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Gene regulatory network and its constituent transcription factors that control nitrogen-deficiency responses in rice

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Summary

- Increase in the nitrogen (N)-use efficiency and optimization of N response in crop species are urgently needed. Although transcription factor-based genetic engineering is a promising approach for achieving these goals, transcription factors that play key roles in the response to N deficiency have not been studied extensively.
- Here, we performed RNA-seq analysis of root samples of 20 Asian rice (*Oryza sativa*) accessions with differential nutrient uptake. Data obtained from plants exposed to N-replete and N-deficient conditions were subjected to coexpression analysis and machine learning-based pathway inference to dissect the gene regulatory network required for the response to N deficiency.
- Four transcription factors, including members of the G2-like and bZIP families, were predicted to function as key regulators of gene transcription within the network in response to N deficiency. Cotransfection assays validated inferred novel regulatory pathways, and further analyses using genome-edited knockout lines suggested that these transcription factors are important for N-deficiency responses *in planta*.
- Many of the N deficiency-responsive genes, including those encoding key regulators within the network, were coordinately regulated by transcription factors belonging to different families. Transcription factors identified in this study could be valuable for the modification of N response and metabolism.

Introduction

Nitrogen (N) is an important determinant for amounts of photosynthesis and plant productivity (Makino, 2011; Ågren & Weih, 2012), and increasing amounts of N fertilizers have been applied to agricultural fields over the last century (Tilman *et al.*, 2002). However, < 50% of the applied N is absorbed and utilized by plants, and excess N fertilizers have led to environmental hazards through the production of volatile N oxides and outflow of nitrate ions from agricultural fields (Good *et al.*, 2004; Coskun *et al.*, 2017). Therefore, the breeding of crop varieties with high N-use efficiency is essential. However, N-use efficiency is a complex trait involving a number of processes (Xu *et al.*, 2012), and manipulation of a single gene encoding an enzyme or transporter associated with N use often fails to improve the N-use efficiency (Good *et al.*, 2004). On the other hand, genetic engineering targeting transcription factors that simultaneously regulate a number of N use-associated genes could be a promising approach to improve the N-use efficiency (Ueda & Yanagisawa, 2018). Rice (*Oryza sativa* L.) is an important target of such genetic engineering because N fertilizer application in rice fields is one of the main causes of environmental pollution (Coskun *et al.*, 2017; Yu *et al.*, 2019).

Plants adapt to the amount of N available in the soil by modulating several physiological traits, including root architecture and N uptake, as well as their transcriptome. Changes in the expression levels of N use-associated genes, such as genes involved in nutrient transport and metabolism, in response to the environmental N status, are well documented in previous studies (Rawat *et al.*, 1999; Zhuo *et al.*, 1999; Wang *et al.*, 2003). Recent studies also revealed that N use-associated genes are regulated by a complex transcriptional network composed of a number of transcription factors (Gaudinier *et al.*, 2018; Varala *et al.*, 2018; Ueda & Yanagisawa, 2019). Unlike the simple one-to-one regulatory relationship between a gene and a transcription factor, a transcriptional regulatory network allows plants to integrate various types of signals generated by sensing internal and external factors, transmitting the integrated information to regulatory systems for diverse biological processes and coordinately regulating these processes (Ingalls, 2013; Rouached & Rhee, 2017). The transcriptional regulatory network that controls N use is highly complex, as it needs to regulate a variety of N use-associated physiological

processes precisely. Therefore, untangling the network and identifying key regulators in the network are difficult.

Recently, several studies in Arabidopsis (*Arabidopsis thaliana*) have attempted to reveal the transcriptional network that regulates the response to different amounts of N sources, based on comprehensive transcriptome analyses and systems biology approaches (Gaudinier *et al.*, 2018; Varala *et al.*, 2018; Brooks *et al.*, 2019). These investigations suggest that a handful of key regulators probably orchestrate the expression of N source-responsive genes. Detailed molecular investigations also elucidated the presence of complex feedback regulation (Vidal *et al.*, 2010; Kiba *et al.*, 2018; Maeda *et al.*, 2018). However, the transcriptional network associated with N use was mostly analyzed in the model plant, Arabidopsis. More importantly, most of these studies focused on the response of plants upon exposure to a N supply, and less attention has been paid to the transcriptional network underlying N-eficiency responses, even though different sets of genes are suggested to respond to increase or decrease in the amount of external nitrate (Coneva *et al.*, 2014). Thus, key regulatory pathways and central transcription factors that regulate N-ficiency responses have been hardly identified, and our understanding of the gene regulatory network that controls the N-deficiency responses in plants is not sufficient.

A basic strategy to reconstruct a response network is to analyze the transcriptome in multiple samples representing different states of the network (Hecker *et al.*, 2009; Haque *et al.*, 2019). Transcriptomes reflecting different states of the network are obtained by introducing environmental perturbation (Coneva *et al.*, 2014; Goel *et al.*, 2018) or using spatially diverse samples (Ishimaru *et al.*, 2019). Varying the genetic background is another method used to produce different states of the network. In fact, differences in the regulation of nutrient uptake and other physiological processes in response to nutrient availability have been observed among genetically diverse populations of several crop plants, including rice (Famoso *et al.*, 2011; Oono *et al.*, 2013; Hu *et al.*, 2015; Matthus *et al.*, 2015; de Abreu Neto *et al.*, 2017; Shrestha *et al.*, 2018). Therefore, transcriptome analysis of rice accessions with diverse genetic backgrounds may shed light on the regulatory relationships among various components of the network.

Here, we show N deficiency-induced changes in the root transcriptome of 20 Asian rice accessions, which were selected on the basis of differences in N uptake and N deficiency-induced reduction of inorganic phosphate (Pi) uptake. Coexpression analysis identified critical groups of coexpressed genes and their hub genes involved in N-deficiency responses. Furthermore, the regulatory network constructed through a machine-learning approach suggested that the products of hub genes function as key transcription factors in the gene regulatory network that controls N-deficiency responses. Several inferred regulatory relationships were further validated through *in vivo* cotransfection assays, and analyses in CRISPR/Cas9-mediated knockout plants corroborated the importance of the identified transcription factors in induction and/or maintenance of N-deficiency responses. Thus, our findings reveal novel regulatory pathways and strong transcription factor candidates that control N-use efficiency in rice.

Materials and Methods

Plant materials and growth conditions

A collection of 119 Asian rice accessions, comprising 69 worldwide and 50 Japanese germplasms (Kojima *et al.*, 2005; Ebana *et al.*, 2008), were used for the analysis of nutrient uptake. CRISPR/Cas9-mediated knockout plants were generated from calli derived from scutella of the Japanese rice cv Nipponbare through agrobacterium (*Agrobacterium tumefaciens* (strain EHA105))-mediated transformation (Toki, 1997; Toki *et al.*, 2006) using the pZH_OsU6gRNA_PubiMMCas9 vector (Mikami *et al.*, 2015). A single guide RNA or multiple guide RNAs for simultaneous knockout of multiple loci was inserted into pZH_OsU6gRNA_PubiMMCas9 (details of vector construction are provided in Supporting Information Methods S1). Nucleotide sequences of the target loci in T0 plants were analyzed using the DSDecode website (Liu *et al.*, 2015), and T1 seeds of T0 plants carrying biallelic mutations at target loci were used for further experiments. The top three possible off-target sites predicted by the CRISPR-P 2.0 website (Liu *et al.*, 2017a) were sequenced to confirm that no undesired editing occurred (Fig. S1).

Surface-sterilized seeds were placed on a sheet of paper towel wetted with deionized water (defined as day 1) and incubated in the dark at 27°C for 4 d. On day 5, seedlings were transferred to a mesh Styrofoam tray and floated on 0.1 mM $CaCl_2$ and 12 μM Fe-EDTA solution (Wu *et al.*, 2017) and grown under a 14 h : 10 h, light : dark photoperiod at 28°C : 25°C in a growth chamber. During the period from day 5 to day 8, plants were gently covered with aluminum foil to avoid exposure to strong light. On day 9, uniform seedlings were transplanted in a perforated Styrofoam tray and floated on half-strength nutrient solution (Yoshida *et al.*, 1976). Before transplanting, the endosperm was excised to eliminate potential artifacts as a result of genotype-dependent differences in the N and P content of the grain (Hori *et al.*, 2016; Wang *et al.*, 2017). On day 12, half-strength nutrient solution was replaced by full-strength nutrient solution containing 2 mM MES-KOH (pH 5.7), 1.42 mM NH_4NO_3, 100 μM NaH_2PO_4, 0.5 mM K_2SO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 36 μM Fe-EDTA, 9 μM $MnCl_2$, 18.5 μM H_3BO_3, 0.16 μM $CuSO_4$, 1.5 μM $ZnSO_4$ and 0.07 μM Na_2MoO_4. After 3–4 d (i.e. on day 15 or 16), each seedling was transferred to a light-impermeable dark culture tube (50 ml volume) and grown in N-free full-strength nutrient solution supplemented with 1.42 mM NH_4NO_3 (N-replete treatment) or 0.07 mM NH_4NO_3 (N-deficient treatment) for 3 d. The nutrient solution in each culture tube was renewed daily. The light intensity was maintained at 140 μmol m^{-2} s^{-1} from day 8 to 12 and at 200 μmol m^{-2} s^{-1} from day 12 onward. All samples for nutrient uptake and gene expression analyses were harvested within 3–4 h on day 18 or 19 during the light period.

Nutrient uptake analyses

The uptake of Pi and N was measured using ^{32}P-labeled Pi and ^{15}N-labeled nitrate or ammonium, respectively, as described in

Methods S1. Plants grown under both N-replete and N-deficient conditions were used for Pi uptake analysis, whereas the uptake of N was analyzed only using plants grown under N-replete conditions.

RNA extraction and quantitative reverse transcription-PCR

Extraction of total RNA, cDNA synthesis, and real-time PCR were performed as described previously (Ueda *et al.*, 2020). Gene-specific primers used are listed in Table S1. *OsC3H38* (Os05g0564200) was used as an internal reference gene, because the RNA-seq data indicated that it was one of the most stably expressed genes across different genotypes and treatments (Table S2).

Library construction and RNA-seq analysis

The integrity of total RNA was verified by agarose gel electrophoresis. Library construction and RNA-seq analysis were conducted as described previously (Nagano *et al.*, 2015). A total of 95 libraries were multiplexed and sequenced on the Illumina HiSeq 2500 platform. Raw sequence reads were processed as described previously (Kamitani *et al.*, 2016). The clean reads were mapped on the reference and quantified using the RSEM software (Li & Dewey, 2011). A total of 2366 857–7880 953 reads were mapped to the Nipponbare reference genome IRGSP-1.0 (Kawahara *et al.*, 2013) per sample (Table S3). We note that comparable mapping rates were obtained for *japonica* (83.8%) and *indica* (83.3%) subspecies, but that only reads mapped to the reference genome of Nipponbare were subjected to further analyses. Normalized reads per million (RPM) values were used for further analyses, unless stated otherwise.

Genes whose expression levels were significantly altered by the N-deficiency treatment in the reference accession Nipponbare were determined using the raw count data with the EDGER package (Robinson *et al.*, 2010). The likelihood-ratio test was performed using the glmLRT function. Genes with $|\log_2(\text{fold-change})| > 1$ and false discovery rate (FDR) < 0.05 (Benjamini & Hochberg, 1995) were considered as differentially expressed. A multidimensional scaling plot was created with the plotMDS function in the EDGER package using the 5000 most abundantly expressed genes.

Weighted gene coexpression network analysis (WGCNA)

The expression data of rRNA genes were manually removed before subsequent analyses. The WGCNA package (Langfelder & Horvath, 2008) was used to construct the coexpression network using 16 493 genes with an average normalized RPM value of > 5 reads across 95 samples. A signed network was constructed using the blockwiseModules function, with the following parameters: power = 12; minModuleSize = 20; mergeCutHeight = 0.1; corType = "bicor"; and maxPoutliers = 0.05. A module number and the corresponding module color were assigned to each gene if its coexpression partners could be defined using the abovementioned criteria; otherwise 'grey' modules were used.

Root section-specific gene enrichment analysis

Previous laser microdissection-based root section-specific transcriptome data in Nipponbare (Takehisa *et al.*, 2012) were used to identify genes that were predominantly expressed in the following sections of roots: section I, meristem/division zone; section II, elongation zone; section III, lower part of the root (2–3 mm from the root tip); section IV, upper part of the root (10–20 mm from the root tip); section IV-a, outer part of the root at section IV; section IV-b, central cylinder at section IV. Genes with $P < 0.01$ (Student's *t*-test) and > 5-fold higher expression in comparison with the values in reference sections were selected for further analysis. Section II, maturation zone, sections IV, III, IV-b and IV-a were reference sections for sections I, II, III, IV, IV-a and IV-b, respectively. We note that the outer part of the root includes epidermis, exodermis, and sclerenchyma, whereas the central cylinder includes endodermis, pericycle, and stele. All the gene lists were obtained from the RiceXPro database (http://ricexpro.dna.affrc.go.jp/; last accessed, April 2019) (Sato *et al.*, 2011).

Gene regulatory network inference using the machine-learning approach

The GENIE3 package in R (Huynh-Thu *et al.*, 2010) was used for regulatory network inference using the same expression matrix as that used for the WGCNA analyses with default settings. Details of the analysis pipeline are provided in Methods S1.

Gene ontology enrichment analysis, *cis*-element enrichment analysis, and data visualization

Gene ontology (GO) and *cis*-element enrichment analyses were conducted on the RiceFREND website (http://ricefrend.dna.affrc.go.jp/; last accessed, June 2019) (Sato *et al.*, 2012). Among the GO terms representing at least four genes in the query gene set, GO terms with FDR < 0.05 and more than two-fold enrichment were considered as significantly enriched. In the *cis*-element enrichment analysis, the 1000 bp sequence upstream of the transcription start site of each gene was searched for using previously defined *cis*-elements as the query, and $P < 0.05$ was used as the significance threshold with the Fisher's exact test. The constructed network was visualized with the CYTOSCAPE software (Shannon *et al.*, 2003) using the output files from WGCNA and GENIE3 packages.

Plasmid construction and cotransfection assays

Plasmids were constructed as detailed in Methods S1. Primers used for cloning purposes are listed in Table S1. All PCR products used for plasmid construction were verified by sequencing. Isolation and transfection of rice protoplasts were carried out as reported previously (Bart *et al.*, 2006) (see Methods S1 for details).

Statistical analyses

Analysis of variance (ANOVA) was conducted using the Anova function in the CAR package of the R software (R Core Team,

2017), with type = 'II'. Tukey's honestly significant difference *post hoc* test was conducted in some analyses. Fisher's exact test was carried out using the fisher.test function.

Results

Differences in nutrient uptake among rice accessions

Imaging of Pi uptake using radiolabeled Pi allows nondestructive, rapid, easy, and quantitative high-throughput analysis of a large number of plant samples (Kanno *et al.*, 2016; Sakuraba *et al.*, 2018). Therefore, to reveal differences in N-deficiency response among 119 Asian rice accessions, we examined N deficiency-induced reduction in Pi uptake. Consistent with previous studies (Smith & Jackson, 1987a,b; Hu *et al.*, 2019), Pi uptake was modulated by the 3 d N-deficiency treatment. Furthermore, both Pi uptake activity and sensitivity to N deficiency (represented by a reduction in Pi uptake) varied significantly among the 119 accessions ($P < 0.01$) (Fig. 1a,b; Tables S4, S5). Diversity in nutrient uptake among accessions was further verified by measuring N uptake in 12 accessions (five *indica* and seven *japonica*) that displayed differences in Pi uptake and sensitivity to N deficiency. The uptake of both ammonium and nitrate differed significantly among these 12 accessions (Fig. 1c; Table S5). Although ammonium uptake was higher than nitrate uptake in each accession, the ratio of ammonium to nitrate uptake also differed among accessions, ranging from 2.7 to 6.9 (Fig. 1d). These results suggest that genetic background differentially affects nutrient uptake and N-deficiency response in rice.

Identification of coexpressed gene clusters using a variety of Asian rice accessions

To reveal the transcriptional network underlying N-deficiency response, we performed RNA-seq analysis of the root tissues of 20 Asian rice accessions, including 11 accessions whose N uptake was quantified. Both Pi-uptake activity and sensitivity to N deficiency of the selected accessions widely differed from one another (Table S4), and the selected accessions were distributed across different subpopulations of rice (Garris *et al.*, 2005). Since nutrients are absorbed via roots, we used root tissues for RNA-seq analysis. A total of 95 RNA samples were prepared from 20 accessions grown in N-replete or N-deficient medium ($n = 2$–3 for each N condition in each accession). The multidimensional scaling plot indicated that the genotype and N abundance in the growth medium were the major factors explaining the variation in RNA-seq data among different samples (Fig. 2a). In Nipponbare, N-deficiency treatment upregulated 184 genes and downregulated 340 genes (Table S6). These genes included *a priori* N deficiency-responsive genes, such as *OsDUR3* (Os10g0580400; encodes urea transporter; Wang *et al.*, 2012), *ALLANTOINASE* (*OsALN*: Os04g0680400; Lee *et al.*, 2018), *ACT DOMAIN CONTAINING PROTEIN KINASE 1* (*OsACTPK1*: Os02g0120100; Beier *et al.*, 2018), and *UREIDE PERMEASE 1* (*OsUPS1*: Os12g0503000; Lee *et al.*, 2018), indicating that our experimental condition successfully induced typical N-deficiency responses.

To define clusters of coexpressed genes (hereafter referred to as 'modules'), a gene coexpression network analysis of 16 493 abundantly expressed genes was performed using the WGCNA package (Langfelder & Horvath, 2008) (Fig. 2b; Table S7). The resultant coexpression network was composed of 16 modules harboring 35–2821 genes (Fig. 3). As subspecies and N abundance in the growth medium were the two major factors accounting for differences in the transcriptome (Fig. 2a), we calculated the effects of both these factors on the expression of the eigengene for each module (Fig. 3b) because the eigengene, defined as the first principal component of the genes within a module, depicts the overall expression pattern of each module (Langfelder & Horvath, 2007). Expression levels of the eigengenes for modules M3 (brown), M5 (green), M9 (magenta), M11 (greenyellow), M15 (midnightblue) and M16 (lightcyan) were significantly lower under N-deficient conditions than under N-replete conditions, indicating that the genes in these modules were repressed by N deficiency. On the other hand, expression level of the eigengene for module M1 (turquoise) was significantly higher under N deficiency. These modules are hereafter referred to as 'N-deficiency response modules'. Among these, modules M1, M3 and M5 seemed to largely contribute to N-deficiency responses, because a large proportion (65%) of N deficiency-responsive genes in Nipponbare clustered in one of these modules (Tables S6, S7).

Several modules were enriched in genes associated with specific GO terms (Fig. 3a; Table S8). For instance, module M5, of which eigengene expression was strongly repressed by N deficiency (Fig. 3b), was enriched in genes associated with 'nitrogen compound biosynthesis', 'carbohydrate metabolism', or 'transporter activity' GO term (Table S8). Interestingly, module M16 was enriched with the GO term 'photosynthesis' and contained many genes encoding proteins for photosystem I and II, probably reflecting chloroplast development and 'greening' in rice roots (Armstrong & Armstrong, 1994).

Expression levels of the eigengenes of N-deficiency response-unrelated modules (M2, M4, M6–M8, M10, M12–M14) in *indica* subspecies were compared with those in *japonica* subspecies. The results indicated that modules M2 (blue), M4 (yellow), M7 (black), M8 (pink), M12 (tan) and M13 (salmon) are enriched with genes whose expression levels were significantly different between *indica* and *japonica* subspecies, suggesting that these modules represent subspecies-dependent differences in gene expression profiles. Six out of seven N-deficiency response modules had enriched GO terms, while only two of these modules had enriched GO terms (Table S8), indicating that many of these modules comprise functionally diverse genes.

Identification of hub genes in N-deficiency response modules

We further explored the hub genes in each coexpression module using the module membership value (kME), which is defined as the Pearson's correlation coefficient between the expression of a

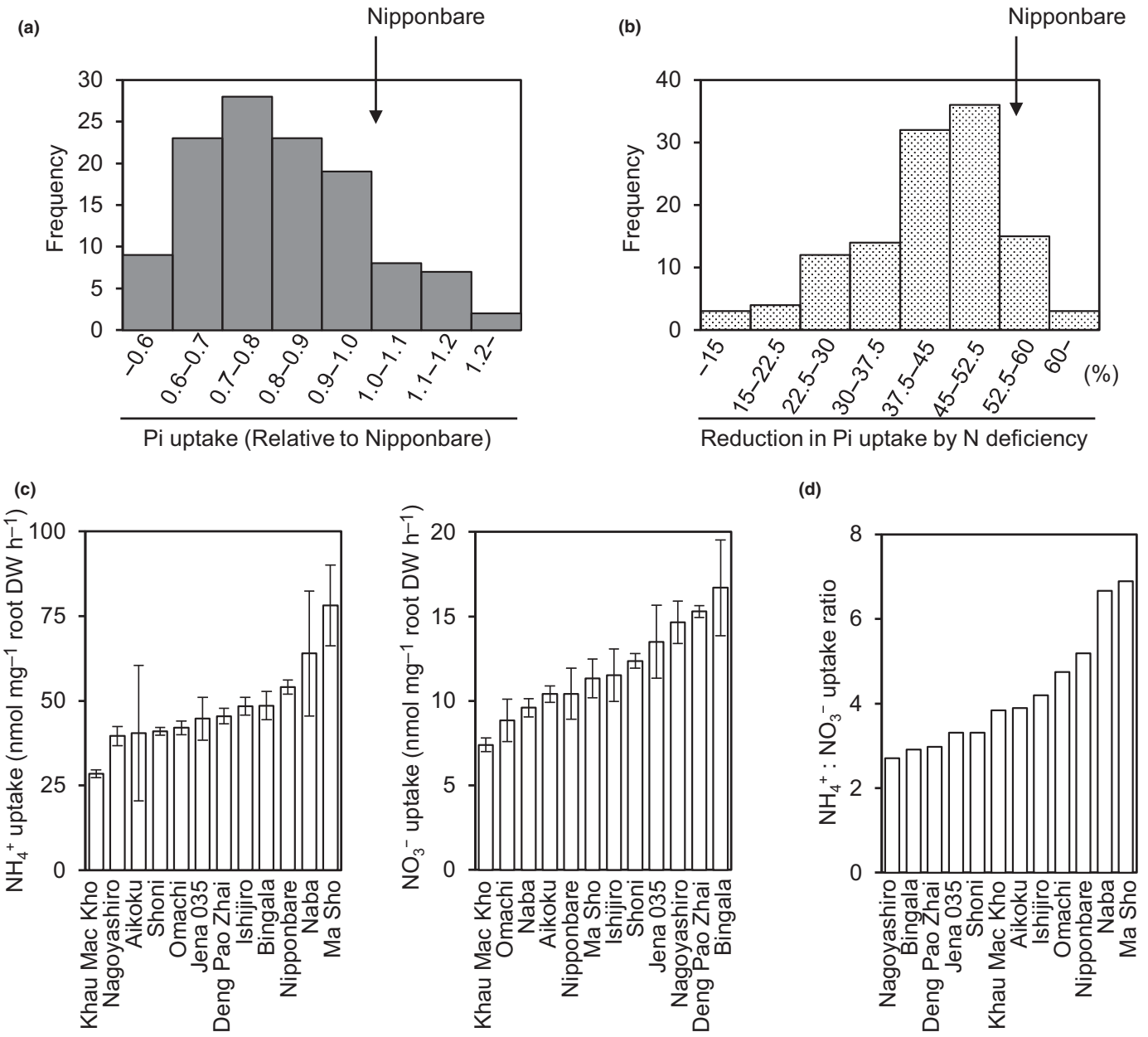


Fig. 1 Diversity in nutrient uptake among Asian rice accessions. (a) Histogram showing the distribution of Pi uptake in 119 rice accessions. Total ^{32}P-labeled inorganic phosphate (Pi) in a plant was divided by its root DW. Pi uptake in each accession was expressed relative to the reference accession Nipponbare. The median value of Pi uptake ($n = 4$–6) from two independent experiments was calculated for each accession. (b) Histogram showing the distribution of the reduction in Pi uptake as a result of N deficiency in 119 accessions. Total ^{32}P-labeled Pi in a plant was divided by its root DW under each condition and used for the calculation of N deficiency-induced percentage reduction in Pi uptake in each accession. The median value of two independent experiments ($n = 4$–6) was defined as the amount of Pi uptake under the respective condition. (c) Uptake of ammonium and nitrate ions in 12 selected rice accessions. The ammonium or nitrate uptake of a plant was divided by its root DW. Data represent means ± SD ($n = 3$). (d) Ratio of ammonium : nitrate uptake in 12 selected accessions. The ratio was calculated using the mean values of ammonium and nitrate uptake for each accession. In (a, b), the arrow indicates the data point of the reference accession Nipponbare.

gene and a given module eigengene (Langfelder & Horvath, 2008). Multiple hub genes that play important roles in the maintenance of the module and network structure are frequently identified in a single module (Li *et al.*, 2015; Mine *et al.*, 2018; van Dam *et al.*, 2018). We also identified multiple hub genes (|kME| > 0.8 with any of the module eigengenes) in each module and some of them encoded transcription factors (Table S9). Among the N-deficiency response modules (M1, M3, M5, M9, M11, M15 and M16), a total of 24 transcription factor-encoding hub genes were identified, all of which are associated with M1, M3, or M5 (Fig. 4). Among these genes, Os03g0764600 and Os07g0119300 encoded HRS/NIGT1 transcription factors, whereas some other hub genes encoded LBD, NAC, MYB and bZIP transcription factors (Fig. 4; Table S9). These transcription

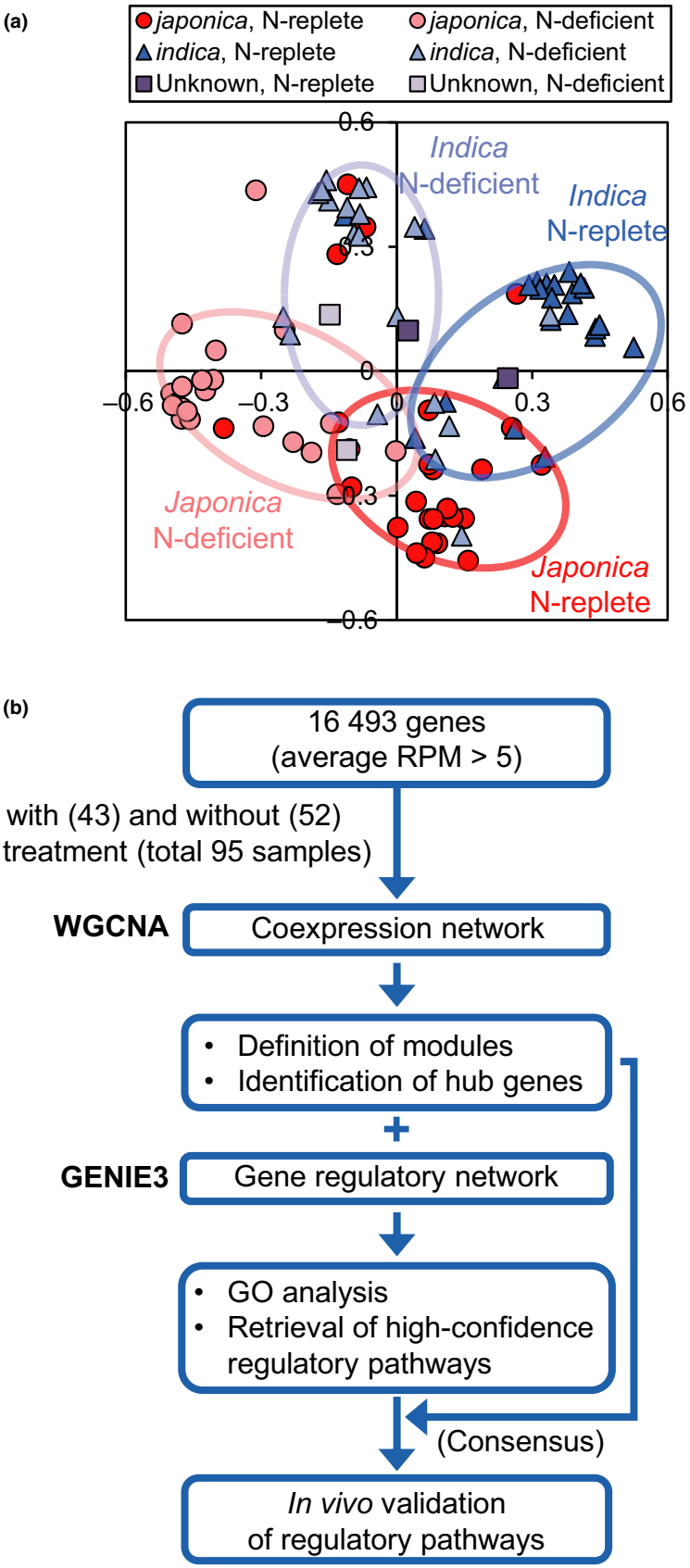


Fig. 2 Overview of RNA-seq and outline of data analyses. (a) Multidimensional scaling plot showing gene expression patterns in 95 rice samples. The most abundantly expressed 5000 genes were used to create the plot. *x*- and *y*-axes indicate the first and second principal components, respectively. One accession (Calotoc) that was assigned to neither *indica* nor *japonica* subspecies is represented by boxes. (b) Outline of network analyses using RNA-seq data from 95 rice samples. A total of 16 493 genes with an average of more than five reads per million (RPM) across 95 samples were used for downstream analyses. The WGCNA package was used to perform coexpression analyses, based solely on the expression levels of these 16 493 genes. Hub genes were identified from the coexpression networks and later prioritized for *in vivo* validation. Subsequently, the GENIE3 package was used to build the gene regulatory network by specifying 796 transcription factors as explanatory factors for the expression of other genes. Then, gene ontology (GO) enrichment analysis of the putative target genes of the top 50 most highly connected transcription factors in the gene regulatory network was performed to obtain high-confidence regulatory pathways. Finally, the regulatory relationships inferred by *in silico* analyses were validated by *in vivo* analyses.

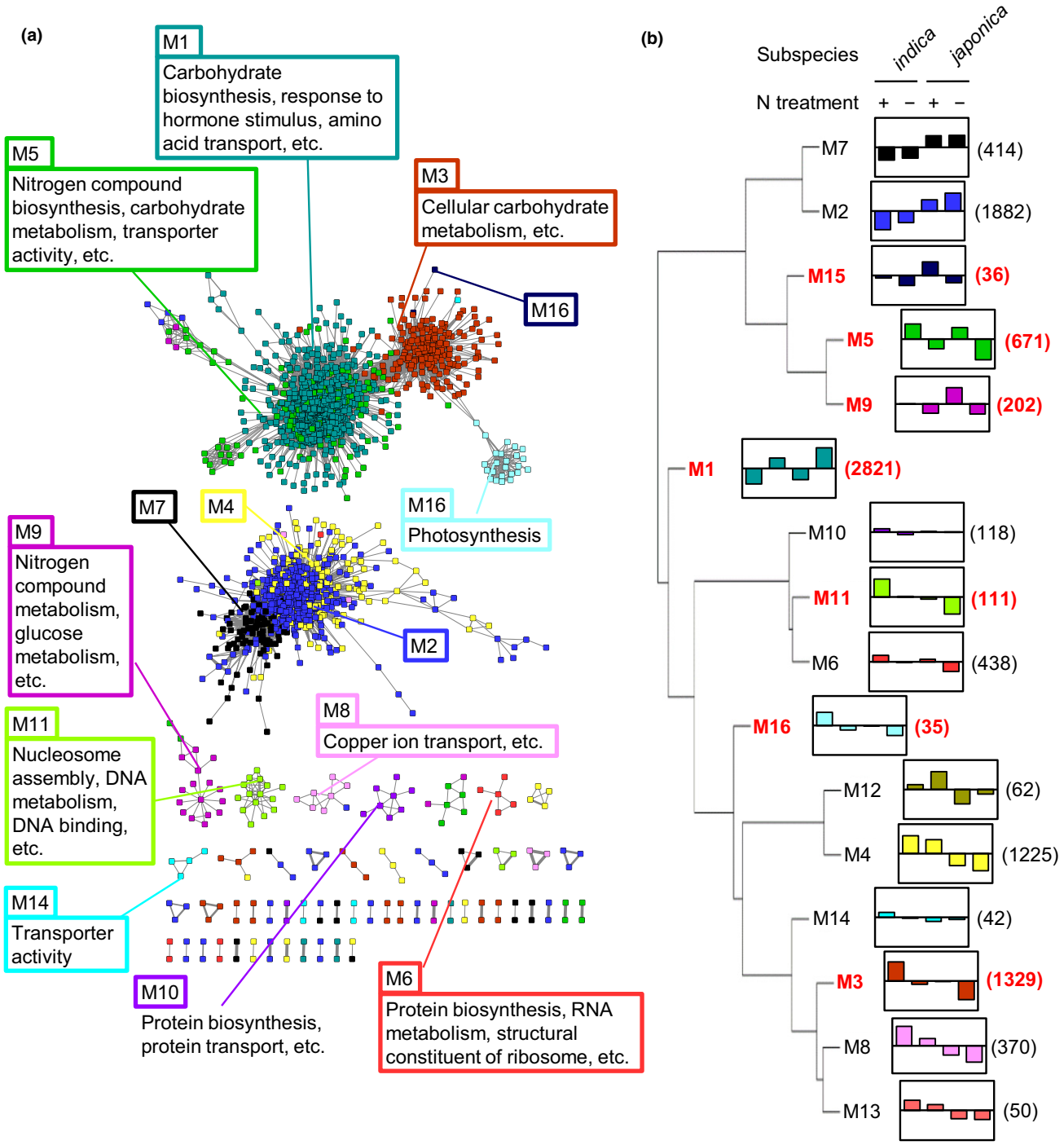


Fig. 3 Coexpression network constructed by WGCNA. (a) Overview of the coexpression network. RNA-seq analysis was performed with 95 root samples from *japonica* and *indica* rice accessions grown under the N-replete or N-deficient condition, and the 'signed' network was constructed on the basis of positive correlations between expression patterns of coexpressed genes. Each node represents a gene, and coexpressed genes are connected with a gray edge. Genes belonging to different coexpression modules (M1–M16) are indicated in different colors. Representative GO terms enriched in each module are indicated. For simplicity, only the genes with adjacency > 0.05 are shown, and modules that do not contain genes with adjacency > 0.05 were omitted. (b) Dendrogram showing the relationship among module eigengenes. Because each eigengene of modules M1–M16 in the coexpression network in (a) represents the expression pattern of each module, degrees of relatedness among expression patterns of the eigengenes were analyzed. Numbers in parentheses indicate the number of genes in each module. The expression of the eigengene for each coexpression module defined in (a) was calculated for each sample, averaged for different sample groups (i.e. *indica*, N-replete; *indica*, N-deficient; *japonica*, N-replete; and *japonica*, N-deficient), and plotted in the bar graph. The average of the eigengene expression across all the samples was set to zero in each module, which is indicated by a middle horizontal line in each bar graph. Higher or lower expression of the eigengene of each sample group, compared with the average across all samples, was indicated in the vertical axis direction in each bar graph. The vertical axis in all bar graphs was set to the same scale. One accession (Calotoc), not assigned to either subspecies, was omitted from the calculation of average eigengene expression. For each module, Student's *t*-test was conducted between the values of eigengene expression under the N-replete and N-deficient conditions, irrespective of subspecies. When the resultant *P*-value was below the Bonferroni-corrected significance level ($P = 3.12 \times 10^{-3}$), the module was considered as an N-deficiency response module and indicated in red color.

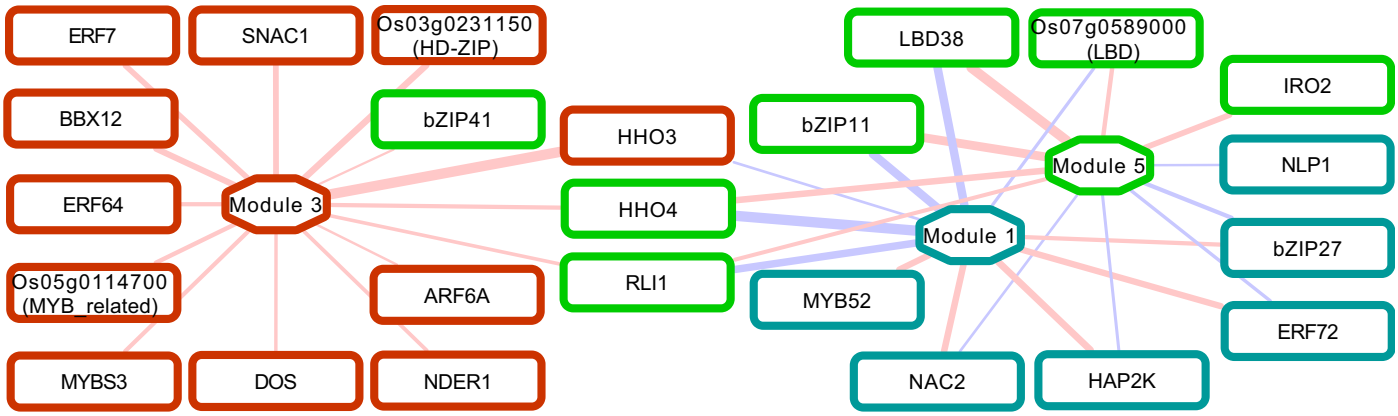


Fig. 4 Hub gene network derived from the coexpression network in rice root. Pearson's correlation coefficient between all genes vs module eigengene expression was calculated, which was defined as 'kME'. Genes with |kME| > 0.8 were defined as hub genes. Eingengenes and identified hub transcription factor genes of modules M1, M3, and M5 are indicated in turquoise, brown, and green color frames, respectively. Positive and negative correlations between the expression of eigengenes and that of hub transcription factor genes are shown with pale red and blue lines, respectively.

factors may play pivotal roles in transcriptional regulation and plant adaptation under N-deficient conditions. The HRS/NIGT1 transcription factors are a subgroup of the G2-like GARP-type transcription factors (Sawaki *et al.*, 2013; Yanagisawa, 2013; Medici *et al.*, 2015; Kiba *et al.*, 2018; Maeda *et al.*, 2018). On the basis of phylogenetic analysis of rice and Arabidopsis HRS/NIGT1 transcription factors, Os03g0764600 and Os07g0119300 were named HRS1 HOMOLOG 3 (OsHHO3) and OsHHO4, respectively (Figs 4, S2a). We also named the uncharacterized gene Os01g0603300, which encodes a MYB-related transcription factor, as OsNDER1 (N Deficiency Response 1) and further investigated its function.

Importantly, several transcription factors were suggested to connect three major N-deficiency response modules (M1, M3, and M5); OsHHO3, OsHHO4, and REGULATOR OF LEAF INCLINATION 1 (OsRLI1: Os04g0665600) (Ruan *et al.*, 2018) connected M1 with M3, and several transcription factors, including OsbZIP11 (Os01g0859500) (Nijhawan *et al.*, 2008) and LATERAL ORGAN BOUNDARIES DOMAIN 38 (OsLBD38: Os03g0609500), connected M1 with M5. This suggests that these transcription factors are particularly important to maintain the coexpression network structure and N deficiency-triggered transcriptional regulation.

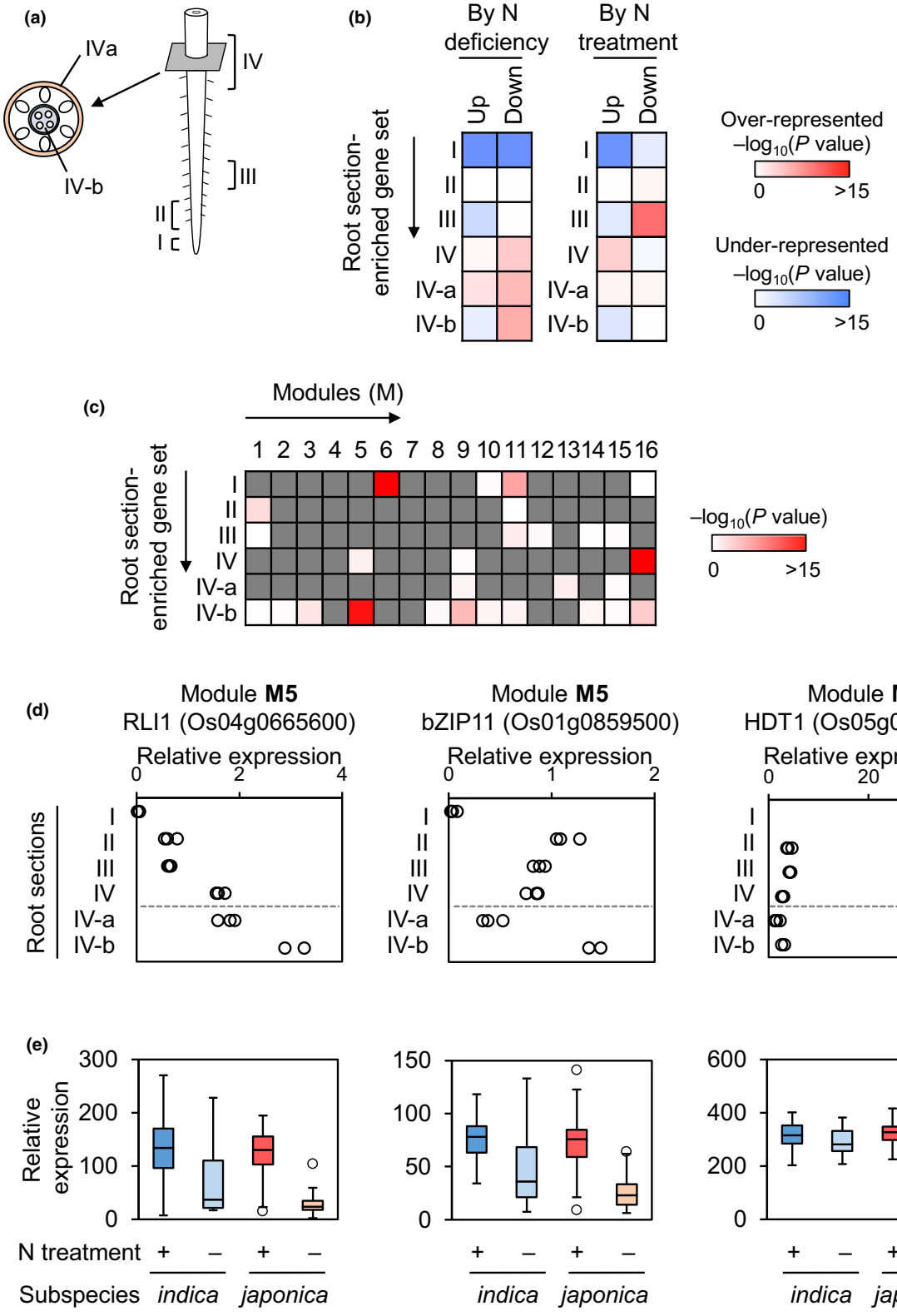
Genes with root zone-specific expression and enrichment in particular coexpression modules

The root tissue consists of various types of cells with distinct physiological functions and unique transcriptome (Jean-Baptiste *et al.*, 2019). To obtain evidence that coexpression modules are relevant to these distinct functions, we investigated whether genes predominantly expressed in certain sections of the root were over-represented in coexpression modules. Before this analysis, we first confirmed that the list of genes predominantly expressed in a particular section of the root (Takehisa *et al.*, 2012) over-represents or under-represents N-responsive genes in Nipponbare (Fig. 5a,b). N deficiency-responsive genes were under-represented among the genes predominantly expressed in the root meristem/division zone (section I) and over-represented among the genes predominantly expressed in the upper part of the root (section IV). Furthermore, N supply-repressive genes expressed in the root tissues of Nipponbare (Obertello *et al.*, 2015) were over-represented among the genes predominantly expressed in the lower part of the root (section III), while N supply-inducible genes were under-represented among those expressed in the meristem/division zone (section I). These results suggest that changes in the rice transcriptome in response to N deficiency vary among various root sections.

Next, we investigated whether coexpression modules were rich in genes predominantly expressed in a certain section of the root (Fig. 5c). The results revealed that genes predominantly expressed in central cylinders (section IV-b) were over-represented in module M5 (Fig. 5c). As nutrient transporters and channels expressed in central cylinders are frequently involved in xylem loading and root-to-shoot translocation (Poirier *et al.*, 1991; Gaymard *et al.*, 1998; Takano *et al.*, 2002), this observation suggests that module M5 is at least partly associated with N availability-dependent alterations in nutrient translocation and distribution. On the other hand, module M6 was rich in genes predominantly expressed in the meristem/division zone (section I) (Fig. 5c). Module M6 did not include any N deficiency-responsive gene, consistent with the observation that N deficiency-responsive genes are under-represented in the meristem/division zone (section I) (Fig. 5b). Representatives for transcription factor genes in modules M5 and M6 (*RLI1*, *bZIP11* and *HDT1*) indeed showed spatially distinct expression patterns in roots and differential responsiveness to N availability (Fig. 5d,e).

Inferring the gene regulatory network by machine-learning

WGCNA is an effective approach to obtain an overview of a coexpression network and identify hub genes in the network; however, clues regarding the regulatory relationships cannot be obtained through this approach alone. Thus, a machine-learning



approach using the GENIE3 package (Huynh-Thu *et al.*, 2010) was adopted to infer the regulatory relationships between various transcription factors and other genes, using the same sample set as in WGCNA (Fig. 2b). An analytical pipeline (Methods S1) suggested 29 transcription factors as strong candidates for influential transcription factors for the N-deficiency response network (Fig. 6a; Tables S10, S11). We note that this analysis can capture nonlinear relationships but does not infer the direction of

regulation. The coexpression modules defined by WGCNA were recognizable even in the gene regulatory network, clarifying the relationships between coexpression modules and regulatory pathways. The genes in modules M1, M3 and M5 were located in the central part of the gene regulatory network (Fig. 6a). The genes encoding these 29 transcription factors were not necessarily genes that are most strongly activated or repressed by N deficiency in Nipponbare (Table S6), suggesting significance of network analysis for identification of putative influential transcription factors.

Identification of key transcriptional regulators in the gene regulatory network

Analysis of the gene regulatory network revealed a considerable overlap of target genes among the 29 putative influential transcription factors, particularly OsHHO3, OsHHO4, OsRLI1, OsbZIP11 and OsLBD38 (Fig. 6b). Interestingly, the top four transcription factors, namely OsHHO3, OsHHO4, OsRLI1 and OsbZIP11, were also predicted to regulate each other (Table S11), suggesting complex feedback regulation among these key regulators. Furthermore, a total of 129 genes, including *OsNAR2.1* (Os02g0595900: encodes nitrate transporter-interacting protein; Yan *et al.*, 2011), *OsNRT2.3* (Os01g0704100: encodes high-affinity nitrate transporter; Feng *et al.*, 2011), and *GLUTAMINE SYNTHETASE 1;1* (*OsGS1;1*: Os02g0735200; Tab uchi *et al.*, 2005) were predicted to be regulated together by OsHHO3, OsHHO4, OsRLI1 and OsbZIP11. On the other hand, other putative influential transcription factors were predicted to affect different sets of target genes. For example, target genes of a C_2H_2-type zinc finger transcription factor (Os05g0114400) and OsONAC1 (Os09g0509100) hardly overlapped with those of OsHHO3, OsHHO4, OsRLI1 and OsbZIP11 (Fig. 6b), suggesting that these transcription factors modulate different aspects of N-deficiency responses.

Promoter analysis using the RiceFREND database (Sato *et al.*, 2012) revealed that the promoters of target genes of NIN-LIKE PROTEIN 1 (OsNLP1: Os03g0131100) (Table S11) were enriched with the *cis*-element TGRCYCTT, which is the binding sequence for Arabidopsis NLP family proteins (Konishi & Yanagisawa, 2014). Similarly, the GAATC motif, one of the *cis*-elements recognized by the HRS/NIGT1 family transcription factors including rice Nitrate-Inducible, GARP-type Transcriptional Repressor 1 (OsNIGT1) (Sawaki *et al.*, 2013; Yanagisawa, 2013; Maeda *et al.*, 2018), was over-represented in the promoters of putative OsHHO4 target genes (Table S11). These observations further support that the machine-learning approach captured *bona fide* regulatory relationships.

In vivo validation of the regulatory network inference

To perform *in vivo* validation of the inferred regulatory relationships, we performed co-transfection assays using protoplasts isolated from Nipponbare seedlings. A reporter plasmid harboring the firefly luciferase (LUC) gene under the control of the 1.5 kb promoter of a putative target gene was cotransfected with an expression vector harboring *OsbZIP11*, *OsRLI1* or *OsHHO3* cDNA. First, we focused on the mutual regulation of these transcription factors (Fig. 7a; Table S11). Cotransfection assays validated some of these predicted regulatory relationships and also revealed the presence of autorepression (Fig. 7b). The effects of OsHHO3 on *OsbZIP11* and *OsRLI1* promoters and vice versa were less obvious, which is consistent with the observation that *OsbZIP11* and *OsRLI1* were associated with module M5 but weakly associated with module M3 in the coexpression network, whereas *OsHHO3* was associated with module M3 but not with module M5 (Fig. 4).

Next, we examined the effects of OsbZIP11, OsRLI1, and OsHHO3 on the promoter activity of predicted common N-related target genes, namely, *OsNAR2.1*, *OsNRT2.3* and *OsGS1;1*. The *OsNAR2.1* promoter was suppressed by OsbZIP11, OsRLI1 and OsHHO3, while the *OsNRT2.3* promoter was clearly repressed by OsbZIP11 and OsRLI1 but less clearly by OsHHO3. The *OsGS1;1* promoter was evidently repressed by only OsbZIP11 (Fig. 7c). These results supported the regulatory relationships inferred by the network analysis and also suggested that OsbZIP11, OsRLI1 and OsHHO3 function as transcriptional repressors, although we cannot totally rule out the possibility that these transcription factors function as transcriptional activators on some target genes. Because putative targets selected

Fig. 5 Enrichment of genes predominantly expressed in certain sections of roots in coexpression modules. (a) Root sections of rice used to retrieve predominantly expressed genes from a previous report (Takehisa *et al.*, 2012). Section I, meristem/division zone; section II, elongation zone; section III, lower part of the root (2–3 mm from the root tip); section IV, upper part of the root (10–20 mm from the root tip); section IV-a, outer part of the root at section IV; section IV-b, central cylinder at section IV. (b) Heatmaps indicating the over-representation and under-representation of N deficiency- or supply-responsive genes in the list of genes predominantly expressed in certain rice root sections (I–IV, IV-a, IV-b). Genes up- or downregulated by N deficiency were defined in the current study using the whole root of Nipponbare (see Supporting Information Table S6). Genes up- or downregulated by N supply were defined as those listed by a previous study in the whole root of Nipponbare (Obertello *et al.*, 2015). Fisher's exact test was conducted to determine the significance of the overlap between the list of genes predominantly expressed in different root sections and the N-responsive gene list. Over-represented and under-represented pairs are shown in red and blue, respectively. (c) Heatmaps indicating the over-representation of genes enriched in specific root sections and genes in coexpression modules. Over-represented and under-represented pairs are shown in red and gray, respectively. (d) Expression levels of *RLI1*, *bZIP11* and *HDT1* in different sections of rice roots. Expression levels of *RLI1*, *bZIP11* and *HDT1* (transcription factor genes with high kME values for module M5 or M6) in different root sections were obtained from a previous report (Takehisa *et al.*, 2012). Each circle represents a data point from different samples. Originally reported 'normalized $\log_2$-transformed data' were used to calculate the actual values of expression. (e) Box plots showing expression levels of *RLI1*, *bZIP11* and *HDT1* in root samples from *japonica* and *indica* accessions. A horizontal line in the middle of each box represents the median, and upper and lower ends of each box represent the upper and lower quantiles, respectively. Circles below and above the whisker indicate the minimum and maximum values, respectively, that did not fall within the range of the whisker. One accession (Calotoc), not assigned to either subspecies, was omitted from the calculation.

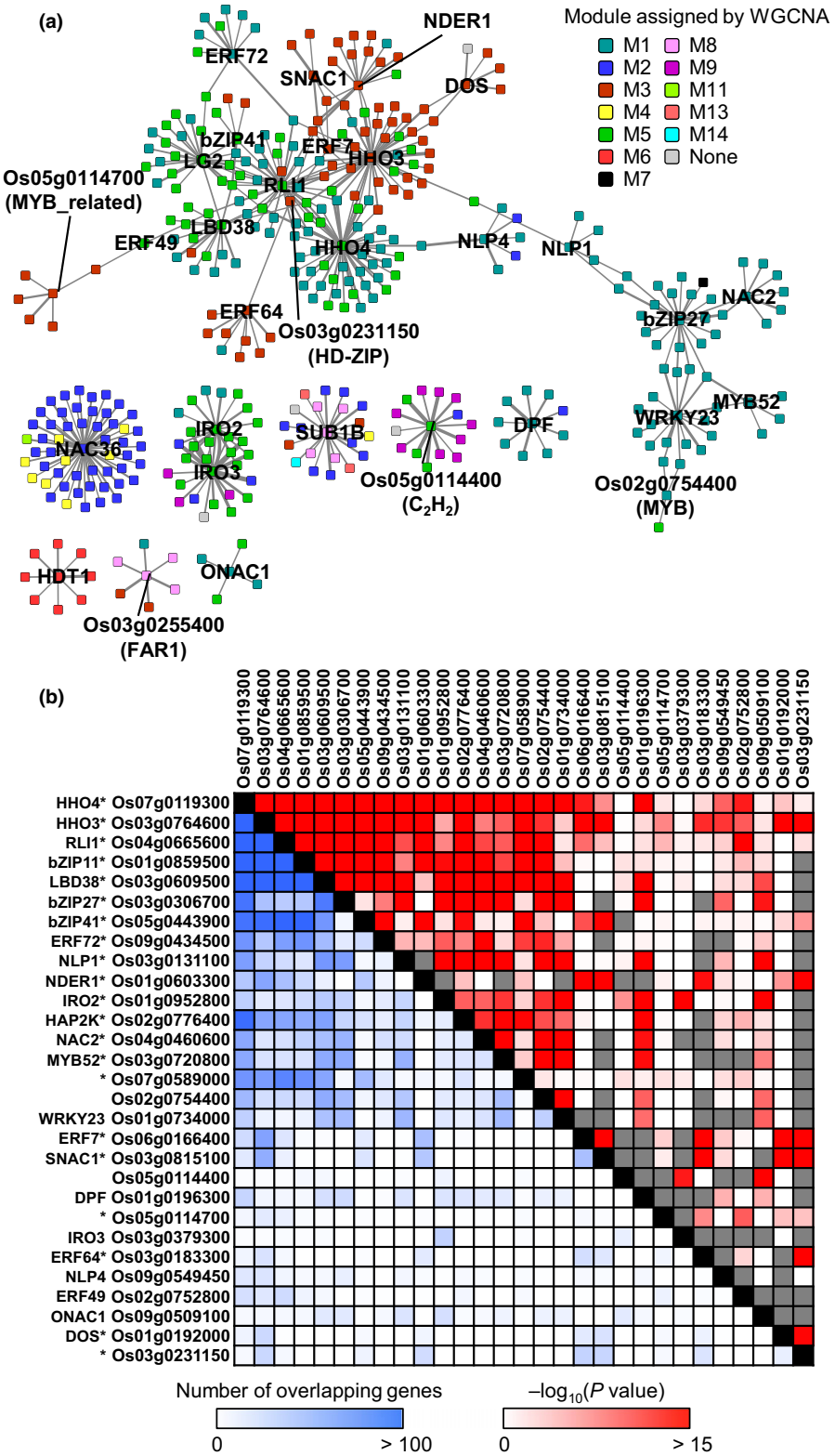


Fig. 6 Gene regulatory network constructed using GENIE3. (a) Overview of the high-confidence gene regulatory network in rice root. Each node represents a gene, and genes exhibiting a regulatory relationship are connected with an edge. Genes belonging to different coexpression modules (as determined by WGCNA) are indicated in different colors that correspond to those of coexpression modules. Identical colors have been used in Figs 3 and 6 to indicate genes belonging to the same coexpression modules. For simplicity, the gene regulatory network drawn here was limited to high-confidence edges only among the top 1000 raw outputs from GENIE3. (b) Overlaps among the predicted target genes of 29 influential transcription factors in the constructed gene regulatory network. Genes connected with a transcription factor among the top 10 000 edges were defined as putative targets. Significance of the overlap was determined by Fisher's exact test. The upper triangle indicates $-\log_{10}(P\text{-values})$, and the lower triangle indicates the number of overlapping putative target genes. Asterisks (*) indicate transcription factors encoded by hub genes in the coexpression network constructed by WGCNA.

by network analysis include direct and indirect targets, weak or opposite effects in cotransfection assays may suggest indirect relationships. Alternatively, analyzed transcription factors may regulate transcription via interactions with regions outside the used promoter sequences. A weak or no effect of OsHHO3 on the *OsNRT2.3* and *OsGS1;1* promoters agrees with the finding that *OsHHO3* is only weakly associated with the eigengene expression of module M1, which harbor *OsNRT2.3* and *OsGS1;1* (Fig. 4).

Besides the genes directly involved in N uptake and metabolism, *OsACTPK1* encoding N use-associated protein

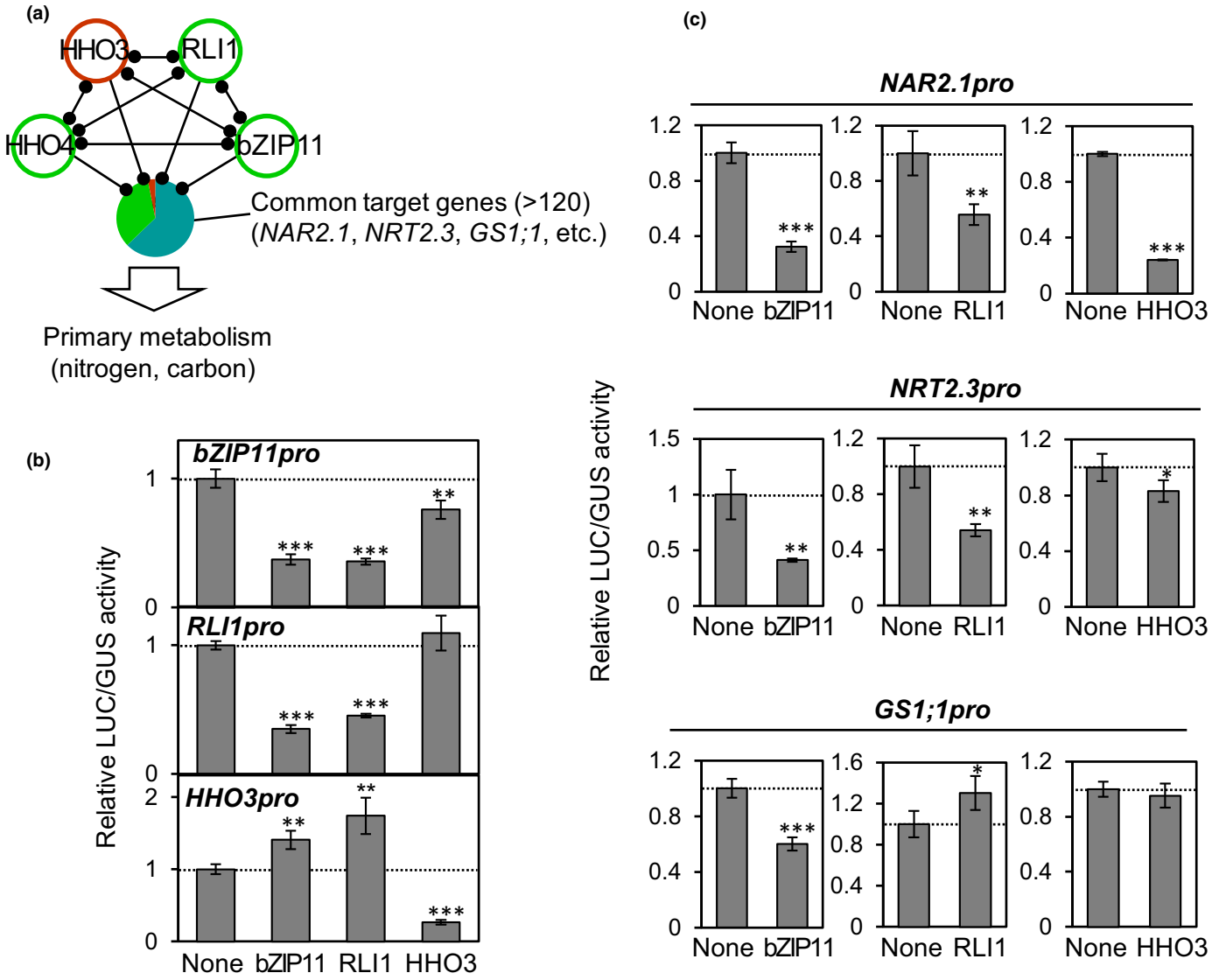


Fig. 7 *In vivo* validation of the regulatory pathways involving OsbZIP11, OsRLI1, OsHHO3 and OsHHO4. (a) Schematic representation of mutual regulatory relationships and putative common target genes of OsHHO3, OsHHO4, OsbZIP11 and OsRLI1. As the direction of regulation is not inferred by the network analysis, predicted regulation by the connected transcription factors are indicated by black lines with dots at their ends. Transcription factors belonging to modules M3 and M5 are indicated with brown and green circles, respectively. The pie chart indicates the ratio of genes in modules M1 (turquoise), M5 (green), and M3 (brown) of the coexpression network. Genes with the GO term 'primary metabolism' were enriched among the common target genes of OsbZIP11, OsRLI1, OsHHO3 and OsHHO4. (b) *In vivo* validation of the mutual regulatory relationships among OsbZIP11, OsRLI1 and OsHHO3 in cotransfection assays. (c) *In vivo* validation of the regulation of putative common target genes of OsbZIP11, OsRLI1 and OsHHO3 in cotransfection assays. In (b, c), reporter plasmids containing the firefly luciferase (LUC) gene and an internal control plasmid containing the β-glucuronidase (GUS) gene were cotransfected into rice protoplasts with either an effector plasmid containing *OsbZIP11*, *OsRLI1* or *OsHHO3* cDNA or an empty vector (none). Relative LUC activity obtained with the empty plasmid (none) was set to 1. Data represent means ± SD ($n = 4$). Two-tailed Student's *t*-test was performed to determine the significance of the effect of the analyzed transcription factor (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

kinase (Beier *et al.*, 2018) was predicted to be under the control of OsNDER1, a hub gene of module M3 (Fig. 4), as well as OsbZIP11, OsRLI1 and OsHHO3 (Fig. 8a; Table S11). Consistent with the prediction, these transcription factors significantly affected activity of the *OsACTPK1* promoter (Fig. 8b). The activation of the *OsACTPK1* promoter by HHO3 could be a result of indirect regulation by HHO3 or possible activator activity of HHO3. The analysis also validated the regulatory relationship between OsNDER1 and the promoter of *OsRLI1* and *OsHHO3* (Fig. 8b).

The N status is known to affect various biological and metabolic pathways, including carbohydrate and iron (Fe) metabolism in plants (Raven, 1988; Paul & Stitt, 1993; Wang *et al.*, 2003; Goel *et al.*, 2018). Consistently, the regulatory network inferred regulations of several key genes involved in these processes, such as *SUCROSE SYNTHASE 1* (*OsSUS1*: Os03g0401300) and *OsSUS2* (Os06g0194900) (Hirose *et al.*, 2008), and a basic helix–loop–helix transcription factor-encoding gene involved in Fe signaling, *OsIRO2* (Os01g0952800) (Ogo *et al.*, 2006). The network also suggested that OsRLI1 and OsbZIP11 are novel regulatory factors

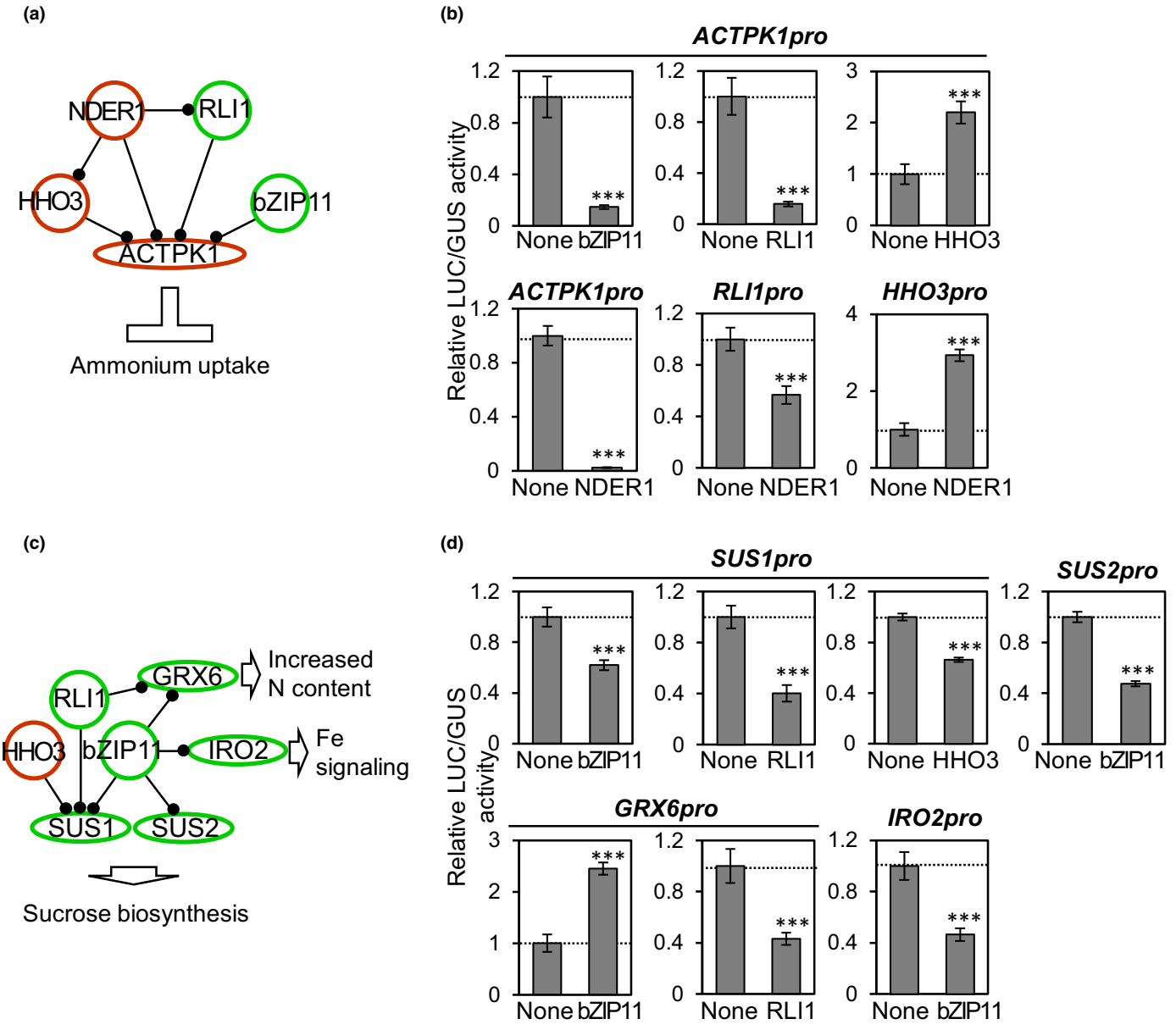


Fig. 8 *In vivo* validation of newly inferred regulatory pathways. (a, b) Schematic representation of the inferred regulatory pathways of *OsACTPK1* by OsbZIP11, OsRLI1, OsHHO3 and OsNDER1 (a), and *in vivo* validation of the regulatory pathway by cotransfection assays (b). OsNDER1 encodes a MYB-related protein named in this study, and *OsACTPK1* encodes an N use-associated protein kinase. (c, d) Schematic representation of the inferred regulatory pathways involved in the coordination of carbohydrate and Fe metabolism with N use by OsbZIP11, OsRLI1 and OsHHO3 (c), and *in vivo* validation of the regulatory pathway by cotransfection assays (d). *OsSUS1* and *OsSUS2* encode sucrose synthases 1 and 2, respectively; *OsIRO2* encodes a basic helix–loop–helix transcription factor; *OsGRX6* encodes a CC-type glutaredoxin. In (a, c), since the direction of regulation is not inferred by the network analysis, predicted regulation by the connected transcription factors are indicated by black lines with dots at their ends. The arrow and the '⊥' sign indicate promotion and repression of the indicated biological pathways, respectively. Factors belonging to modules M3 and M5 are indicated with brown and green circles, respectively. In (b, d), reporter plasmids (containing the LUC reporter gene) and an internal control plasmid (containing GUS reporter gene) were cotransfected into rice protoplasts with either an effector plasmid containing *OsbZIP11*, *OsRLI1*, *OsHHO3* or *OsNDER1* cDNA or an empty vector (none). Relative LUC activity obtained with the empty plasmid (none) was set to 1. Data represent means ± SD ($n = 4$). Two-tailed Student's *t*-test was performed to analyze the significance of the effect of each transcription factor on the activity of the target promoter (***, $P < 0.001$).

for *GLUTAREDOXIN 6* (*OsGRX6*: Os01g0667900) involved in the homeostatic regulation of N use (El-Kereamy *et al.*, 2015) (Fig. 8c). The result of *in vivo* validation supported these inferred regulatory relationships (Fig. 8d). These results suggest a novel connection between carbohydrate, Fe, and N metabolism, and the mechanism underlying their coordinated regulation.

In planta validation of involvement of two OsbZIP transcription factors and OsRLI1 in N-deficiency responses

For *in planta* analysis of contribution of the identified transcription factors to N-deficiency responses, genome-edited rice plants were generated with the CRISPR/Cas9 system. We obtained a

double knockout line for OsbZIP11 and its closest homolog, OsbZIP41, which was another influential transcription factor within the gene regulatory network (Fig. S2b; Table S10), and a knockout line of OsRLI1. All deletion or insertion by genome editing caused frameshift mutations, which probably did not affect the transcription of knockout genes but produced nonfunctional proteins (Fig. S3a). First we examined the expression levels of genes encoding critical influential transcription factors in the network (Fig. 9a). As most evident effects, N deficiency-induced reductions in expression levels of *OsbZIP11* and *OsRLI1* were observed in the wild-type but not in *bzip11/41* and *rli1* mutant lines. Although N deficiency-induced reductions in expression levels of *OsHHO3/4* and *OsbZIP41* were observed in both the wild-type and mutant lines, such reductions were attenuated in mutant lines. These results corroborate the mutual repressive effects of these transcription factors that were shown by cotransfection assays (Fig. 7b) and suggested that loss of the functions of these transcription factors led to perturbation of the N-deficiency response network. Next, we analyzed the expression levels of several predicted common target genes of OsbZIP11 and OsRLI1 (Fig. 9b). The expression of *OsNAR2.1* was hardly affected by the mutations, while expression levels of *OsNRT2.3*, *OsGS1;1* and *OsACTPK1* were substantially different in the wild-type and the mutants under the N-deficient condition. Collectively, *in planta* validation also corroborated our hypothesis that these transcription factors are required for gene regulation for N-deficiency responses.

Discussion

Gene regulatory network for N-deficiency responses

Previous transcriptional network analyses suggested several influential transcription factors that affect N-deficiency responses. In poplar (*Populus tremula* × *Populus alba*), it was proposed that the NAC transcription factor, PtaNAC1, and the ERF transcription factor, PtaRAP2.11, are important for the regulation of root transcriptome under nitrate deficiency (Wei *et al.*, 2013; Dash *et al.*, 2015). In rice, *OsNAC36* was identified as a hub gene in a coexpression module that responds to short-term nitrate deprivation in roots (Coneva *et al.*, 2014). However, only a limited number of transcription factors have been proposed to be important under N-deficient conditions, and their target genes largely remain obscure. On the contrary, the transcriptional network involved in the response to N supply has been analyzed in great detail, particularly in Arabidopsis, resulting in the identification of several important hub transcription factors. For instance, in Arabidopsis, CIRCADIAN CLOCK ASSOCIATED 1 (Gutierrez *et al.*, 2008), SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (Krouk *et al.*, 2010), AtNAC4 (Vidal *et al.*, 2013), and AtTGA1/4 (Alvarez *et al.*, 2014) play important roles within the transcriptional cascade triggered by nitrate or organic N supply. Coexpression analyses also suggested the role of HRS/NIGT1 and TGA transcription factors in the response to nitrate supply in the roots of wheat (*Triticum aestivum* L.) (Dissanayake *et al.*, 2019). Another investigation in rice also suggested that these transcription factor families play vital roles in the response to N source supply (Obertello *et al.*, 2015). These investigations suggest that key transcription factors that regulate the response to N supply partially overlap, although they may differ depending on the plant species, tissue type, N source, and N treatment duration.

Our analyses strongly suggest that G2-like HRS/NIGT1 and TGA bZIP transcription factors play important roles in N-deficiency responses, similar to their roles in N-supply responses. However, as discussed in detail in the next section, these transcription factors were not orthologous to those identified in the analysis of N-supply responses in Arabidopsis. Moreover, we identified previously unrecognized transcription factors, such as OsNDER1. These results, together with a previous notion that plants probably employ different transcriptional cascades for the response to N supply and N deficiency (Coneva *et al.*, 2014), suggest that analyses of both N-deficiency responses and N-supply responses are necessary to fully understand the mechanisms that optimize N use in plants.

Key transcription factors regulating N-deficiency responses in rice

The current study identified two HRS/NIGT1 transcription factors (OsHHO3 and OsHHO4), a TGA transcription factor (OsbZIP11), and OsRLI1 as strong candidates for key regulators of N-deficiency responses, which probably function as transcriptional repressors (Figs 6, 7). In Arabidopsis, HRS/NIGT1 transcription factors (AtNIGT1.1-AtNIGT1.4) and TGA transcription factors (AtTGA1 and AtTGA4) are involved in nitrate-inducible gene expression. The *HRS/NIGT1* gene family in Arabidopsis is composed of four nitrate-inducible genes (*AtNIGT1.1–1.4*) and three noninducible genes (*AtHHO4–6*) (Scheible *et al.*, 2004; Maeda *et al.*, 2018). AtNIGT1 transcriptional repressors function as feedback regulators in the repression of nitrate-inducible genes (Maeda *et al.*, 2018), and NIGT1-mediated repression is abolished under N-deficient conditions to induce N-deficiency responses (Kiba *et al.*, 2018). However, OsHHO3 and OsHHO4 are phylogenetically more similar to AtHHO5 and AtHHO6 than to NIGT1 proteins. Furthermore, although the OsNIGT1 protein is the most closely related to AtNIGT1 (Fig. S2a) and functions as a negative regulator of N use (Sawaki *et al.*, 2013), OsNIGT1 was not selected as a key regulator in the N-deficiency network. Furthermore, similar to the case of OsHHO3 and OsHHO4, we also note that OsbZIP11 and OsbZIP41 are homologous but not orthologous to AtTGA1 and AtTGA4 which play a central role in maintaining the coexpression network that regulates nitrate supply-induced responses in Arabidopsis (Alvarez *et al.*, 2014). These suggest that similar but distinct proteins play major roles in the responses to N supply and N deficiency. Alternatively, homologous, but not orthologous, transcription factors may act as functional homologs in the responses to N availability in Arabidopsis and rice. Indeed, functional diversification within a transcription factor family between monocot and dicot species has been suggested recently (Borrill *et al.*, 2019; Dissanayake *et al.*, 2019).

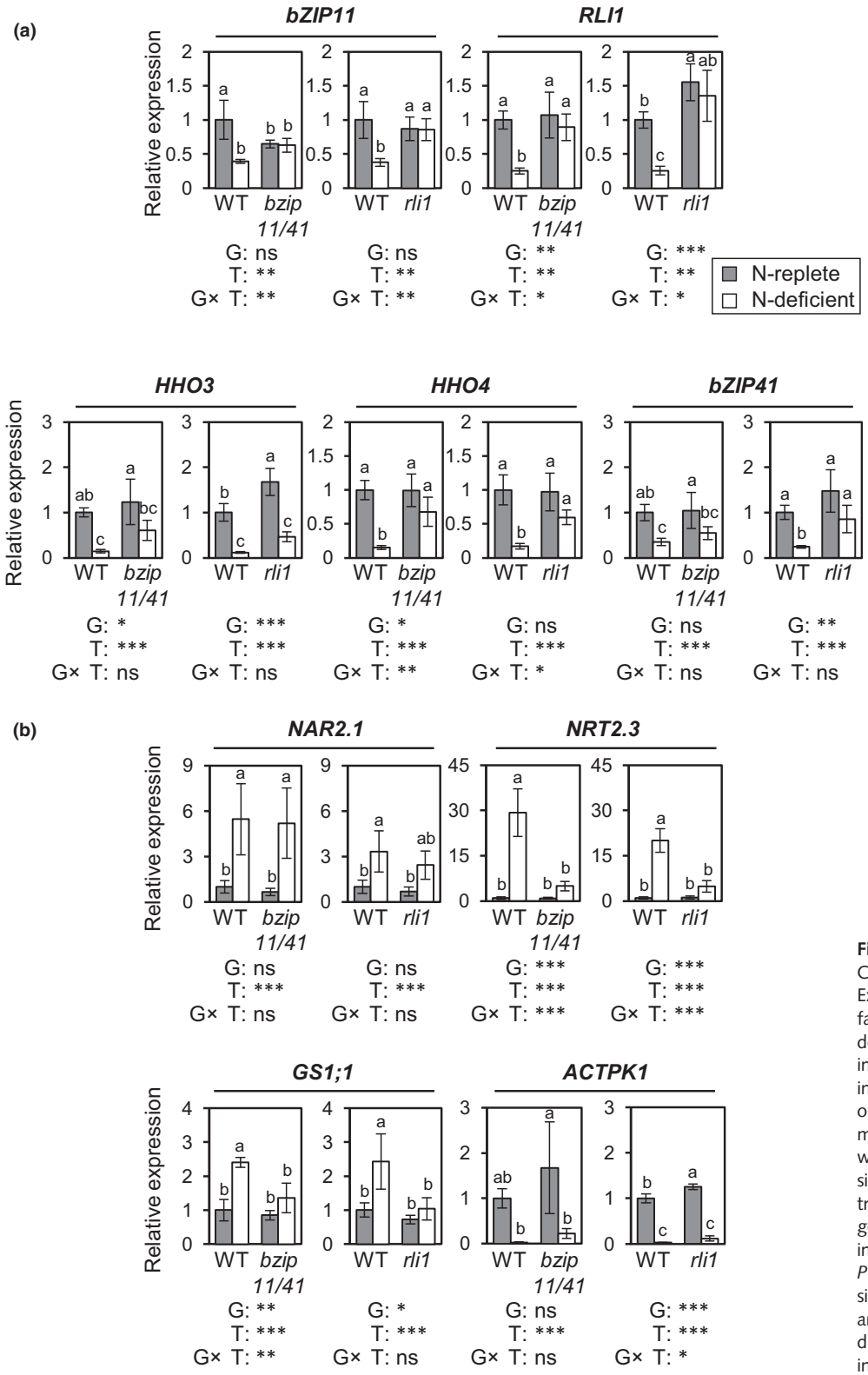


Fig. 9 Gene expression analysis in roots of CRISPR/Cas9-mediated knockout rice lines. Expression of genes encoding transcription factors (a) suggested to be involved in N-deficiency response and proteins (b) involved in the regulation of N uptake and metabolism in roots of plants grown under the N-replete or N-deficient condition. Data represent means ± SD ($n = 4$–6). Two-way ANOVA was conducted for each panel, and significance of the effects of genotype (G), treatment (T), and the interaction between genotype and treatment (G × T) are indicated (ns, $P \geq 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Tukey's honestly significant difference test was conducted, and mean values that are significantly different from each other ($P < 0.05$) are indicated by different letters.

The influential transcription factors identified in this study could possibly be used to develop cereal cultivars with improved N-use efficiency via genetic engineering. To date, among these transcription factors, only OsRLI1 has been characterized, suggesting its physiological role in shoots (Ruan *et al.*, 2018). Our current analysis using the *rli1* knockout line shed light on its novel physiological functions in roots. Furthermore, *in planta* evaluation of OsbZIP11 and OsbZIP41 also indicated their critical roles in N-deficiency responses. Thus, further investigations of their roles in N-use efficiency and grain yield in soil-grown rice

plants would establish the efficacy of genetic engineering with these transcription factors for improving plant productivity. From this viewpoint, it is worth noting that OsNLP1 and OsNLP4 (Os09g0549450) were also identified as influential transcriptional regulators for N-deficiency responses (Figs 6b, S2c). Consistent with our prediction that OsNLP1 regulates *OsNRT1.1B* and *OsAMT1;1* (Table S11), it was recently proposed by transgenic rice plant-based analysis that OsNLP1 and OsNLP4 are involved in the regulation of N-related genes, including *OsNRT1.1B* and *OsAMT1;1*, grain yield, and N-use efficiency (Alfatih *et al.*, 2020; Wu *et al.*, 2020). As the NLP family proteins are key regulators of nitrate supply-inducible responses in Arabidopsis (Konishi & Yanagisawa, 2013, 2014; Marchive *et al.*, 2013; Liu *et al.*, 2017b), these findings also suggest a critical role of nitrate signaling in N-deficiency responses in rice, although rice prefers ammonium over nitrate as the N source (Fig. 1d) (Sasakawa & Yamamoto, 1978).

Conclusions and future perspectives

In vivo and *in planta* analyses of influential transcription factors found by coexpression analysis and machine learning-based pathway inference revealed interconnectivity among the key factors in the network for N-deficiency responses and their autoregulation. For instance, the mutually repressive relationship between OsbZIP11 and OsRLI1 was found. Owing to this relationship, *OsbZIP11* and *OsRLI1* expression were enhanced in the *rli1* and *bzip11/41* mutant plants under the N-deficient condition, respectively. It has been shown that the mutually repressive relationship between components in a response system increases the amplitude of response without affecting the threshold to a signal (Ishihara & Shibata, 2008). Furthermore, autoregulation of regulatory genes reduces variability in their expression levels (Becskei & Serrano, 2000). Thus, interconnectivity and autoregulation found in the current study could underlie steep and robust responses to N deficiency in rice roots. Further analysis of this interconnectivity and autoregulation could pave the way to improving plant productivity under the N-deficient conditions.

Although root tissues are the interface between plants and the surrounding soil environment and are responsible for nutrient uptake, previous studies suggested the importance of aboveground tissues in determining the gene expression profile under N deficiency (Ohkubo *et al.*, 2017) and overall N-use efficiency via internal recycling and translocation of N sources (Vinod & Heuer, 2012). Thus, gene regulatory network analysis and identification of putative key regulators in shoots may reveal more comprehensive mechanisms associated with N-deficiency responses. On the other hand, as post-translational modifications such as phosphorylation, in addition to transcriptional regulation, severely influence gene regulatory networks, complementary molecular biological analyses of these transcription factors are warranted to obtain more profound insights into the relationship among these potential key N regulators. Such analyses of transcription factors identified in the current study would provide an opportunity to engineer N-deficiency responses in crops in the future.

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Author contributions

YU and SY conceived and designed the research. YU, NO, AT, TK and YK performed the experiments. AJN, MM and SY supervised the experiments. YU, KK, AT and AJN analyzed the data. YU and SY wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Putative off-target sites for the guide RNA at respective loci.

Fig. S2 Phylogenetic analysis of the HRS/NIGT1, TGA, and NLP family transcription factors of *Arabidopsis thaliana* and rice (*Oryza sativa*).

Fig. S3 Sequence analysis of target loci in the mutant lines.

Methods S1 Additional methodological details.

Table S1 List of primers used in this study.

Table S2 Coefficient of variation of the expression of commonly used internal reference genes.

Table S3 Summary of RNA-seq samples and mapped reads per sample.

Table S4 Uptake patterns of Pi and N sources and sensitivity to N deficiency in 119 Asian rice accessions.

Table S5 Results of the analysis of variance (ANOVA) of parameters related to nutrient uptake.

Table S6 List of genes differentially expressed between N-replete and N-deficient conditions in Nipponbare root.

Table S7 Normalized values of reads per million (RPM) and module assignment of each gene.

Table S8 Gene ontology (GO) enrichment analysis of modules from the coexpression network.

Table S9 List of hub genes in the N-deficiency response modules.

Table S10 Transcription factors constituting the high-confidence gene regulatory network.

Table S11 List of predicted regulatory pathways in the high-confidence gene regulatory network.

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