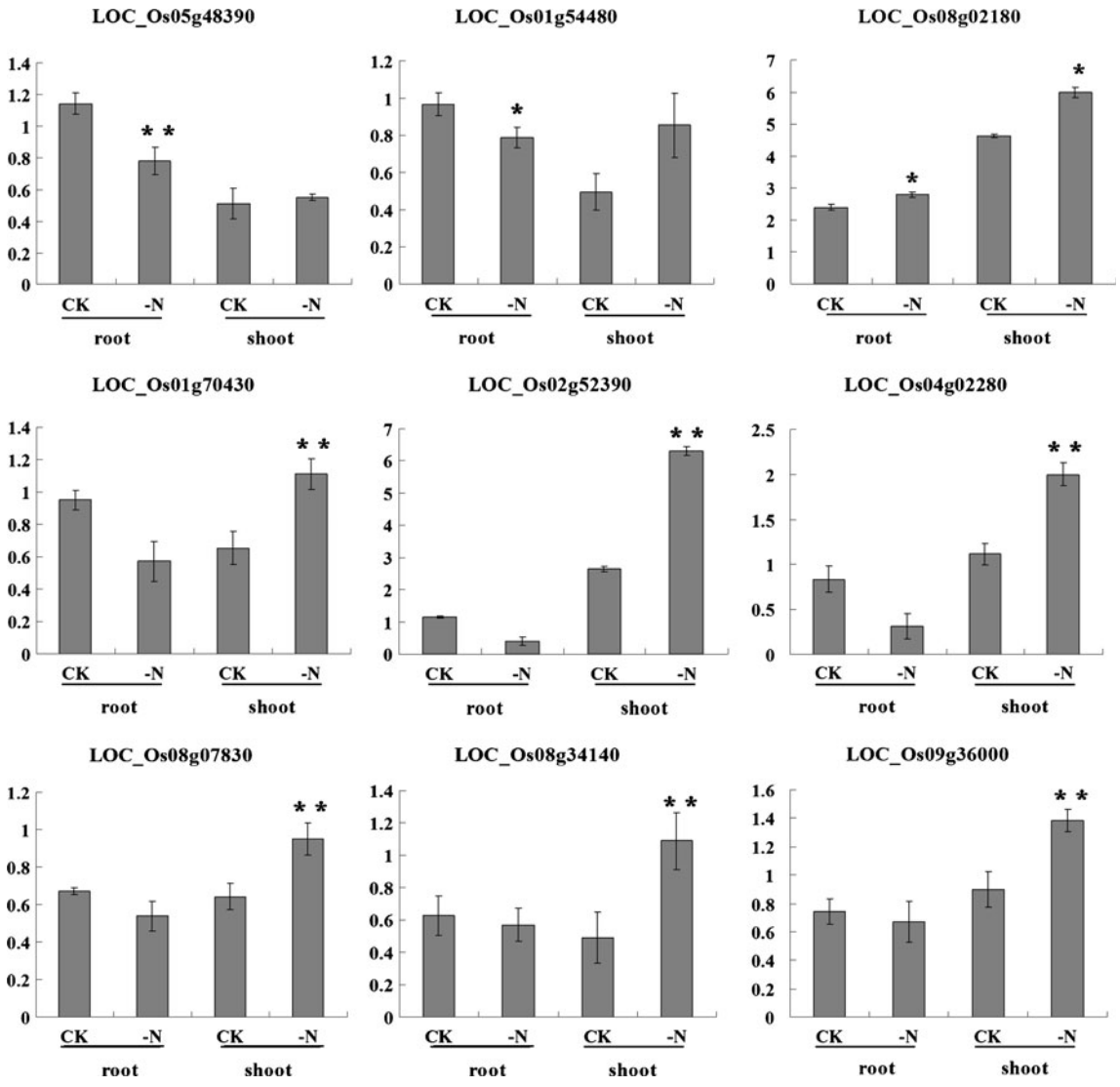


Figure 5. Relative expression analysis of the predicted target genes of osa-miR399i (LOC_Os05g48390, LOC_Os01g54480) and osa-miR530 (LOC_Os08g02180, LOC_Os01g70430, LOC_Os02g52390, LOC_Os04g02280, LOC_Os08g07830, LOC_Os08g34140, LOC_Os09g36000) in root and shoot by qRT-PCR. CK: no N starvation treatment; -N: 7 days after N starvation. Y-axis means the relative expression level of predicted target genes mentioned above; values are mean ± SD from three independent biological replicates. *, **Significant differences at the levels of $P=0.05$ and $P=0.01$, respectively.

nitrogen and carbon metabolism were intrinsically regulated. Wu *et al*. (2003) proposed that Pi starvation resulted in repression of photosynthetic gene expression and photosynthetic activity in leaf cells, which would affect carbon reduction and nitrate assimilation. After 24 h of Pi starvation, genes for Fd-NiR (nitrite reductase), GS (glutamine synthetase) and Fd-GOGAT (glutamate synthase) were repressed, while genes for GDH (glutamate dehydrogenase) were induced by the reduction of carbon and organic nitrogen in plants to provide NH_4^+ for the GS/GOGAT cycle (Wu *et al*. 2003). Similarly, in our study, N starvation affected carbon utilization and fixation, photosynthesis and carbohydrate metabolic processes. Additionally, N starvation also affected lipid transport and metabolism, sulphate transport and reduction, which proposed potential interactions between N and C/P/S assimilation.

4.3 *Conservative regulatory networks respond to N starvation responses in rice and Arabidopsis*

Comparative analysis displayed many conserved genes differentially expressed both in rice and *Arabidopsis*. For example, genes encoding N metabolic proteins, hormone

responsive proteins, cytochromes, protein kinases and domain/motif-containing proteins were differentially expressed in both rice and *Arabidopsis* during N starvation. Besides genes encoding N transporters and reductases, expression levels of several signal transduction genes were also altered after N starvation in both rice and *Arabidopsis*, suggesting that not only structural and functional genes but genes involved in important regulatory networks also respond to N starvation in monocot and dicot plants. Six MADS-box transcription factors were differentially expressed during this experiment but none of them responded to N starvation in root. This is different from the results reported in *Arabidopsis*, in which ANR1, a MADS-box gene that regulates lateral root development, responded to N starvation (Zhang and Forde 1998). However, we found two LOB-domain proteins (LOC_Os01g32770, LOC_Os07g40000) with orthologs in *Arabidopsis* were differentially expressed at 7 days after N starvation in root. LOB-domain proteins were found to be required for adventitious root formation in rice (Liu *et al*. 2005). These results suggested that there are conserved modules in nutrient responses between the two species, despite of differences in the initial signaling pathways. Further comparative analysis of nutrient starvation process with time series experiments of paralleled N starvation will likely facilitate the identification of downstream conserved modules for general stress responses and evaluation of their weightiness.

5. Conclusion

Analysis of genome-wide gene expression profiles in N starvation in rice shoot and root, using Affymetrix Rice Genome Chip, has provided an overview of transcriptional responses to N starvation conditions. Future efforts will be devoted to understand the interaction of these nutrient starvation responsive genes, especially those with conserved functions in both rice and *Arabidopsis*. Experimental alterations of the expression of these genes and miRNAs by overexpression, RNA interference and promoter analysis have been demonstrated to be useful strategies and are being used for characterizing their functions to gather valuable information for nitrogen-use efficiency improvement of rice crop.

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