Early molecular events associated with nitrogen deficiency in rice seedling roots

Ping-Han Hsieh, Chia-Cheng Kan, Hsin-Yu Wu, Hsiu-Chun Yang & Ming-Hsiun Hsieh

Nitrogen (N) deficiency is one of the most common problems in rice. The symptoms of N deficiency are well documented, but the underlying molecular mechanisms are largely unknown in rice. Here, we studied the early molecular events associated with N starvation (−N, 1 h), focusing on amino acid analysis and identification of −N-regulated genes in rice roots. Interestingly, levels of glutamine rapidly decreased within 15 min of −N treatment, indicating that part of the N-deficient signals could be mediated by glutamine. Transcriptome analysis revealed that genes involved in metabolism, plant hormone signal transduction (e.g. abscisic acid, auxin, and jasmonate), transporter activity, and oxidative stress responses were rapidly regulated by −N. Some of the −N-regulated genes encode transcription factors, protein kinases and protein phosphatases, which may be involved in the regulation of early −N responses in rice roots. Previously, we used similar approaches to identify glutamine-, glutamate-, and ammonium nitrate-responsive genes. Comparisons of the genes induced by different forms of N with the −N-regulated genes identified here have provided a catalog of potential N regulatory genes for further dissection of the N signaling pathways in rice.

Rice is a staple food for almost half of the world’s population. The production of rice, especially in Asian countries, is important in food security. The Green Revolution rice cultivars developed in 1960’s, which constitute most of the rice varieties grown today, require large amounts of nitrogen (N) fertilizers to produce high yields. However, the production of N fertilizer requires a lot of energy. Furthermore, only 20–30% of the applied N fertilizer is taken up by the rice plant. Most of the N fertilizers applied to rice are lost to the air or water, which causes substantial environmental problems. Thus, the use of N fertilizer is costly to farmers and the environment. The current agricultural practices are not economically and environmentally sustainable. Therefore, considerable efforts have been directed toward improvement of N management and development of new rice varieties with better N use efficiency in the past decades to ensure sustainable agriculture.

Despite decades of study, the improvement of N use efficiency in crop plants is still one of the scientific “Grand Challenges” in the 21st century. To face this challenge, we need to have a better understanding of the genetics behind N uptake, transport, metabolism, and remobilization in crop plants, especially when N is limited in the environment. Since N is a major constituent of amino acids, nucleic acids, chlorophyll, ATP, coenzymes, plant hormones, and secondary metabolites, N deficiency affects all aspects of plant function, from metabolism to resource allocation, growth and development. To cope with N deficiency, plants have evolved complex morphological, physiological, and biochemical adaptations to the adverse environments. For instance, plants will increase its capacity to acquire N by stimulating root growth relative to shoot growth in response to N deficiency. The expression of high affinity nitrate and ammonium transporter genes was induced by N starvation (−N). Furthermore, the remobilization of stored N and the release of ammonium via the biosynthesis of phenylpropanoids were stimulated by N deprivation. It is evident that plants have evolved regulatory systems to adjust metabolism, conserve resources and activate the acclimatory pathways enabling them to adapt to N-deficient conditions. Nevertheless, the molecular mechanisms underlying the N-deficient responses are still largely unknown in plants.

Global gene expression profiling using microarrays or RNA sequencing (RNA-Seq) has been a successful approach to study the molecular aspects of nutrient and stress responses. For instance, microarrays were used in several studies to identify nitrate-responsive genes in Arabidopsis and rice. Ammonium is believed to be the...
major N source for paddy rice. Transcriptome analysis using microarray or RNA-Seq has been applied to identify ammonium-responsive genes in rice. Similarly, N-responsive genes have been identified by transcriptome analyses in rice. These studies have provided catalogs for the identification of potential N regulatory genes.

Indeed, transcriptome analysis followed by reverse genetic study has successfully identified several N regulatory genes in plants. For instance, the LBD37/38/39 transcription factor genes were identified as nitrate-responsive genes in Arabidopsis. Further genetic studies demonstrated that LBD37/38/39 are regulators of N responses in Arabidopsis. The expression of Os02g0325600 encoding nitrate-inducible GARP (GOLDEN2, ARR-B, Par1) transcriptional repressor 1 (NIGT1) was specifically induced by nitrate. The Arabidopsis NIGT1 homolog is involved in the regulation of nitrate and phosphate signals at the root tip. More recently, the Os02g0120100 gene encoding ACT domain-containing protein kinase 1 (ACTPK1), a homolog of Arabidopsis serine/threonine/tyrosine kinase 46 (STY46), was identified by transcriptome analysis of ammonium-responsive genes in rice roots. Further genetic and biochemical studies demonstrated that ACTPK1 can phosphorylate and inactivate AMT1;2, a major ammonium transporter, under ammonium-sufficient conditions.

Since the availability of nutrients in the soil is directly perceived by roots, we aim to uncover the early molecular events associated with N deficiency in rice roots. This study primarily focused on the analysis of amino acids and identification of differentially expressed genes (DEGs) in response to N deficiency. All of the DEGs identified by microarray analysis were verified by quantitative (q)RT-PCR, and only the verified genes were further used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Interestingly, these analyses revealed that genes involved in carbon (C) and N metabolism, "plant hormone signal transduction" and "transporter activity" were enriched in N-regulated genes. In addition, several novel N regulatory genes, including those encode transcription factors, protein kinases and protein phosphatases, were identified here. These newly identified N regulatory genes may play important roles in the regulation of N-deficient responses in rice roots.

In addition to N, we previously used the same platform with similar criteria to identify ammonium nitrate (+N)-, glutamine (+Gln)-, and glutamate (+Glu)-regulated genes in rice roots. These studies were conducted with similar setups, which allowed us to perform data comparisons across different experiments. We have identified at least 34 N-sensitive genes, whose expression was rapidly induced by +N and quickly repressed by N. In addition to genes involved in nitrate/nitrite assimilation, ferredoxin reduction, and the pentose phosphate pathway, the identified N-sensitive genes include several well-known N regulatory genes, such as LBD37 (Os02g0445700, Os07g0589000), LBD38 (Os03g0609500) and BT2 (Os01g0908200, BTB/POZ and TAZ domain-containing protein 2). These results implicate that some of the novel N-sensitive genes may be involved in the regulation of N starvation and/or responses in rice. Further studies on the N-regulated genes or N-sensitive genes identified here may provide new solutions to increase N use efficiency in rice.

Results

Effects of N starvation on the growth of rice seedlings. Compared with the green and healthy seedlings grown in +N hydroponic solution, the 10-day-old rice seedlings grown in N medium have chlorotic leaves, thinner and longer roots (Fig. 1A). The shoot length, root length, and chlorophyll content of the +N- and N-treated rice seedlings are shown in Fig. 1B,C. The inhibition of shoot growth and promotion of root growth were also observed in 10-day-old rice seedlings treated with N for 2, 5, and 7 days (Supplementary Fig. S1). It is known that N can stimulate primary root growth by enhancing cell elongation as well as cell division. These results implicated that N deficiency could induce changes at cellular, biochemical, and molecular levels to affect plant growth and development.

Effects of N starvation on amino acid content in rice roots. To examine the effects of N on amino acid content in 10-day-old rice seedlings, we measured the amounts of free amino acids in the roots after N treatment for 15 min to 4 h. Glutamine, glutamate, asparagine and aspartate are the most abundant amino acids in rice seedlings. Interestingly, levels of glutamine were rapidly reduced during the time course of N starvation. The amount of free glutamine was reduced approximately 50% during the first 15 min of N treatment (Fig. 2, Gln). By contrast, levels of glutamate were not reduced until 4 h after N treatment (Fig. 2, Glu). The amount of aspargine did not change significantly during the time course of N treatment (Fig. 2). Levels of aspartate increased slightly within 1 h, and started to decrease after 4 h of N treatment (Fig. 2). The amounts of the other amino acids did not change significantly during the time course of N treatment (Supplementary Fig. S2).

Identification of genes rapidly respond to nitrogen starvation. We used microarray analysis to identify DEGs in the roots of 10-day-old rice seedlings treated with N for 1 h. The expression of 288 genes were differentially regulated (N/-N, 144 up and 144 down) with 2-fold cutoff. Quantitative (q)RT-PCR analysis was used to examine the expression of all 288 genes identified by microarray analysis. The results confirmed that N induced the expression of 116 genes and repressed the expression of 98 genes within 1 h with 2-fold cutoff. The expression patterns of these genes during the time course of N treatment are shown in Supplementary Figs S3 and S4. The N-induced 116 genes and the N-repressed 98 genes are listed in Tables 1 and 2, respectively. These results revealed that approximately 81% of the up-regulated genes and 68% of the down-regulated genes identified by microarray analysis were reproducible in the independent qRT-PCR experiment. Most of the disqualified genes were up- or down-regulated by approximately 2-fold in the microarray analysis.

GO and KEGG enrichment analyses of early N starvation-induced genes. AgriGO (http://bioinfo.cau.edu.cn/AgriGO/) was used for GO enrichment analysis of the 116 genes induced by N. In biological process, the GO terms "cellular amino acid and derivative metabolic process", "cellular ketone metabolic process", "organic acid metabolic process", "cellular nitrogen compound metabolic process", and "amine metabolic process" were enriched.
significantly enriched (Fig. 3A). In molecular function, the GO terms “ion/cation/metal ion binding” were significantly enriched (Fig. 3B). No GO terms were enriched in the category of cellular component. The information of genes enriched in GO analysis is provided in Supplementary Table S1. In addition, KEGG pathway analysis of the 116 −N-induced genes revealed that “plant hormone signal transduction (ko04075)”, “carotenoid biosynthesis (ko00906)”, “plant-pathogen interaction (ko04626)”, “linoleic acid metabolism (ko00591)”, and “arginine and proline metabolism (ko00330)” were enriched. The information of genes enriched in these KEGG pathways is provided in Supplementary Table S2. Together, the GO and KEGG enrichment analyses suggest that −N rapidly induced the expression of genes involved in N remobilization and plant hormone signal transduction in rice roots.

Analysis of −N-induced genes involved in metabolism, plant hormone signal transduction, and oxidative response. In the category of biological process, GO enrichment analysis identified 9 genes encoding enzymes involved in metabolic processes. These enzymes are arogenate dehydratase/prephenate dehydratase 6 (ADT6, Os10g0523700) of phenylalanine biosynthesis, phenylalanine ammonia-lyase (PAL3, Os02g0626600 and PAL4, Os02g0627100) of the phenylpropanoid pathway, methionine gamma-lyase (MGL, Os10g0517500) of methionine catabolism, acetylornithine aminotransferase (ACOAT, Os07g0461900) and ornithine decarboxylase 1 (ODC1, Os09g0543400) of arginine biosynthesis, acyl-[acyl-carrier-protein] desaturase 7 (SAD, Os08g0200100, an Arabidopsis SAD2 homolog), α-carboxyl anhydrase 7 (α-CA7, Os08g0470200), and GTP diphosphokinase (calcium-activated RelA-SpoT homolog 2 [CRSH2], Os05g0161500). CRSH2 is a Ca^{2+}-activated (p)ppGpp synthetase that has been proposed to integrate the Ca^{2+} and (p)ppGpp signaling pathways in rice. The expression patterns of these metabolic genes during the time course (0–24 h) of −N treatment are shown in Fig. 4A.

The genes identified in the KEGG pathway “carotenoid biosynthesis (ko00906)”, Os09g0555500 encoding phytoene synthase 3 (PSY3), Os03g0125100 encoding beta-carotene hydroxylase 1 (BCH1), and Os08g0472800 encoding abscisic acid 8′-hydroxylase 2 (ABA8OX2), are also involved in the biosynthesis and metabolism of plant hormone ABA. The expression patterns of these genes during the time course of −N treatment are...
shown in Fig. 4B. The genes enriched in the KEGG pathway “linoleic acid metabolism (ko00591), including Os08g0508800 encoding chloroplastic lipoxygenase 7, a homolog of Arabidopsis LOX2, and Os03g0738600 encoding linoleate 9S-lipoxygenase 2, a homolog of Arabidopsis LOX1, are involved in the biosynthesis of plant hormone jasmonic acid (JA). The expression of these JA biosynthesis genes was rapidly induced by −N (Fig. 4B).

In addition, the expression of several genes related to oxidative stress was also rapidly induced by −N (Table 1 and Supplementary Fig. S3). For instance, the expression of Os06g0521500, Os05g0135400, and Os06g0522300 encoding peroxidase (POX) and Os07g0468100 encoding glutathione S-transferase (GSTU1) was rapidly induced by −N (Fig. 4C). Galactinol synthase (GolS) is the key enzyme for the synthesis of raffinose family oligosaccharide in plants43. In addition to their roles as osmoprotectants, galactinol and raffinose have been shown to protect plants from oxidative damage44. Interestingly, we found that the expression of GolS1 (Os03g0316200) and GolS2 (Os07g0687900) was rapidly and strongly induced by −N in rice roots (Fig. 4C). These results implicated that oxidative stress might be one of the early events associated with N deprivation in the roots of rice seedlings.

Identification of early N starvation-induced transcription factor genes. Of the 116 −N-induced genes, at least 12 genes encode transcription factors. The expression patterns of these transcription factor genes during the time course of −N treatment are shown in Fig. 5A. The Os03g0183500 gene encodes an uncharacterized plant-specific FCS-like zinc finger protein FLZ2445,46. The expression of FLZ24 was rapidly and strongly induced by −N, which peaked at 15 min during the time course of −N treatment (Fig. 5A). The homeodomain-leucine zipper (HD-ZIP) gene HOX22 (Os04g0541700) has been shown to affect ABA biosynthesis and regulate drought and salt responses through ABA-mediated signaling pathways in rice47. The basic helix-loop-helix transcription factor gene bHLH120 (Os09g0455300) corresponds to the quantitative trait locus qRT9 that controls root thickness and root length in upland rice48. Previous studies revealed that the expression of HOX22 and bHLH120 was strongly induced by polyethylene glycol, salt, and ABA47,48. Interestingly, the expression of HOX22 and bHLH120 was also rapidly and strongly induced by −N (Fig. 5A). The Os12g0156100 gene encodes a homolog of Arabidopsis NAC90. The expression of Os12g0156100 (NAC90) was strongly induced by −N after treatment for 1–4 h (Fig. 5A).

The plant-specific TIFY/JAZ (jasmonate-zim domain) transcription factors are key regulators of JA signaling pathways49. It has been demonstrated that the expression of TIFY11a (JAZ9, Os03g0180800), TIFY11c (JAZ11, Os03g0180900) and TIFY11e (JAZ13, Os10g0391400) was strongly induced by JA49. Interestingly, −N also rapidly induced the expression of these key transcription factor genes for JA signaling (Table 1). The expression patterns of TIFY11a (JAZ9, Os03g0180800), TIFY11c (JAZ11, Os03g0180900) and TIFY11e (JAZ13, Os10g0391400) during
| No. | Locus ID      | Fold change | Gene description                                      |
|-----|--------------|-------------|-------------------------------------------------------|
| 1   | Os12g0189300 | 9.5         | Carboxyvinyl-carboxyphosphonate phosphorylmutase       |
| 2   | Os12g01356100| 5.5         | NAC domain-containing protein 90 (NAC90)              |
| 3   | Os06g0725200 | 5.5         | GDSL esterase/lipase (GELP90)                        |
| 4   | Os08g0137800 | 5.1         | Mavicyanin, phytocyanin                               |
| 5   | Os07g0127500 | 4.6         | Pathogenesis-related protein PRB1-2                  |
| 6   | Os03g0667500 | 4.1         | Fe(II) transport protein 1 (OsIRT1)                  |
| 7   | Os02g0626600 | 4.1         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 8   | Os09g0555500 | 4.0         | Phytosynthase 3 (PSY3)                               |
| 9   | Os09g0543900 | 3.9         | Agmatine cyanuramyltransferase-2                     |
| 10  | Os09g0537401 | 3.9         | Unknown                                              |
| 11  | Os01g0595600 | 3.7         | Probable esterase KARRIKIN-INSENSITIVE 2 (KA12)      |
| 12  | Os12g0556100 | 3.5         | NAC domain-containing protein 90 (NAC90)             |
| 13  | Os06g0725200 | 3.5         | GDSL esterase/lipase (GELP90)                        |
| 14  | Os09g0555500 | 3.4         | Pathogenesis-related protein PRB1-2                  |
| 15  | Os03g0667500 | 3.4         | GDSL esterase/lipase (GELP90)                        |
| 16  | Os02g0626600 | 3.4         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 17  | Os09g0555500 | 3.3         | Pathogenesis-related protein PRB1-2                  |
| 18  | Os03g0667500 | 3.3         | GDSL esterase/lipase (GELP90)                        |
| 19  | Os02g0626600 | 3.3         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 20  | Os09g0555500 | 3.2         | Pathogenesis-related protein PRB1-2                  |
| 21  | Os03g0667500 | 3.2         | GDSL esterase/lipase (GELP90)                        |
| 22  | Os02g0626600 | 3.2         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 23  | Os09g0555500 | 3.1         | Pathogenesis-related protein PRB1-2                  |
| 24  | Os03g0667500 | 3.1         | GDSL esterase/lipase (GELP90)                        |
| 25  | Os02g0626600 | 3.1         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 26  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 27  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 28  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 29  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 30  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 31  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 32  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 33  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 34  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 35  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 36  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 37  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 38  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 39  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 40  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 41  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 42  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 43  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 44  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 45  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 46  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 47  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 48  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 49  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 50  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 51  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 52  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 53  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |
| 54  | Os03g0667500 | 3.0         | GDSL esterase/lipase (GELP90)                        |
| 55  | Os02g0626600 | 3.0         | Phenylalanine ammonia-lyase 3 (PAL3)                 |
| 56  | Os09g0555500 | 3.0         | Pathogenesis-related protein PRB1-2                  |

Continued
| No. | Locus ID | Fold change | Gene description |
|-----|----------|-------------|------------------|
| 57  | Os07g0633400 LOC_Os07g43970 | 2.4 | IQ domain-containing protein IQM2 |
| 58  | Os04g0365100 LOC_Os04g29580 | 2.3 | Wall-associated receptor kinase 37 (WAK37) |
| 59  | Os09g0325700 LOC_Os09g15670 | 2.3 | Protein phosphatase 2C 68 (PP2C68) |
| 60  | Os12g0227500 LOC_Os12g12600 | 2.3 | Dirigent-like protein |
| 61  | Os08g0347000 LOC_Os08g25850 | 2.3 | Unknown |
| 62  | Os12g0150200 LOC_Os12g05440 | 2.3 | Cytochrome P450 94C1 |
| 63  | Os04g0517500 LOC_Os04g3710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 64  | Os12g0245700 LOC_Os12g14220 | 2.3 | Unknown |
| 65  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 66  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 67  | Os08g0347000 LOC_Os08g25850 | 2.3 | Unknown |
| 68  | Os12g0245700 LOC_Os12g14220 | 2.3 | Unknown |
| 69  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 70  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 71  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 72  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 73  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 74  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 75  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 76  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 77  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 78  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 79  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 80  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 81  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 82  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 83  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 84  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 85  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 86  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 87  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 88  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 89  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 90  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 91  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 92  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 93  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 94  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 95  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 96  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 97  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 98  | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 99  | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 100 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 101 | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 102 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 103 | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 104 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 105 | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 106 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 107 | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 108 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |
| 109 | Os01g0826400 LOC_Os01g61080 | 2.3 | WRKY transcription factor 33 (WRKY33) |
| 110 | Os04g0517500 LOC_Os04g43710 | 2.3 | Phosphoenolpyruvate carboxylase kinase 3 (PPCK3) |

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previously shown to be down-regulated by potassium (K) deficiency\(^57,58\). In addition, the protein kinase 29 (CIPK29) was rapidly induced by −N in rice roots. The cell wall-associated receptor kinases (WAKs) are primarily involved in the regulation of plant cell wall functions such as pathogen response, binding to pectin to control cell expansion, morphogenesis and development\(^55,56\). The expression of CIPK29 was rapidly induced by −N in rice roots (Fig. 5B). Interestingly, the expression of CIPK29 was previously shown to be down-regulated by potassium (K) deficiency\(^57,58\). In addition, the Os12g0189300 gene encodes a homolog of Arabidopsis SARD1 that is involved in salicylic acid (SA) signaling pathways. The expression of SARD1 (Os08g306300) was also induced by −N in rice roots (Fig. 5A).

We previously used microarray and qRT-PCR analyses to identify 158 genes that were rapidly induced by −N and +N treatments in rice roots (Table 1). To verify this result, we used 10-day-old rice seedlings to conduct −N and +N time course treatments. Total RNA extracted from these samples was used for qRT-PCR analysis to examine the expression of these genes identified in the microarray data. The expression of genes listed here was up-regulated by nitrogen starvation (−N/+N) for more than 2-fold in the qRT-PCR analysis.

### Table 1. List of genes rapidly induced by nitrogen starvation in rice roots.

| No. | Locus ID | Fold change | Gene description |
|-----|----------|-------------|------------------|
| 111 | LOC_Os01g04590 | 2.0 | Unknown, DUF789 domain-containing |
| 112 | LOC_Os02g05060 | 2.0 | Auxin-induced protein X15 |
| 113 | LOC_Os06g05700 | 2.0 | Probable receptor-like protein kinase (RLK) |
| 114 | LOC_Os06g18900 | 2.0 | Unknown |
| 115 | LOC_Os04g58100 | 2.0 | Homeobox-leucine zipper protein HOX22 |
| 116 | LOC_Os05g34600 | 2.0 | Protein LURP-one-related 5 |

| No. | Locus ID | Fold change | Gene description |
|-----|----------|-------------|------------------|
| 117 | LOC_Os01g04590 | 2.0 | Unknown, DUF789 domain-containing |
| 118 | LOC_Os02g05060 | 2.0 | Auxin-induced protein X15 |
| 119 | LOC_Os06g05700 | 2.0 | Probable receptor-like protein kinase (RLK) |
| 120 | LOC_Os06g18900 | 2.0 | Unknown |
| 121 | LOC_Os04g58100 | 2.0 | Homeobox-leucine zipper protein HOX22 |
| 122 | LOC_Os05g34600 | 2.0 | Protein LURP-one-related 5 |

The activity of phosphoenolpyruvate carboxylase (PEPC), a key enzyme of primary metabolism of higher plants, is regulated by PEPC kinase (PPCK). The expression of PPCK3 (Os04g0365100) was rapidly induced by −N in rice roots (Fig. 3B). The expression of Os04g0365100 encoding calcineurin B-like protein (CBL) interacting protein kinase 39 (CIPK39) was rapidly induced by −N (Fig. 3B). Interestingly, the expression of CIPK29 was previously shown to be down-regulated by potassium (K) deficiency\(^57,58\). In addition, the Os12g0189300 gene encoding carboxyvinyl-carboxyphosphonate phosphorylmutase was rapidly and strongly induced by −N (Table 1). The only protein phosphatase gene found to be rapidly induced by −N is Os09g0325700 that encodes protein phosphatase 2C 68 (PP2C68). The rice PP2C68 is a homolog of Arabidopsis HAI1/2/3 (highly ABA-induced PP2C protein 1/2/3). It is not clear if ABA can induce the expression of PP2C68. Nevertheless, −N can induce the expression of PP2C68 in rice roots. The expression patterns of PP2C68 during the time course of −N treatment are shown in Fig. 5A.

**Analysis of genes rapidly induced by −N and +N.** We previously used microarray and qRT-PCR analyses to identify 158 genes that were rapidly induced by +N (1.43 mM ammonium nitrate, 30 min) in the roots of hydropponically grown rice seedlings\(^34\). We compared the 116 genes up-regulated by −N (Table 1) with the 158 genes induced by +N and found that the expression of 3 genes, Os01g0705200 encoding a late embryogenesis-abundant protein, Os08g0473900 encoding an α-amylose, and Os10g0576600 encoding a tetratricopeptide repeat (TPR) protein was induced by both −N and +N treatments in rice roots (Fig. 6A and Table 3). To verify this result, we used 10-day-old rice seedlings to conduct −N and +N time course treatments. Total RNA extracted from roots of these samples was used for qRT-PCR analysis to examine the expression of Os01g0705200, Os08g0473900, and Os10g0576600. The results revealed that these genes responded to +N and −N rapidly and transiently (Fig. 6B–D). In general, the expression of these genes was induced by +N and −N after 15 min to 4 h, and back to control levels after 24 h (Fig. 6B–D).

**GO and KEGG enrichment analyses of early N starvation-repressed genes.** In addition to −N-induced genes, we also performed GO and KEGG enrichment analyses on the 98 genes repressed by −N. In biological process, the GO terms such as “metal ion transport” and “glucose metabolic process” were significantly enriched (Fig. 7A). In molecular function, the GO terms “transporter activity” and “inorganic cation transport activity” were also significantly enriched (Fig. 7B).
| No.  | Locus ID   | Fold change | Gene description                        |
|-----|------------|-------------|-----------------------------------------|
| 1   | Os02g0770800 | −16.7       | Nitrates reductase [NAD(P)H]             |
| 2   | Os05g0114400 | −13         | Zinc finger transcription factor, ZOSS-02|
| 3   | Os08g0468100 | −7.5        | Nitrates reductase [NAD(P)H] 1           |
| 4   | Os11g0184900 | −7.4        | NAC domain-containing protein 5 (NAC5)  |
| 5   | Os10g0461200 | −7          | Uroporphyrinogen-III C-methyltransferase|
| 6   | Os03g0684700 | −6.8        | Unknown, integral membrane HPP family protein |
| 7   | Os08g0210600 | −6.4        | Fructose-bisphosphate aldolase           |
| 8   | Os05g0194900 | −6.2        | ATP-dependent 6-phosphofructokinase 4 (PFK04) |
| 9   | Os03g0695500 | −6.1        | LOB-domain-containing protein 38 (LBD38) |
| 10  | Os09g0507900 | −5.9        | EF-hand domain-containing protein        |
| 11  | Os03g0126900 | −5.8        | Unknown, putative AtZIP domain-containing protein |
| 12  | Os08g0113900 | −5.7        | Unknown, putative AtZIP domain-containing protein |
| 13  | Os11g0184900 | −5.6        | Ferredoxin, root R-B1                    |
| 14  | Os04g0506800 | −5          | Sialyltransferase-like protein 3 (STLP3)  |
| 15  | Os09g0484900 | −5          | Tonoplast dicarboxylate transporter (TDT) |
| 16  | Os07g0589000 | −4.8        | NBR-ARC and LRR domain-containing protein, RPM1-like |
| 17  | Os12g0199000 | −4.6        | NB-ARC and LRR domain-containing protein, RPM1-like |
| 18  | Os04g0656500 | −4.5        | Myb family protein                       |
| 19  | Os07g0147500 | −4.4        | UDP-glucose:protein N-acetyltransferase 2 |
| 20  | Os05g0194900 | −4          | RING-type E3 ubiquitin-protein ligase ELS-like |
| 21  | Os09g0545280 | −3.8        | OsSAUR39 - Auxin-responsive SAUR gene family member |
| 22  | Os11g0184900 | −3.8        | CROCETIN GLUCOSYLTRANSFERASE-2 (CGL2)     |
| 23  | Os11g0184900 | −3.7        | Zinc-finger-FLZ domain-containing protein 14 (FLZ14) |
| 24  | Os02g0620600 | −3.6        | Ammonium transporter 1 member 2 (AMT1:2) |
| 25  | Os02g0756600 | −3.5        | Protein EXORDIUM                        |
| 26  | Os02g0120100 | −3.5        | Serine/threonine-protein kinase STY46     |
| 27  | Os02g0325600 | −3.4        | Nitrate-inducible, GARP-type transcriptional repressor 1 (NIGT1) |
| 28  | Os12g0113500 | −3.3        | CBL-interacting protein kinase 14 (CIPK14) |
| 29  | Os10g0578000 | −3.3        | Plastidial glycolate/glycerate translocase 1 (PGI1) |
| 30  | Os01g0180200 | −3.3        | BTB/POZ and TAZ domain-containing protein 2 (BT2) |
| 31  | Os05g0011600 | −3.2        | Protein phosphatase 2 C 46 (PP2C46)       |
| 32  | Os03g0764600 | −3.1        | O-methyltransferase (OMT)                 |
| 33  | Os04g0656500 | −3.1        | MYB family protein                       |
| 34  | Os05g0401500 | −3          | ZOG-Fe(II) oxygenase superfamily         |
| 35  | Os05g0443700 | −3          | Unknown, syntaxin 6 N-terminal domain-containing protein |
| 36  | Os05g0472400 | −3          | Zinc transporter 9 (ZIP9)                 |
| 37  | Os05g0507000 | −2.9        | Putative F-box protein PP2-B12            |
| 38  | Os08g0506800 | −2.9        | GDSL esterase/lipase 2 (GELP2)           |
| 39  | Os06g0535200 | −2.9        | RING-H2 finger protein ATL74              |
| 40  | Os06g0566300 | −2.9        | Zinc transporter 10 (ZIP10)               |
| 41  | Os05g0380250 | −2.8        | Unknown                                  |
| 42  | Os01g0180200 | −2.8        | Histone deacetylase 3 (HDAC3)            |
| 43  | Os01g0180200 | −2.8        | Probable serine incorporator (Serinc)     |
| 44  | Os03g0784700 | −2.8        | Ferredoxin – NAPD reductase (FRN)         |
| 45  | Os03g0445500 | −2.7        | LOB-domain-containing protein 37 (LBD37)  |
| 46  | Os04g0649500 | −2.7        | Unknown                                  |
| 47  | Os04g0649600 | −2.7        | Unknown                                  |
| 48  | Os04g0649600 | −2.7        | Unknown                                  |
| 49  | Os04g0669800 | −2.7        | Dioxygenase for auxin oxidation (DAO)     |
| 50  | Os12g0204100 | −2.7        | Aquaporin nodulin 26-like intrinsic membrane protein NIP3:5 |
| 51  | Os02g0807000 | −2.6        | Phosphoenolpyruvate carboxylase kinase 1 (PPCK1) |
| 52  | Os04g0525070 | −2.6        | Senescence regulator-like protein         |
| 53  | Os06g0631100 | −2.6        | Glutamine dumper 6 (GDU6)                |

Continued
transmembrane transporter activity” were significantly enriched (Fig. 7B). In cellular component, the GO term “membrane” was significantly enriched (Fig. 7C). These results suggest that rapid changes in glucose metabolism to decrease the amounts of C skeleton for N assimilation and the reduction of various transporter activities are among the early events of N deprivation in rice roots. The information of N-repressed genes enriched in GO analysis is provided in Supplementary Table S3.

KEGG pathway enrichment analysis of the 98 —N-repressed genes indicated that “pentose phosphate pathway (ko00030)”, “photosynthesis (ko01195)”, “nitrogen metabolism (ko00910)”, “carbon metabolism (ko01200)”, “fructose and mannose metabolism (ko00051)”, “carbon fixation in photosynthetic organisms (ko00710)”, “glutathione metabolism (ko00480)” and “biosynthesis of amino acids (ko01230)” were enriched. These results suggest that the expression of genes involved in N assimilation and production of C skeletons for amino acid

| No. | Locus ID                  | LOC_Os06g46980 | Fold change | Gene description           |
|-----|---------------------------|----------------|-------------|----------------------------|
| 57  | LOC_Os06g46980            | 2.6            | Unknown     |
| 58  | LOC_Os06g472700           | 2.6            | Zinc transporter 5 (ZIP5) |
| 59  | LOC_Os06g473400           | 2.6            | Bowman-Birk type trypsin inhibitor (BBI1) |
| 60  | LOC_Os02g527100           | 2.6            | Alpha-amylose 1 (AMY1)    |
| 61  | LOC_Os05g016400           | 2.6            | Leucine rich repeat domain-containing protein |
| 62  | LOC_Os01g589100           | 2.5            | EamA domain-containing drug/metabolite transporter (DMT) |
| 63  | LOC_Os01g286000           | 2.5            | Exocyst complex component EXO70A1 |
| 64  | LOC_Os11g294000           | 2.4            | 6-phosphogluconate dehydrogenase (6PGDH) |
| 65  | LOC_Os11g306000           | 2.4            | O-methyltransferase       |
| 66  | LOC_Os06g477500           | 2.4            | Tyrosine-sulfated glycoprotein receptor 1; leucine-rich repeat receptor-like protein kinase |
| 67  | LOC_Os01g2548400          | 2.4            | Nitrite reductase         |
| 68  | LOC_Os05g371400           | 2.4            | Ferredoxin-6, chloroplastic |
| 69  | LOC_Os04g587100           | 2.4            | Ornithine–CoA ligase; acyl-activating enzyme 3 (AAE3) |
| 70  | LOC_Os05g4019400          | 2.4            | Putative disease resistance protein RGA3 |
| 71  | LOC_Os04g473600           | 2.4            | Prolyl endopeptidase      |
| 72  | LOC_Os07g233500           | 2.3            | Glucose-6-phosphate dehydrogenase (G6PDH) |
| 73  | LOC_Os04g403700           | 2.3            | Protein phosphatase 2 C 39 (PP2C39) |
| 74  | LOC_Os01g337000           | 2.3            | Unknown, conserved peptide uORF-containing transcript |
| 75  | LOC_Os03g0990000          | 2.3            | Leucine rich repeat domain-containing protein |
| 76  | LOC_Os08g106300           | 2.3            | Zinc transporter 4 (ZIP4) |
| 77  | LOC_Os08g363100           | 2.2            | Cytochrome P450 76M5-like |
| 78  | LOC_Os03g062200           | 2.2            | Unknown, mTERF domain-containing protein |
| 79  | LOC_Os04g082900           | 2.2            | Zinc finger protein STAR3-like, ZOS4-04 |
| 80  | LOC_Os09g0279900          | 2.2            | Annexin D8               |
| 81  | LOC_Os02g519700           | 2.1            | Protein EXORDIUM         |
| 82  | LOC_Os03g063700           | 2.1            | Putative gamma-glutamylcyclotransferase |
| 83  | LOC_Os01g252700           | 2.1            | Jacalin-like plant lectin domain-containing protein |
| 84  | LOC_Os01g406000           | 2.1            | NRT1/ PTR FAMILY 6.3 (NPF6.5) |
| 85  | LOC_Os01g180990           | 2.1            | Unknown                 |
| 86  | LOC_Os06g218000           | 2.1            | Indole-3-acetate O-methyltransferase 1 (IAMT1)-like |
| 87  | LOC_Os07g028000           | 2.1            | MYB family protein       |
| 88  | LOC_Os02g030400           | 2.1            | Nicotianamine aminotransferase A |
| 89  | LOC_Os09g298200           | 2.1            | KZIP transcription factor S3 |
| 90  | LOC_Os03g139500           | 2.1            | Actin-depolymerizing factor 5 (ADF5) |
| 91  | LOC_Os01g542400           | 2.1            | PDDEXX nuclease-like family of unknown function |
| 92  | LOC_Os02g324500           | 2.1            | Unknown                 |
| 93  | LOC_Os01g197500           | 2.1            | ATP-dependent 6-phosphofructokinase 6 (PFK01) |
| 94  | LOC_Os03g228100           | 2.1            | Unknown                 |
| 95  | LOC_Os05g275800           | 2.1            | Wound-induced WI12 family protein |
| 96  | LOC_Os01g665440           | 2.1            | Unknown                 |
| 97  | LOC_Os04g525500           | 2.1            | S-adenosylmethionine-dependent methyltransferase |
| 98  | LOC_Os05g340300           | 2.1            | NRT1/ PTR FAMILY 3.1-like (NPF) |

Table 2. List of genes rapidly repressed by nitrogen starvation. Total RNA extracted from roots of 10-day-old rice seedlings (+N) or treated with nitrogen starvation for 1 h (−N) was used for microarray analysis. Quantitative RT-PCR analysis was used to verify the expression of genes identified in the microarray data. The expression of genes listed here was down-regulated by nitrogen starvation (−N/+N) for more than 2-fold in the qRT-PCR analysis.
biosynthesis is rapidly repressed by $-\text{N}$. The information of genes enriched in these pathways is provided in Supplementary Table S4. These GO and KEGG enrichment analyses highlight the importance of coordinated regulation of C and N metabolism in response to changes of N nutrients in rice seedlings.

**Analysis of metabolic and transporter genes rapidly repressed by $-\text{N}$**. It is expected that $-\text{N}$ will repress the expression of genes related to nitrate/nitrite assimilation, ferredoxin reduction, and the pentose phosphate pathway. Indeed, we found that the expression of Os02g0770800 and Os08g0468100 encoding nitrate reductase (NIA), Os01g0357100 encoding nitrite reductase (NIR), Os01g0860601 encoding ferridoxin (Fd), and Os03g0784700 encoding ferredoxin-NADP reductase (FNR) was rapidly repressed by $-\text{N}$ (Fig. 8A). The major function of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) of the oxidative pentose phosphate pathway is to generate NADPH for the assimilation of inorganic N and fatty acid biosynthesis. The expression of G6PDH (Os07g0406300) and 6PGDH (Os11g0484500) was also rapidly repressed by $-\text{N}$ (Fig. 8A). In addition to G6PDH and 6PGDH, genes involved in “cellular carbohydrate metabolic process (GO:0044262)” include Os05g0194900 encoding ATP-dependent 6-phosphofructokinase 4 (PFK4), a key enzyme of the glycolysis pathway, Os08g0120600 encoding fructose-bisphosphate aldolase (FBA), and Os04g0506800 encoding sialyltransferase-like protein 3 (STLP3) (Supplementary Table S3). The expression patterns of these genes during the time course of $-\text{N}$ treatment are shown in Fig. 8A.

In addition to genes involved in C and N metabolism, the expression of genes involved in the transport and allocation of C and N metabolites was also rapidly repressed by $-\text{N}$ (Supplementary Table S3). For instance, the expression of AMT1;2 (Os02g0620600) encoding a key ammonium transporter was rapidly and strongly repressed by $-\text{N}$ (Fig. 8B). The expression of Os09g0484900 encoding a tonoplast dicarboxylate transporter (TDT), Os12g0204100 encoding glutamine dumper 6 (GDU6), Os10g0578800 encoding plastidial glycolate/glycerate translocator 1 (PLGG1) and Os01g0803300 encoding a drug/metabolite transporter (DMT) was also rapidly repressed by $-\text{N}$ (Fig. 8B). Interestingly, we also found that the expression of four zinc transporter genes, ZIP4 (Os08g0207500), ZIP5 (Os05g0472700), ZIP9 (Os05g0472400), and ZIP10 (Os06g0566300), was rapidly repressed by $-\text{N}$ in rice roots (Fig. 8B).

The rice nitrate transporter NRT1.1B/NPF6.5 has been demonstrated to play an important role in the regulation of N use efficiency. Interestingly, the expression of Os10g0554200 (NRT1.1B/NPF6.5) and Os05g0411100 (NPF) was rapidly repressed by $-\text{N}$ (Table 2 and Fig. 8C). By contrast, the expression of another two nitrate transporter genes, Os02g0699000 (NPF) and Os04g0597600 (NPF7.4), was rapidly induced by $-\text{N}$ (Table 1 and Fig. 8C). It has been demonstrated that $-\text{N}$ can induce the expression of high affinity nitrate transporter genes and repress the expression of low affinity nitrate transporter genes. It is possible that Os02g0699000

\[ \text{Figure 3. Gene ontology (GO) analysis of nitrogen starvation ($-\text{N}$)-induced genes. AgriGO (http://bioinfo.cau.edu.cn/agriGO/) was used to analyze the $-\text{N}$-induced genes in 10-day-old rice seedling roots (false discovery rate, FDR < 0.05). Of the three structured networks, the $-\text{N}$-induced genes are significantly enriched in the GO categories of biological process (A), and molecular function (B). The $-\text{N}$-induced genes were not enriched in the GO categories of cellular component.} \]
(NPF) and Os04g0597600 (NPF7.4) have higher affinity to nitrate than Os10g0554200 (NRT1.1B/NPF6.5) and Os05g0411100 (NPF). Nevertheless, the physiological and biochemical features of these nitrate transporters have yet to be further characterized.

Identification of genes that are sensitive to the availability of N in rice roots. Venn diagram analysis of the 98 genes down-regulated by −N and the 158 genes up-regulated by +N identified 34 overlapped genes (Fig. 9A). The expression of these N-sensitive genes was rapidly induced by +N and quickly repressed by −N. A complete list of these 34 genes is shown in Table 4. As expected, genes related to nitrate/nitrite assimilation, ferredoxin reduction, and the pentose phosphate pathway are very sensitive to the availability of N in the growth medium (Table 4). The BT2 (Os01g0908200) gene encoding a negative regulator of N use efficiency and several prominent candidate genes for the regulation of N response, including LBD37 (Os03g0445700, Os07g0589000), and LBD38 (Os03g0609500), are among the 34 genes identified here (Table 4). Still, we have identified several
novel genes encoding potential N regulatory proteins, which may be involved in the regulation of N metabolism and/or signaling in rice roots.

To verify the expression of these 34 N-sensitive genes, total RNA extracted from roots of 10-day-old rice seedlings treated with +N or −N for 0, 15 min, 1 h, 4 h, and 24 h was used for qRT-PCR analysis. The results of these +N and −N time course experiments confirmed that the expression of these 34 genes was rapidly induced by +N and quickly repressed by −N (Fig. 9B,C and Supplementary Fig. S5). For instance, the expression of the N regulatory gene BT2 was rapidly regulated by +N and −N, but in the opposite way (Fig. 9B). The 34 N-sensitive genes include at least 7 transcription factor genes, Os03g0609500 (LBD38), Os03g0445700 (LBD37), Os07g0589000 (LBD37), Os05g0114400 (ZOS5-02), Os11g0184900 (NAC5), Os07g0119300 (MYB) and Os03g0764600 (MYB), and one protein kinase gene, Os12g0113500 (CIPK14). The expression patterns of these genes during the time course of +N and −N treatments are shown in Fig. 9C.

The expression patterns of genes related to nitrate/nitrite assimilation, ferredoxin reduction, and the pentose phosphate pathway during the +N and −N time course treatments are shown in Supplementary Fig. S5. The assimilation of N is highly dependent on the availability of C skeletons derived from glycolysis and the TCA cycle. In addition to N metabolic and regulatory genes, the expression of Os05g0194900 encoding ATP-dependent 6-phosphofructokinase 4 (PFK04), a key enzyme of the glycolysis pathway, was rapidly induced by +N and quickly repressed by −N (Supplementary Fig. S5). The expression of 5 transporter genes, including Os06g0633100 encoding glutamine dumper 6 (GDU6), Os09g0484900 encoding tonoplast dicarboxylate transporter (TDT), Os03g0684700 encoding an integral membrane HHP family protein (HHP), Os08g0207500 encoding zinc

Figure 5. Expression of transcription factor and protein kinase/phosphatase genes induced by nitrogen starvation. (A) Transcription factor genes. (B) Protein kinase/phosphatase genes. RNA samples from roots of 10-day-old rice seedlings treated with nitrogen starvation for 0, 15 min, 1 h, 4 h, and 24 h were analyzed by qRT-PCR. The expression level of each gene in the control sample (0 h) was set at 1. Relative expression represents the fold change of the target gene relative to that of the control. Data are mean ±SD of 3 biological replicates.
transporter 4 (ZIP4) and Os06g0566300 encoding zinc transporter 10 (ZIP10), was up-regulated by +N and down-regulated by −N (Supplementary Fig. S5). The Os04g0475600 gene encoding dioxygenase for auxin oxidation (DAO) is involved in catalyzing the irreversible oxidation of active indole-3-acetic acid (IAA) to biologically inactive 2-oxindole-3-acetic acid (oxIAA)60. Interestingly, the expression of DAO (Os04g0475600) was rapidly regulated by +N and −N treatments (Supplementary Fig. S5). Actin-depolymerizing factors (ADFs) are involved in the regulation of actin assembly, which affects cell growth, expansion, proliferation and differentiation. We have identified that one of the ADF genes, ADF5 (Os03g0243100), is a N-sensitive gene (Supplementary Fig. S5). This implicates that the N status may rapidly and directly regulate cell growth and differentiation via the reorganization of cytoskeletons in rice roots. In addition to BT2, the ubiquitin-mediated proteolytic degradation machinery has been shown to modulate N responses in Arabidopsis61. Interestingly, the expression of Os05g0360400 encoding RING-type E3 ubiquitin-protein ligase EL5-like was rapidly regulated by the availability of N in the growth medium (Supplementary Fig. S5).

Table 3. List of genes rapidly induced by nitrogen starvation and ammonium nitrate in rice roots.

| Locus ID     | Fold change | Gene description                      |
|--------------|-------------|---------------------------------------|
| Os01g0705200 | 3.2         | Late embryogenesis abundant protein, group 3 |
| Os08g0473900 | 2.4         | α-amylase isozyme 3D                   |
| Os10g0576600 | 2.2         | TPR protein                            |

transporter 4 (ZIP4) and Os06g0566300 encoding zinc transporter 10 (ZIP10), was up-regulated by +N and down-regulated by −N (Supplementary Fig. S5).

The Os04g0475600 gene encoding dioxygenase for auxin oxidation (DAO) is involved in catalyzing the irreversible oxidation of active indole-3-acetic acid (IAA) to biologically inactive 2-oxindole-3-acetic acid (oxIAA)60. Interestingly, the expression of DAO (Os04g0475600) was rapidly regulated by +N and −N treatments (Supplementary Fig. S5). Actin-depolymerizing factors (ADFs) are involved in the regulation of actin assembly, which affects cell growth, expansion, proliferation and differentiation. We have identified that one of the ADF genes, ADF5 (Os03g0243100), is a N-sensitive gene (Supplementary Fig. S5). This implicates that the N status may rapidly and directly regulate cell growth and differentiation via the reorganization of cytoskeletons in rice roots. In addition to BT2, the ubiquitin-mediated proteolytic degradation machinery has been shown to modulate N responses in Arabidopsis61. Interestingly, the expression of Os05g0360400 encoding RING-type E3 ubiquitin-protein ligase EL5-like was rapidly regulated by the availability of N in the growth medium (Supplementary Fig. S5).

In addition, the expression of Os03g0823400 encoding a Bowman-Birk type trypsin inhibitor (BBTI) was rapidly induced by +N and quickly repressed by −N (Supplementary Fig. S5). We previously found that the expression of BBTI was also rapidly induced by glutamine and glutamate22,23. Trypsin inhibitor is usually associated with defense response85. It is not clear why the expression of this particular BBTI (Os03g0823400) gene is tightly regulated by the availability of N in the growth medium. The Os05g0443700 gene, one of the 7 unknown function genes identified here, encodes a syntaxin 6 N-terminal domain-containing protein, which is commonly found in various SNARE proteins involved in endosomal transport86. The rapid response of Os05g0443700 to +N and −N treatments (Supplementary Fig. S5) suggest that the encoded protein may be involved in cell trafficking associated with N metabolism and/or signaling. The expression patterns of the other 6 unknown function genes (Os04g0640900, Os01g0621900, Os03g0838900, Os04g0649500, Os04g0649600, Os01g0747300) during the time course of +N and −N treatments are shown in Supplementary Fig. S5.
Analysis of −N-repressed transcription factor genes. In addition to the 7 N-sensitive transcription factor genes shown in Fig. 9C, we have identified at least 6 more genes encoding transcription factors or nuclear proteins, including Os04g0665600 (MYB), Os02g0325600 (NIGT1), Os02g0214900 (HDAC3, HISTONE DEACETYLASE 3), Os04g0165200 (ZOS4-04), Os09g0474000 (bZIP53) and Os09g0433800 (FLZ14), that were rapidly repressed by −N. NIGT1 is a N regulatory protein. The enzyme histone deacetylase 3 (HDAC3) may have a global effect on gene expression via chromosome modification. The functions of Os04g0665600 encoding a MYB family protein, Os04g0165200 encoding a zinc-finger protein (ZOS4-04), Os09g0474000 encoding basic leucine zipper 53 (bZIP53) and Os09g0433800 encoding FCS-like zinc finger protein 14 (FLZ14) are unknown. The expression patterns of these 6 genes during the time course of −N treatment are shown in Fig. 10A. The expression of Os04g0665600 (MYB) and Os02g0325600 (NIGT1) was strongly and continuously repressed by −N (Fig. 10A). By contrast, the expression of Os09g0474000 (bZIP53) and Os09g0433800 (FLZ14) was only transiently repressed within 1 h of −N treatment (Fig. 10A).

Analysis of −N-repressed protein kinase/phosphatase genes. We identified at least 4 protein kinase and 2 phosphatase genes that were rapidly repressed by −N in rice seedling roots (Table 2). CIPK14 (Os12g0113500) is a N-sensitive gene that is rapidly regulated by +N and −N treatments as shown in Fig. 8B. The expression patterns of the other protein kinase/phosphatase genes, e.g. Os02g0120100 (ACTPK1), Os02g0807000 (PPCK1), Os06g0692600 (RLK), Os04g0430701 (PP2C39), and Os05g0111800 (PP2C46), during the time course of −N treatment are shown in Fig. 10B. ACTPK1 (Os02g0120100) has been demonstrated to phosphorylate and inactivate the ammonium transporter AMT1;2 in rice seedling roots under sufficient ammonium conditions. The Os06g0692600 gene encodes phosphoenolpyruvate carboxylase kinase 1 (PPCK1) that plays an important role in the regulation of phosphoenolpyruvate carboxylase (PEPC) and plant metabolism. The functions of the other protein kinases/phosphatases and/or their roles in the regulation of N response have yet to be characterized in rice.

Genes rapidly regulated by N starvation, glutamine, and glutamate. In addition to ammonium nitrate, we previously used transcriptome analysis followed by RT-PCR or qRT-PCR verification to identify genes that were rapidly regulated by glutamine (+Gln, 2.5 mM, 30 min) or glutamate (+Glu, 2.5 mM, 30 min) in rice roots. In the +Gln study, we only identified 35 up-regulated genes, whereas 122 up- and 4 down-regulated genes were identified in the +Glu study. None of the 35 +Gln-induced genes were
induced by −N (Supplementary Fig. S6A). By contrast, 10 of the 35 Gln-induced genes were rapidly repressed by −N (Supplementary Fig. S6B and Supplementary Table S5). Interestingly, 5 of the 10 +Gln-induced and −N-repressed genes encode transcription factors, e.g. ZOS5-02 (Os05g0114400), NAC5 (Os11g0184900), LBD37 (Os07g0589000), LBD37 (Os03g0445700), and MYB (Os07g0119300) (Supplementary Table S5). The other 5 +Gln-induced and −N-repressed genes are CIPK14 (Os12g0113500), GDU6 (Os06g0627100), PAL4 (Os07g0119300), BBTO13 (Os03g0823400), and Os09g0482800 encoding an EF-hand domain-containing protein (Supplementary Table S5).

Interestingly, the expression of 9 +Gln-induced and −N-repressed genes, except Os09g0482800 encoding an EF-hand domain-containing protein, was also rapidly induced by +N (Table 4). These results suggest that part of the inorganic N signaling pathways may be mediated via glutamine, and some of the transcription factors/ regulatory proteins identified here may be involved in these processes in rice seedlings.

Figure 8. Quantitative RT-PCR analysis of genes repressed by nitrogen starvation (−N) in rice roots. (A) Carbon and nitrogen metabolic genes. (B) Transporter genes. (C) Nitrate transporter genes. RNA samples from roots of 10-day-old rice seedlings treated with −N for 0, 15 min, 30 min, 1 h, 4 h and 24 h were used for qRT-PCR analysis. The expression of nitrate transporter genes Os10g0554200 (NRT1.1B/NPF6.5) and Os05g04111100 (NPF) was rapidly repressed by −N, whereas the expression of Os02g0699000 (NPF) and Os04g0597600 (NPF7.4) was rapidly induced by −N. The expression level of each gene in the control sample (0 h) was set at 1. Relative expression represents the fold change of the target gene relative to that of the control. Data are mean ±SD of 3 biological replicates.
Os01g0666000 encoding lipid phosphate phosphatase 2, Os03g0318400 encoding aspartic proteinase nepenthesin-1, Os01g0705200 encoding a late embryogenesis abundant protein, Os03g0194600 encoding cytochrome b561 and DOMON domain-containing protein, and Os06g0292400 encoding an unknown function protein. Exogenous Glu has been implicated to induce defense response33,64. It is possible that +Glu and −N may share some components related to stress and/or defense response. For instance, the Os08g0508800 gene encodes a LOX2 homolog that may play an important role in the biosynthesis of JA in rice. The expression of Os08g0508800 (LOX2) was commonly induced by +Glu33 and −N (Fig. 4B). It will be interesting to further investigate if JA, as well as the other +Glu- and −N-induced genes, are involved in the interaction between +Glu and −N signaling pathways.

By contrast, comparison between the −N-repressed and +Glu-induced genes revealed that the expression of NAC5 (Os11g0184900), LBD37 (Os07g0589000), MYB (Os07g0119300), BBT13 (Os03g0823400), TDT (Os09g0484900), and Os09g0482800 encoding an EF-hand domain-containing protein was rapidly regulated by +Glu and −N (Supplementary Fig. S7B and Table S7). Interestingly, 4 of the 6 +Glu-induced and −N-repressed genes, e.g. NAC5 (Os11g0184900), LBD37 (Os07g0589000), MYB (Os07g0119300) and BBT1 (Os03g0823400), were also commonly induced by +N and +Gln32–34. The Os09g0482800 gene encoding an EF-hand domain-containing protein is only shared by +Glu and +Gln, whereas the TDT (Os09g0484900) gene

Figure 9. Identification of nitrogen-sensitive genes in rice seedling roots. (A) Venn diagram analysis of genes repressed by nitrogen starvation (−N) and induced by ammonium nitrate supplementation (+N)34. The expression of the 34 overlapped genes was rapidly induced by +N and quickly repressed by −N. RNA samples from roots of 10-day-old rice seedlings treated with +N or −N for 0, 15 min, 30 min, 1 h, 4 h and 24 h were used for qRT-PCR to analyze the expression of BT2 encoding a nitrogen regulatory protein (B), and transcription factor/protein kinase genes (C). The expression level of each gene in the control sample (0h) was set at 1. Relative expression represents the fold change of the target gene relative to that of the control. Data are mean ±SD of 3 biological replicates.
In rice roots.

In addition to genes involved in C and N metabolism, the expression of genes related to “plant hormone signal and 32% of the down-regulated genes identified by microarray analysis did not pass the verification by qRT-PCR. In both experiments were used for GO and KEGG enrichment analyses. Approximately 19% of the up-regulated genes identified by microarray analysis were further verified by qRT-PCR, and only those genes that had fold-change greater than 2 (\( \Delta N+ \)) approach to identify genes that were rapidly regulated by \( \Delta N \) in rice. None of the 4 transcription factors NAC5 (Os11g0184900), LBD37 (Os07g0589000) and MYB (Os07g0119300) are potential N regulatory proteins in rice. None of the 4 up-regulated genes overlapped with the genes up- or down-regulated by \( \Delta N \).

### Discussion

Transcriptomic analysis using microarray or RNA-Seq has been applied to identify genes that are differentially regulated by \( \Delta N \) in rice25–27. However, these studies were either performed with a relatively long time of N deprivation or the identified genes were not verified by independent experiments. Here, we used a more stringent approach to identify genes that were rapidly regulated by \( \Delta N \) in rice roots. All genes identified by microarray analysis were further verified by qRT-PCR, and only those genes that had fold-change greater than 2 (\( \Delta N+ \)) in both experiments were used for GO and KEGG enrichment analyses. Approximately 19% of the up-regulated and 32% of the down-regulated genes identified by microarray analysis did not pass the verification by qRT-PCR. In addition to genes involved in C and N metabolism, the expression of genes related to “plant hormone signal transduction and “transporter activity” is rapidly regulated by \( \Delta N \) in rice roots.

The expression of genes related to nitrate/ammonium uptake, nitrate/nitrite assimilation, ferredoxin reduction, the pentose phosphate pathway, and glucose metabolic process was rapidly repressed by \( \Delta N \) (Table 2, Fig. 7 and Supplementary Fig. S4). By contrast, the expression of genes involved in the release of ammonium, including \( PAL3 \) (Os02g0626600), \( PAL4 \) (Os02g0627100), and \( MGL \) (Os10g0517500) was rapidly induced by \( \Delta N \) (Figs 3 and 4).

**Table 4.** List of nitrogen-sensitive genes. Genes listed here are up-regulated by ammonium nitrate (\( +N/\Delta N \)) and down-regulated by nitrogen starvation (\( -N/\Delta N \)) for more than 2-fold after 30 min-1 h of treatments. Numbers of fold change are derived from the microarray data.

| No. | Locus            | Fold change | Gene description                       |
|-----|------------------|-------------|----------------------------------------|
| 1   | Os03g0970800     | LOC_Os03g25310 | 65.8 | -16.7 | Nitrate reductase [NAD(P)H]       |
| 2   | Os01g0860601     | LOC_Os01g64120 | 42.2 | -5.6  | Ferredoxin, root R-B1             |
| 3   | Os01g0631200     | LOC_Os01g44050 | 32.3 | -7.0  | Uroporphyrinogen-III C-methyltransferase |
| 4   | Os03g0609500     | LOC_Os03g41330 | 30.5 | -6.1  | LOB domain-containing protein 38 (LBD38) |
| 5   | Os03g0684700     | LOC_Os03g48030 | 25.8 | -6.8  | Integral membrane HPP family protein (HPP) |
| 6   | Os09g0484900     | LOC_Os09g31130 | 15.7 | -5.0  | Tonoplast dicarboxylate transporter (TDT) |
| 7   | Os03g0445700     | LOC_Os03g33090 | 15.0 | -2.7  | LOB domain-containing protein 37 (LBD37) |
| 8   | Os05g0114000     | LOC_Os05g02390 | 14.0 | -13.0 | Zinc finger transcription factor, ZOS5-02 |
| 9   | Os03g0640900     | LOC_Os04g54840 | 13.4 | -2.7  | Ferredoxin+ - NADP reductase (FRN) |
| 10  | Os11g0184900     | LOC_Os11g08210 | 11.5 | -7.4  | NAC domain-containing protein 5 (NAC5) |
| 11  | Os07g0119300     | LOC_Os07g02800 | 9.4  | -2.1  | MYB family transcription factor |
| 12  | Os07g0589000     | LOC_Os07g40080 | 8.5  | -4.8  | LOB domain-containing protein 37 (LBD37) |
| 13  | Os03g0784700     | LOC_Os03g57120 | 8.5  | -2.8  | Ferredoxin- - NADP reductase (FRN) |
| 14  | Os03g0764600     | LOC_Os03g55590 | 8.0  | -2.4  | Nitrate reductase |
| 15  | Os01g0357100     | LOC_Os01g25484 | 8.0  | -2.4  | Nitrate reductase |
| 16  | Os03g0243100     | LOC_Os03g13950 | 6.5  | -2.1  | Actin-depolymerizing factor 5 (ADF5) |
| 17  | Os05g0360400     | LOC_Os05g29710 | 5.9  | -4.0  | RING-type E3 ubiquitin-protein ligase EL5-like |
| 18  | Os12g0113500     | LOC_Os11g22240 | 5.4  | -3.5  | CBL-interacting protein kinase 14 (CIPK14) |
| 19  | Os07g0406300     | LOC_Os07g22350 | 5.1  | -2.3  | Glucose-6-phosphate dehydrogenase (G6PDH) |
| 20  | Os03g0823400     | LOC_Os03g60840 | 5.0  | -2.6  | Bowman-Birk type trypsin inhibitor 13 (BBTI13) |
| 21  | Os06g0633100     | LOC_Os06g42660 | 4.9  | -2.6  | Glutamine dumper 6 (GDU6) |
| 22  | Os05g0443700     | LOC_Os05g37150 | 4.8  | -3.0  | Unknown, syntaxin 6 N-terminal domain protein |
| 23  | Os06g0566000     | LOC_Os06g37010 | 4.5  | -2.9  | Zinc transporter 10 (ZIP10) |
| 24  | Os11g0484500     | LOC_Os11g29400 | 4.3  | -2.4  | 6-phosphogluconate dehydrogenase (6PGDH) |
| 25  | Os04g0475600     | LOC_Os04g39980 | 3.1  | -2.7  | Dioxygenase for auxin oxidation (DAO) |
| 26  | Os05g0194900     | LOC_Os05g10650 | 2.9  | -6.2  | ATP-dependent 6-phosphofructokinase 4 (PFK04) |
| 27  | Os01g0621900     | LOC_Os01g43370 | 2.9  | -2.3  | Unknown, conserved peptide uORF-containing transcript |
| 28  | Os03g0838900     | LOC_Os03g62240 | 2.9  | -2.2  | Unknown, mTERF domain-containing protein |
| 29  | Os05g0443500     | LOC_Os05g37140 | 2.9  | -2.4  | Ferredoxin-6, chloroplastic |
| 30  | Os08g0207500     | LOC_Os08g10630 | 2.6  | -2.3  | Zinc transporter 4 (ZIP4) |
| 31  | Os12g0908200     | LOC_Os12g68020 | 2.4  | -3.3  | BTR/POZ and TAZ domain-containing protein 2 (BT2) |
| 32  | Os04g0495400     | LOC_Os04g55600 | 2.3  | -2.7  | Unknown |
| 33  | Os04g0649600     | LOC_Os04g55610 | 2.3  | -2.7  | Unknown |
| 34  | Os01g0747300     | LOC_Os01g54340 | 2.2  | -2.1  | Unknown, PDDEXK nuclease-like family protein |

**Table 4.** List of nitrogen-sensitive genes. Genes listed here are up-regulated by ammonium nitrate (\( +N/\Delta N \)) and down-regulated by nitrogen starvation (\( -N/\Delta N \)) for more than 2-fold after 30 min-1 h of treatments. Numbers of fold change are derived from the microarray data.
These results suggest that the recycling of ammonium from amino acids is one of the early events during −N in rice seedlings. The release of ammonium from amino acids may provide the initial demand of N during the sudden change from N sufficient to N deficient conditions.

In bacteria, guanosine pentaphosphate and tetraphosphate (p)pGpp play a major role in the stringent response such as nutrient starvation. Bacterial (p)pGpp is synthesized from ATP and GTP/GDP by the RelA and SpoT enzymes, which modulates target enzymes to reduce cell proliferation to conserve resources and activates the acclimatory pathways. Genes encoding RelA and SpoT homologs (RSH) are widespread in plants and algae, which may play an important role in influencing plant growth and stress acclimation. Interestingly, the expression of Os05g0161500 encoding chloroplastic GTP diphosphokinase/calcium-activated RelA-SpoT homolog 2 (CRSH2) was rapidly induced by −N in rice roots (Table 1, Fig. 4A). CRSH2 contains a central RelA-SpoT domain and two EF-hand motifs for calcium binding that may function as a Ca2+−activated (p)pGpp synthetase to integrate the Ca2+ and (p)pGpp signaling pathways. It will be interesting to investigate if CRSH2 and its product (p)pGpp are involved in the acclimatory responses during N deficiency in rice.

The metabolism of C and N is highly interdependent as the assimilation of inorganic N requires C skeletons derived from glycolysis and the TCA cycle. Thus, the production and flux of C skeletons has to be regulated to match the demands under various N conditions. However, how the N status is perceived to regulate C metabolism and flux is unknown. Phosphofructokinase (PFK) catalyzes a key regulatory step of the glycolysis pathway. The expression of Os05g0194900, one of the 15 PFK genes identified in rice, was rapidly induced by +N and quickly repressed by −N in rice roots (Table 4, Supplementary Fig. S5). Dicarboxylate transporters play an important role in the transport and compartmentation of C metabolites. The expression of TDT (Os09g0484900) was co-regulated with PFK04 and N assimilatory genes in response to the availability of N in the environment (Table 4, Supplementary Fig. S5). It is possible that PFK04 is one of the key enzymes that coordinate
C metabolism, and the intracellular dicarboxylate transporter TDT may modulate the levels of dicarboxylate in different cellular compartments in response to the demand of N assimilation in rice roots.

The inter-dependency of C and N metabolism suggests that the signal transduction pathways underlying C and N deficiency may also interact with each other. The regulator proteins CIPK14/CIPK15 have been shown to coordinate the responses to oxygen and sugar deficiency in rice. Interestingly, CIPK14/CIPK15 are N-sensitive genes as the expression of CIPK14/CIPK15 in rice roots was rapidly regulated by the availability of N in the growth medium (Fig. 9). This finding further supports the notion that CIPK14/15 may coordinate the C and N signaling pathways in response to the relative C/N status in rice.

In addition to rapid changes in C and N metabolism, the homeostasis of plant hormones such as IAA, JA, andABA, and their signal transduction pathways may be associated with the early events of N deficiency in rice. It is known that auxin/IAA is involved in the regulation of root system archetithe response to nitrate and N deficiency in Arabidopsis. By contrast, how auxin/IAA regulates the growth and development of rice roots in response to N deficiency is largely unknown. DAO (Os04g0475600) catalyzes the irreversible oxidation of IAA to oxIAA in rice. The discovery that DAO is a N-sensitive gene provides insights into the involvement of IAA oxidation in the modulation of N responses in rice roots. In addition to DAO, the formation of IAAGlucose conjugate or IAA methyl ester (MeIAA) is one of the molecular modifications controlling IAA homeostasis and activity. Interestingly, the expression of Os01g0179600 encoding indole-3-acetate beta-D-glucosyltransferase (IAGLU) and Os06g0323100 encoding indole-3-acetate O-methyltransferase 1 (IAMTI) was rapidly repressed by −N (Table 2, Supplementary Fig. 3A). These results suggest that N deficiency may increase the amount of IAA via decreasing the formation of oxidized- and conjugated-IAA, which in turn activates the IAA signaling pathway. In accordance with this hypothesis, the expression of PIN9 (Os01g08027000), SAUR19 (Os06g07020000), S' AUR36 (Os04g06083000) and Os02g01434300 encoding auxin-induced protein X15 was rapidly induced by −N (Table 1 and Supplementary Fig. S3). Thus, the oxidation and modifications of IAA may play a role in mediating N-deficient responses in rice roots.

In addition to IAA, plant hormones JA and ABA may be also involved in the regulation of −N responses in rice roots. The TIFY11a (JAZ9, Os03g01808000), TIFY11c (JAZ11, Os03g01809000), and TIFY11e (JAZ13, Os10g03914000) genes identified in the categories of "plant hormone signal transduction" and plant-pathogen interaction encode key components of the JA signaling pathway. The Os08g05088000 gene identified in the "linoleic acid metabolism" encodes a LOX2 homolog, which is a key enzyme of the JA biosynthesis pathway. The expression of these genes and Os04g03085000 encoding a 23 kDa jasmonate-induced protein was rapidly induced by −N (Table 1, Figs 4B and 5A and Supplementary Fig. S3). These results indicate that the JA signal transduction pathways are among the early responses associated with N deficiency in rice roots.

The plant hormone ABA is derived from the carotenoid biosynthesis pathway. The −N-induced genes PSY3 (Os09g05555000) and BCH1 (Os03g01251000) are associated with ABA biosynthesis. The rice PSY3 gene family consists of 3 members. PSY1 and PSY2 are involved in light-regulated carotenoid biosynthesis, whereas PSY3 is devoted to abiotic stress-induced ABA formation. The Os03g01251000 gene encoding 3-carotenoid hydroxylase 1 (BCH1) was shown to confer drought and oxidative stress resistance by increasing xanthophylls and ABA in rice. Moreover, the expression of PSY3 and BCH1 is induced by ABA. Interestingly, we found that −N could rapidly and strongly induce the expression of PSY3 and BCH1 (Fig. 4B). The expression of ABA-responsive transcription factor genes HOX22 and GH1210 was also rapidly and strongly induced by −N (Fig. 5A). These results suggest that ABA biosynthesis and signaling are among the early events induced by N deficiency in rice roots. Recently, ABA was shown to regulate auxin homeostasis to promote root hair elongation in rice root tips. It is known that N deficiency will induce cell division as well as cell elongation to promote primary root growth in rice. It is possible that these processes are governed by auxin and the interactions between auxin and ABA in rice.

ROS production was shown to be associated with K, P, and N deficiencies in Arabidopsis. The rapid induction of genes encoding peroxidase or peroxidase-like proteins (Os06g0521500, Os05g0135400, and Os06g05223000) and Os07g0468100 encoding GSTU1 (Fig. 4C) indicates that N deficiency may also cause ROS production in rice roots. In addition, the expression of several oxidative stress-responsive genes was also rapidly induced by −N (Table 1). For instance, the expression of GolS1 (Os03g03162000) and GolS2 (Os07g06879000) encoding galactinol synthase, a key enzyme for the synthesis of raffinose family oligosaccharides to protect plants from oxidative damage, was rapidly and strongly induced by −N (Table 1 and Fig. 4C). These results intimate that the production of ROS and redox signaling pathways are among the early events associated with N deficiency in rice roots.

We have identified several transcription factor genes, including NIGT1, whose expression is rapidly regulated by −N (Figs 5, 9, and 10). The functions of these genes in the regulation of N responses are mostly characterized in the Arabidopsis LBD37/38/39 transcription factors have been demonstrated to regulate N responses. Interestingly, the expression of Os07g0589000, Os03g0457000, and Os03g0609500 encoding LBD37/38 homologs is co-regulated with NIA and NIR in response to changes of N availability (Fig. 9). It is likely that the LBD37/38 homologs also play a key role in the regulation of N responses in rice. The NAC transcription factor is involved in stress tolerance, but its role as an N regulatory protein has yet to be characterized. Further studies on NAC5 may provide insights into the interaction between the N response and stress signaling pathways in rice. In addition to transcription factor genes, we have also identified several protein kinase/phosphatase genes that are rapidly up- or down-regulated by −N (Figs 5, 9, and 10). The functions of these genes, except ACTPK1, in the regulation of N responses are unknown. Further studies on these potential N regulatory genes may provide a new solution to enhance N use efficiency in rice.

Components of the ubiquitin-mediated proteolytic degradation machinery have been shown to modulate N responses in Arabidopsis. The Arabidopsis BTB protein acts as a substrate-specific adapter of an E3 ubiquitin–protein ligase complex (CUL3-RBX1-BTB), which mediates the ubiquitination and subsequent proteasomal degradation of target proteins. One of the Arabidopsis BTB proteins, BT2, has been shown to mediate
multiple responses to nutrients, stresses, and hormones\textsuperscript{35,38,81}. The rice BT2 homolog (Os01g0908200) functions as a negative regulator of nitrate transporter genes and N use efficiency\textsuperscript{35}. It is possible that the ubiquitin-mediated proteolytic degradation machinery using BT2 as a hub may also interconnect N, hormone, and stress signaling pathways in rice. Interestingly, BT2 and Os05g0360400 encoding RING-type E3 ubiquitin-protein ligase EL5-like are N-sensitive genes. The expression of BT2 and EL5-like was co-regulated with NIA and NIR in response to changes of N in the growth medium (Table 4, Fig. 9B and Supplementary Fig. S5). Ubiquitin ligase EL5 has been shown to maintain the viability of root meristems by influencing cytokinin-mediated nitrogen effects in rice\textsuperscript{82}. The function of EL5-like (Os05g0360400) protein has yet to be characterized. It is worthy to further investigate if BT2 and EL5-like (Os05g0360400) are involved in the regulation of N response and the crosstalk among multiple signaling pathways in rice.

GO enrichment analysis revealed that transporter genes were enriched in −N-repressed genes (Fig. 7 and Table 2). Interestingly, some of the −N-repressed transporter genes were also rapidly induced by +N (Table 4). In addition to TDT, the expression of GDU6, ZIP4, ZIP10 and Os03g0684700 encoding an integral membrane HPP family protein was co-regulated with NIA and NIR in response to the availability of N (Supplementary Fig. S5). Glutamine dumpers are plant-specific membrane proteins that are involved in nonselective amino acid export\textsuperscript{83,84}. GDU6 may modulate the transport of amino acids in response to changes of N in the growth medium. Members of integral membrane HPP family are predicted to contain 4 transmembrane domains and a conserved HPP motif (Pfam: PF04982). Some of the Arabidopsis HPP family proteins are nitrate-inducible components of the nitrate transport system of plastids\textsuperscript{85}. It will be interesting to further study if HPP (Os03g0684700) is a nitrate transporter in rice. Zinc is an essential element that functions as a catalytic or structural co-factor in a large number of enzymes and regulatory proteins in plants\textsuperscript{86}. It has been shown that improved N nutrition can enhance zinc uptake and remobilization in plants\textsuperscript{87}. However, it is not clear if the uptake and remobilization of zinc will affect N metabolism. The discovery that ZIP4 and ZIP10 are N-sensitive genes raises an interesting question whether the homeostasis of zinc plays a role in the regulation of N response. Still, we cannot exclude the possibility that some of the zinc transporters may transport zinc as well as the other compounds associated with N metabolism.

We previously used microarray analysis followed by RT-PCR or qRT-PCR verification to identify genes that were rapidly regulated by +N, +Gln, and +Glu in rice roots\textsuperscript{32–34}. Comparison of these results revealed that there were at least 7 genes that were commonly induced by +N, +Gln, and +Glu\textsuperscript{34}. We proposed that these genes may be involved in the regulation of general N responses in rice roots regardless the forms of N source\textsuperscript{41}. Interestingly, 4 of these 7 genes, e.g. LBD37 (Os07g058900), NAC5 (Os11g184900), MYB (Os07g0119300), and BBT13 (Os03g0823400), are among the N-sensitive genes identified here (Fig. 9C and Supplementary Fig. S5). Previously, Gln was found to be rapidly accumulated in the roots of rice seedlings after 15–30 min of +N treatment\textsuperscript{34}. Similarly, Gln also rapidly accumulated after feeding of Glu in rice seedling roots\textsuperscript{33}. Here, we found that levels of Gln were rapidly reduced within 15 min of −N treatment in rice roots (Fig. 2). These results suggest that the endogenous levels of glutamine are very sensitive to the availability of N in the environment. It is conceivable that part of the general N signal may be mediated by Gln. Nonetheless, further studies on the putative N regulatory genes identified here may provide insights into the regulation of N signaling pathways in rice roots.

**Methods**

**Plant material and growth conditions.** The rice plant Oryza sativa L. ssp. japonica cv. TNG67 was used in all experiments. Rice seeds were surface-sterilized and placed in darkness at 30 °C for 3 days. The germinated seedlings were transferred to 5-inch square pots filled with hydroponic solutions and placed in a growth chamber at 30 °C for 7 days under a 12 h light/12 h dark cycle, 200 μmol photons m\textsuperscript{-2} s\textsuperscript{-1} light intensity, and 70% relative humidity. The hydroponic solution recommended by the International Rice Research Institute containing 1.43 mM NH\textsubscript{4}NO\textsubscript{3} was used as +N treatment\textsuperscript{56}, and the same hydroponic solution without the addition of 1.43 mM NH\textsubscript{4}NO\textsubscript{3} was used as −N treatment in all experiments. For −N treatment, 10-day-old rice seedlings grown in +N hydroponic solution were transferred to −N hydroponic solution for 1 h or the indicated time. The nutrient solution was completely renewed every 3 days.

**Leaf chlorophyll measurement.** Leaf chlorophyll measurement was conducted with the Chlorophyll Content Meter (CCM-300, Opti-sciences, NH, USA) as described previously\textsuperscript{34}. Fifteen leaves from 15 rice seedlings grown in +N or −N hydroponic solution were used for the measurement.

**Microarray analysis.** The GeneChip Rice Genome Array (Affymetrix, Santa Clara, CA, USA) was used for transcriptome analysis. The extraction of total RNA from roots of 10-day-old rice seedlings grown in +N or −N (1 h) was conducted as described previously\textsuperscript{48}. RNA samples of three biological replicates from +N- or −N-treated rice seedling roots were submitted to the Affymetrix Gene Expression Service Lab at Academia Sinica (http://ipmb.sinica.edu.tw/afly/) for microarray analysis. The experimental procedures and criteria for selecting −N-regulated genes were performed as described previously\textsuperscript{22}. AgriGO (http://bioinfo.cau.edu.cn/agriGO/) and ExpPath (http://exppath.itps.ncku.edu.tw) were used for GO and KEGG enrichment analysis of the −N-regulated genes, respectively. The nomenclature of genes listed in Tables 1 and 2 is according to the annotation in NCBI (https://www.ncbi.nlm.nih.gov/gene/) or relevant literatures.

**Quantitative RT-PCR analysis of genes responsive to −N or +N treatment.** Total RNA extracted from 10-day-old rice seedlings treated with −N or +N for the indicated time was used for qRT-PCR analysis with 3 biological replicates. The expression of nuclear genes UBC3 (Os02g0634800) and UBQ10 (Os02g0161900) was used to normalize the qRT-PCR data. The primer sequences used in this study are listed in Supplementary Tables S8 and S9. The sequences of CIPK14 (Os12g0013500) and CIPK15 (Os11g0113700) are highly identical. The expression of these two genes cannot be distinguished by qRT-PCR with the primers used in this study.
Analysis of free amino acids in rice roots. The Waters Acquity UPLC system was used to analyze free amino acids extracted from 10-day-old rice seedlings treated with –N for 0–4 h. Amino acid extraction and analysis were performed as described previously.32

Data availability. The microarray datasets generated in this study are available in the NCBI GEO repository GSE109649 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109649). All other data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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**Author Contributions**

M.H.H. conceived this research and designed experiments. P.H.H. C.C.K., H.Y.W. and H.C.Y. performed experiments. P.H.H., C.C.K. and M.H.H. analyzed data and prepared figures. M.H.H. wrote the manuscript. All authors read and approved the final manuscript.

**Additional Information**

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