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Comparative transcriptome analyses under individual and combined nutrient starvations provide insights into N/P/K interactions in rice

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ABSTRACT

Crops often suffer from simultaneous limitations of multiple nutrients in soils, including nitrogen (N), phosphorus (P) and potassium (K), which are three major macronutrients essential for ensuring growth and yield. Although plant responses to individual N, P, and K deficiency have been well documented, our understanding of the responses to combined nutrient deficiencies and the crosstalk between nutrient starvation responses is still limited. Here, we compared the physiological responses in rice under seven kinds of single and multiple low nutrient stress of N, P and K, and used RNA sequencing approaches to compare their transcriptome changes. A total of 13,000 genes were found to be differentially expressed under all these single and multiple low N/P/K stresses, and 66 and 174 of them were shared by all these stresses in roots and shoots, respectively. Functional enrichment analyses of the DEGs showed that a group of biological and metabolic processes were shared by these low N/P/K stresses. Comparative analyses indicated that DEGs under multiple low nutrient stress was not the simple summation of single nutrient stress. N was found to be the predominant factor affecting the transcriptome under combined nutrient stress. N, P, or K availability exhibited massive influences on the transcriptomic responses to starvation of other nutrients. Many genes involved in nutrient transport, hormone signaling, and transcriptional regulation were commonly responsive to low N/P/K stresses. Some transcription factors were predicted to regulate the expression of genes that are commonly responsive to N, P, and K starvations. These results revealed the interactions between N, P, and K starvation responses, and will be helpful for further elucidation of the molecular mechanisms underlying nutrient interactions.

1. Introduction

Plants require 14 essential nutrients of which nitrogen (N), phosphorus (P), and potassium (K) are three most abundant macronutrients for plant growth and development. Plants grown under conditions lacking any of these nutrients will exhibit visual deficiency symptoms and severe growth inhibition (de Bang et al., 2021). N, P, and K are also three most critical nutrient elements for maintaining crop productivity and quality. In order to feed the world's growing population, an increasing amount of N, P, and K fertilizers are being used in agricultural systems (Heuer et al., 2017; Teng et al., 2017). However, over-application of these fertilizers bring adverse impacts on environments, such as soil acidification and eutrophication of water resources (West et al., 2014). In addition, the use efficiency (the total biomass or grain yield produced by one unit of applied fertilizer) of N, P, K fertilizers is very low in agricultural practices. For example, the use efficiency of applied N fertilizers for worldwide cereal production is about 33% (Raun and Johnson, 1999). Crops can only exploit 20–30% of applied P fertilizers, mostly due to the precipitation and mineralization of inorganic phosphate (Pi), which also cause the Pi availability suboptimal for vegetative growth and crop productivity for approximately 70% of arable land (Herrera-Estrella and López-Arredondo, 2016; Heuer et al., 2017). The worldwide K fertilizer use efficiency is around 20% for cereal crops (Dhillon et al., 2019). Moreover, crop plants often

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simultaneously suffer from limitations of multiple nutrients, such as N, P, and K, in agricultural systems (Ismail et al., 2007). Therefore, to develop sustainable and environment-friendly agriculture, it is urgent to reduce the dependency of crop productivity on heavy use of chemical fertilizers by breeding or engineering crops with improved nutrient use efficiency and/or better tolerance to single and/or multiple nutrient starvation stress. To achieve this goal, it is essential to understand the molecular regulatory networks underlying N, P, and K starvation responses in plants.

Many efforts have been made in the physiological and molecular responses of plants to N, P, and K starvations by forward and reverse genetics, and also by multiple omics (e.g., genomics, proteomics, transcriptome, and microRNAome) (Paz-Ares et al., 2022; Tsay et al., 2011; Vidal et al., 2020; Wang, Y. et al., 2021a). Plants mainly take up N in the forms of inorganic ammonium (NH_4^+) and nitrate (NO_3^-), depending on soil conditions and plant species. After the uptake by plant roots, inorganic N is converted to amino acids by a series of biochemical processes that are catalyzed sequentially by nitrate reductase, nitrite reductase, glutamine synthetase (GS), and glutamate synthase (glutamine-2-oxoglutarate aminotransferase, GOGAT) (Xu et al., 2012). Under N starvation conditions, plants exhibit diverse physiological responses, such as stunted shoot growth, leaf chlorosis, reduced branching, decreased leaf expansion, altered root architecture, and early flowering (de Bang et al., 2021). A large number of N-responsive genes have been functionally identified to coordinate N starvation responses, including genes encoding NO_3^- transporter NRTs, NH_4^+ transporter AMTs, protein kinases like CIPKs, and transcription factors like NLPs and NIGTs (Vidal et al., 2020). Plants predominantly absorb P in the form of inorganic orthophosphate $H_2PO_4^-$ and HPO_4^{2-}, depending on soil pH value. To cope with low P stress, plants have evolved a series of developmental and physiological responses, including exudation of organic acids, secretion of acid phosphatases, induction of high-affinity Pi transporters, changes in shoot-to-root biomass ratio, and association with soil microorganisms (Puga et al., 2017). Many genes have identified to play important roles in the low P adaptive response, such as genes encoding Pi transporter PHT1, PHR1 transcription factor, SPX-domain containing proteins, and small RNAs (Chang et al., 2019; Paz-Ares et al., 2022; Wang, L. et al., 2021b). Plants only take up K in the form of K^+. High-affinity K^+ uptake at low external K^+ concentrations is mediated by K^+/H^+ symporters in the HAK/KUP/KT family, whereas voltage-gated K^+ channels (e.g., AtAKT1 in *Arabidopsis thaliana*) mediate low-affinity K^+ uptake at higher external concentrations (Ankit et al., 2022; Wang, Y. et al., 2021a). Plants have evolved sophisticated signal transduction systems to respond to K starvation, which involve signals, such as Ca^{2+}, reactive oxygen species (ROS), and hormones (Shin and Schachtman, 2004; Wang, Y. et al., 2021b). In addition, the molecular mechanism underlying the interactions between N, P, and K nutrients have been started to be uncovered (Hu et al., 2019; Kellermeier et al., 2014; Medici et al., 2019; Nasr Esfahani et al., 2021). Transcriptomic analyses by high-throughput sequencing provide a useful tool for understanding the complex regulatory network of the interaction between different nutrient starvation stresses. Although the transcriptomic responses to individual N, P, and K deficiency have been investigated in plant species, such as Arabidopsis (Forieri et al., 2017; Scheible et al., 2004), rice (Kumar et al., 2021; Yang, S.Y. et al., 2015a; Zhang et al., 2017), maize (Ma et al., 2020), and soybean (Zeng et al., 2018), little attention has been paid to the molecular responses under multiple or combined N, P, and K starvations.

Rice is one of the three major food crops, and is the staple food source for approximately four billion people throughout the world (Chen, R. et al., 2021a). N, P, and K are three most important nutrient elements for rice production. High yields of irrigated rice are dependent on large applications of N, P, and K fertilizers (Ma et al., 2022; Ye et al., 2019), because rice production is globally and seriously constrained by deficiency of nutrients, such as N, P, and K (Ismail et al., 2007). Thus, understanding molecular responses under N, P, and K starvations and their interactions is essential for breeding or engineering rice cultivars with improved tolerance to multiple nutrient starvations. Although the physiological and molecular response to single N, P, or K deficiency has been well documented in rice (Kumar et al., 2021; Ma et al., 2012; Secco et al., 2013; Shin et al., 2018), few study was focused on the combined N, P, and K starvation stresses. The molecular mechanism underlying the interaction among N, P, and K is still largely unclear in rice. In this study, rice was cultivated under eight nutrient conditions including all the single and combined starvations of N, P and K, the physiological and RNA sequencing (RNA-seq) based transcriptomic responses were investigated in order to understand interactions between these macronutrients in both roots and shoots of rice seedlings.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa* L.spp.japonica) seeds from our own lab were surface sterilized and then germinated in water for about two days at 28 °C. Then the uniformed germinated seeds were put on a plate with 96 holes for hydroponic culture (usually one seed in a hole, and half of the holes were evenly filled with seeds). After cultivation in water for 4 days, rice plants were transferred to Yoshida nutrient solution for another 5 days. After that, rice seedlings were used for low nutrient stress treatments. Nutrient solution were changed every three days during the growth period (pH = 5.5). The composition of nutrient solution for various single and multiple low nutrient stresses was shown in Fig. S1. Rice seedlings were grown in a growth chamber under controlled conditions. The light intensity was approximately 250 $\mu molm^{-2}\ s^{-1}$ at shoot height with a day/night cycle of 12 h/12 h at 28 °C/26 °C. The relative humidity was 60%. After low nutrient treatment for 7 days, roots and shoots were collected (4 h after illumination) for physiological and transcriptome analyses. For RNA sequencing, each sample contained three plants. Three biological replicates were included for each treatment. All samples were frozen in liquid nitrogen for 2 h and then stored at −80 °C until RNA extraction.

2.2. Determination of N, P, and K concentrations

Rice roots and shoots were harvested after low nutrient treatments for 7 days. Roots were washed with tap water and rinsed twice with deionized water to remove adhering nutrients. Rice samples were then dried in an oven at 80 °C for 3 days. For the analyses of N, P, and K concentrations, the dry biomass of roots and shoots was ground, and about 0.05–0.10 g sample was used to be digested with H_2SO_4. N concentration was determined with a flow-injection auto-analyzer (Seal AutoAnalyzer AA3). P and K concentrations were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Agilent 710 ICP-OES). Three biological replicates were used for each treatment.

2.3. RNA extraction and sequencing

Total RNA was extracted from the tissue using TRIzol® Reagent (Plant RNA Purification Reagent for plant tissue) according the manufacturer's instructions (Invitrogen, Carlsbad, CA) and genomic DNA was removed using DNase I (TaKaRa, Dalian, China). Then RNA quality was determined by 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA sample (OD260/280 = 1.8–2.2, OD260/230 ≥ 2.0, RIN≥6.5, 28S:18S ≥ 1.0, concentration>1 μg/μL) was used to construct sequencing library. RNA-seq transcriptome librariy was prepared following TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA) using 1 μg of total RNA. Firstly, mRNA was isolated according to polyA selection method by oligo(dT) beads and then fragmented by fragmentation buffer. Secondly, double-stranded cDNA was synthesized using a SuperScript

double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). Then, the synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 300 bp on 2% Low Range Ultra Agarose followed by PCR amplified using Phusion DNA polymerase (NEB) for 15 cycles. After quantified by TBS380, paired-end RNA-seq library was sequenced with the Illumina HiSeq × 10/NovaSeq 6000 sequencer (2 × 150 bp read length). The RNA-seq datasets were available from the NCBI Sequence Read Archive (SRA) under accession number PRJNA902666 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA902666).

2.4. Mapping of RNA-seq reads and identification of differentially expressed genes

The raw paired end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then, clean reads were separately aligned to reference rice genome (http://rice.uga.edu/) with orientation mode using HISAT2 software (Kim et al., 2015). The mapped reads of each sample were assembled by StringTie in a reference-based approach (Pertea et al., 2015). To identify differential expression genes (DEGs) between two different samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. RSEM (http://deweylab.biostat.wisc.edu/rsem/) was used to quantify gene abundances (Li and Dewey, 2011). DEGs with $|\log2FC| > 1$ and P value < 0.05 (DESeq2) were considered to be significantly differentially expressed genes.

2.5. Gene ontology (GO) and KEGG enrichment analysis

GO term enrichment was performed to identify functional enrichment of DEGs by using online tool PlantRegMap (http://plantregmap.gao-lab.org/go.php) (Tian et al., 2020). KEGG enrichment analysis was performed to identify metabolic pathways enriched in DEGs by KOBAS (http://kobas.cbi.pku.edu.cn/home.do). GO and KEGG category with a P-value $\leq$0.05 was regarded as significantly enriched.

2.6. Co-expression regulatory network

Enrichment of transcription factors (TFs) were predicted by PlantRegMap online tool (http://plantregmap.gao-lab.org/tf_enrichment.php)(P-value<0.05)(Tian et al., 2020). The most 15 enriched TFs and targeted genes were visualized by Cytoscape 3.8.

2.7. Quantitative RT-PCR analysis (qRT-PCR)

Total RNA was extracted with the Ultrapure RNA Kit (CWBIO, Beijing, China), and then the cDNA was synthesized with total RNA using reverse transcriptase (HiScript®III All-in-one RT SuperMix Perfect for qPCR) (Vazyme, Nanjing, China) according to the manufacturer's protocol. Quantitative real-time RT-PCR (qRT-PCR) was performed with Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), and amplification was real-time monitored on a real-time PCR system (QuantStudio 3, Applied Biosystems). Gene specific primers were used (Table S1). All reactions were run in triplicate. PCR efficiency was determined by a series of 2-fold dilutions of cDNAs, and the calculated efficiency of all primers was around 2.0. Gene relative expression levels were normalized to two internal control genes, *OsACTIN1* (LOC_Os03g50885) and *OsUBQ2* (LOC_Os02g06640), and presented as $2^{-\triangle\triangle CT}$ to simply the presentation of data (Zeng et al., 2019).

2.8. Statistical analyses

Statistical analyses were performed by two-tailed student's *t*-test or by one-way ANOVA test with SPSS23.0. Data were considered to be significantly different at $P < 0.05$.

3. Results

3.1. Growth and nutrient concentrations of rice seedlings grown under individual and multiple N, P, and K starvation

In order to explore the global gene expression profiles under individual and multiple N, P, and K starvation conditions in rice, 11 day-old seedlings were treated with 7 kinds of single and multiple N, P and K starvation stresses for 7 days (Fig. S1). Shoots and roots showed differences under diverse nutrient stresses (Fig. 1A). The shoot biomass of rice seedlings grown under all single or multiple N/P/K starvations were all significantly lower than that of control, especially for treatments with low N (LN) (Fig. 1B). The root biomass of seedlings under LN and LNPK (combined low N, low P, and low K) were significantly higher than that of control, while the root biomass of combined low P and low K (LPK) was remarkably lower than that of control (Fig. 1C). The root/shoot biomass ratio was increased for most of these nutrient stresses, especially for LN and LNPK (Fig. 1D). With the exception of low P (LP), low K (LK), and LPK, the root length of rice seedlings was all increased by other nutrient starvations (Fig. 1E). The different physiological responses under single or combined N, P, and K starvation suggest the existence of interactions among N, P, and K nutrients.

Concentrations of N, P, and K in rice seedlings treated by these low nutrient stresses were measured. N concentration in roots and shoots was decreased under conditions with low N (including LN, LNP, LNK, and LNPK treatments) (Figs. S2A and B). In addition, LP and LPK treatments reduced N concentration in roots but not in shoots (Figs. S2A and B), suggesting the impact of P and K starvations on the uptake of N. All treatments with low P (including LP, LNP, LPK, and LNPK treatments) significantly reduced P concentrations in both roots and shoots (Figs. S2C and D). LN treatment also reduced the P concentration in roots, while LNK treatment increased the P concentration in shoots, and LK treatment increased the P concentration in both roots and shoots (Figs. S2C and D); these results suggest the influences of N and K starvations on the homeostasis of P. K concentration in roots was reduced under all conditions with low K (including LK, LNK, LPK, LNPK treatments) (Figs. S2E and F). Notably, the K concentration was increased by LP treatment in shoots, and was increased by LNP treatments in both roots and shoots (Figs. S2E and F), suggesting the potential influence of N and P nutrition on the homeostasis of K. Overall, morphological and physiological responses to single and multiple N, P, and K starvations were exhibited, and the nutrient concentrations in roots and shoots confirmed the effectiveness of N, P, and/or K starvations. It is obvious that the effect of multiple nutrient starvations on plant growth is not simply overlapped by the individual nutrient starvation, suggesting the interactions among these nutrients.

3.2. Overview of transcriptome analyses under individual and multiple N, P, and K starvations

Global transcriptome profiles of rice shoots and roots in responses to individual and multiple N, P, and K starvations were analyzed by RNA-seq. A total of 44.7–68.5 million reliable clean reads were obtained from each of the total 48 libraries after excluding the low-quality reads, and most of the clean reads (89.1–93.6%) from each library could be mapped to the rice reference genome (Table S2). The Pearson's correlation (R value) of three biological replicates of each sample was all above 95%, indicating the high reliability of these replicates (Table S3). The expression level of each transcript was calculated according to the transcripts per million reads mapped (TPM), and a total of 55,986 gene loci were detected among all these samples (Tables S4–5).

Differential expression analyses (log2 fold change $\geq$1 or $\leq$ -1, and adjusted P-value $\leq$0.05) showed that a total of 13000 genes (7519 genes in roots, 8914 genes in shoots, and 3433 genes in both roots and shoots)

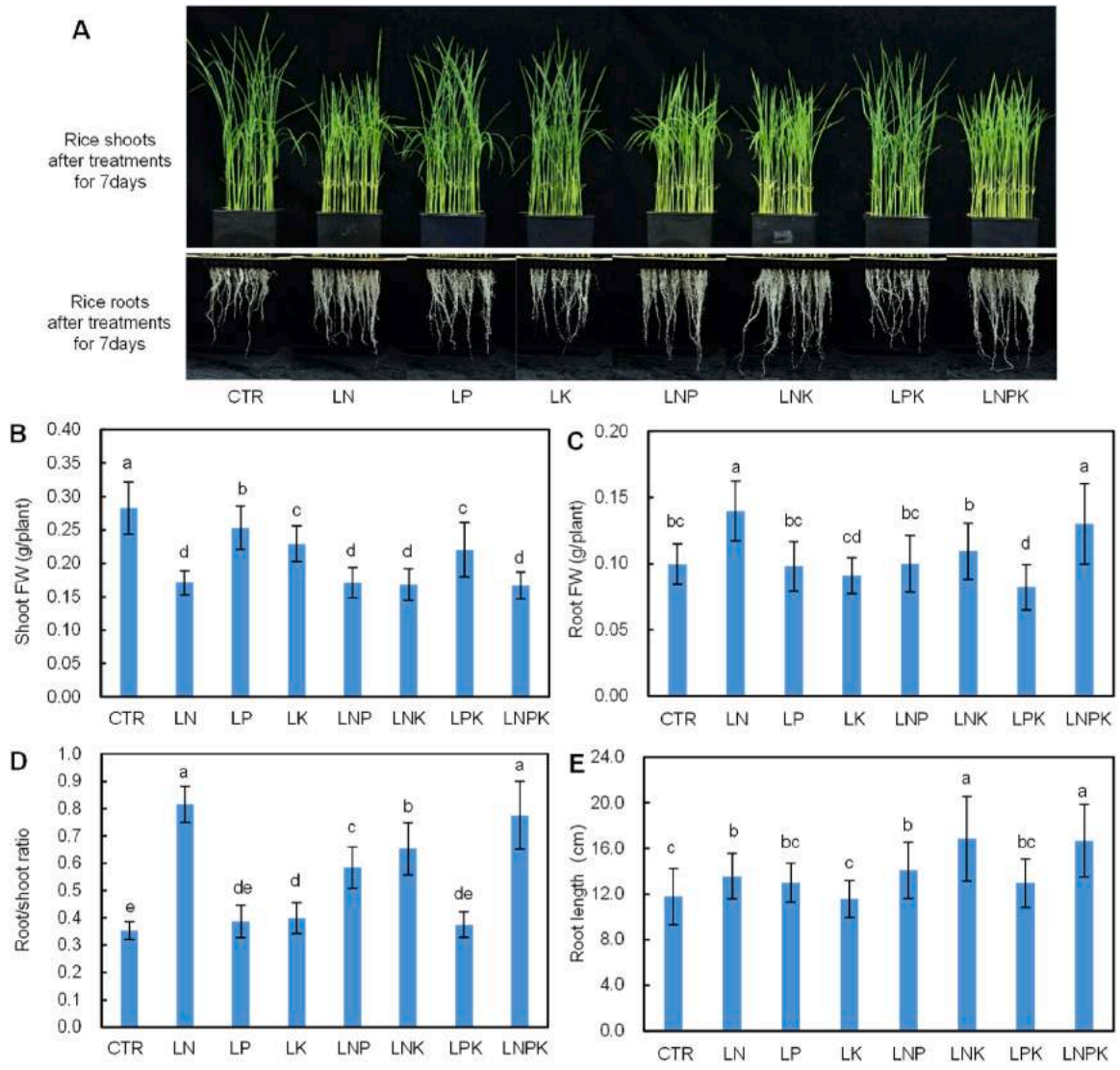
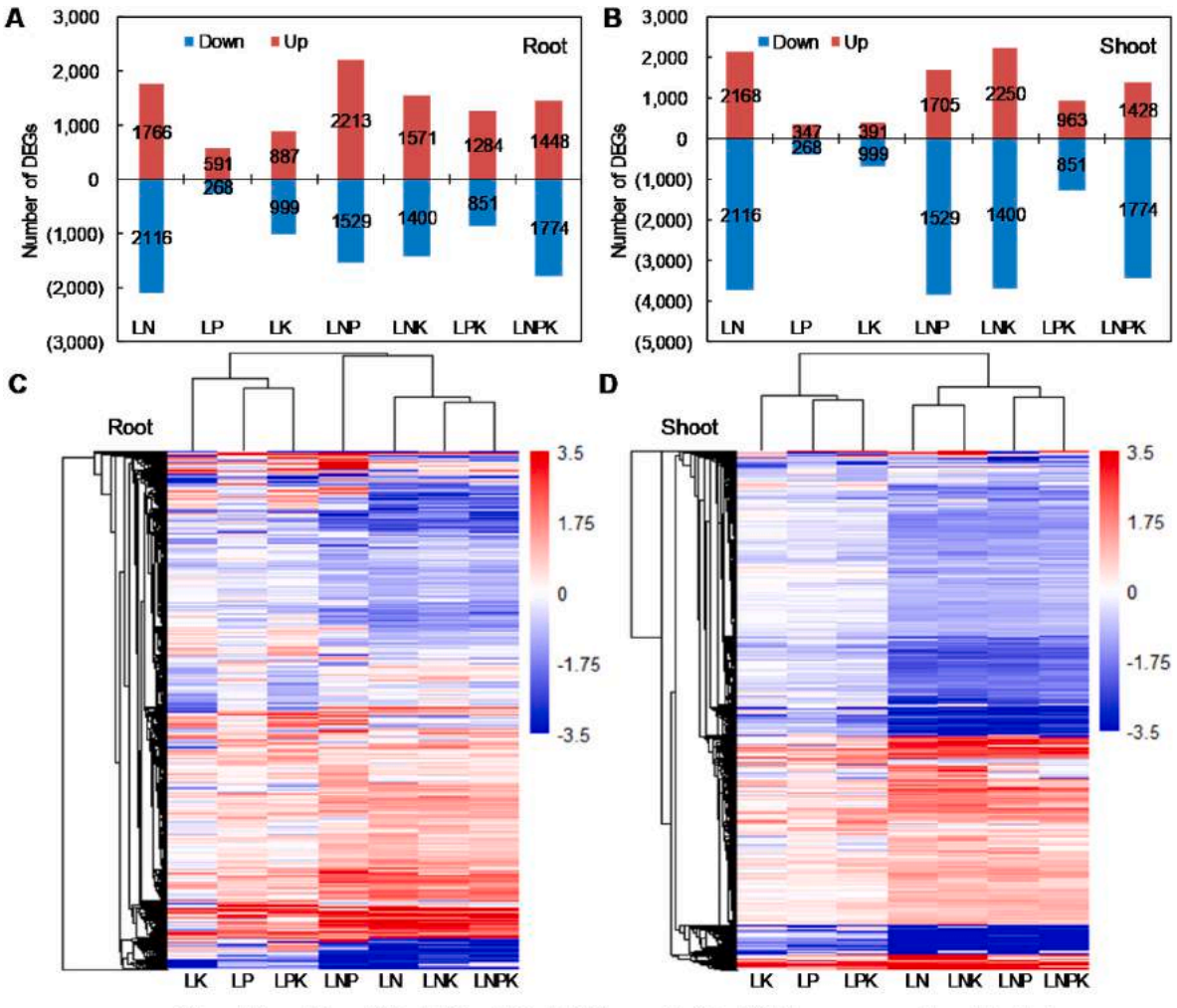


Fig. 1. Physiological responses of rice seedlings to individual and combined nutrient starvations (LN, LP, LK, LNP, LNK, LPK and LNPK). (A) Morphological appearance of shoots and roots of rice seedlings after 7 days treatments of individual and combined nutrient starvations. The fresh weight of shoots (B) and roots (C), root/shoot ratio (D), and root length (E) of rice seedlings after individual and combined nutrient starvation treatments for 7 days. Values represent means ± SD, n = 22. Different letters indicate that the values are significantly different at $P < 0.05$ (one-way ANOVA).

were differentially expressed in roots and/or shoots, under either of the N/P/K nutrient starvation conditions (Tables S5 and 6). The number of differentially expressed genes (DEGs) fluctuated tremendously under each single and multiple low nutrient treatment (Table S6; Fig. 2A and B). For example, the DEGs number under LN in roots was about 4.5 folds and 2.0 folds higher than that under LP and LK, respectively (Fig. 2A and B), but the number of DEGs under LNPK was lower than that of LN in both roots and shoots. But for LPK treatment, the number of DEGs was roughly equal to the summation of DEGs under LP and under LK. A large proportion of DEGs (around 30% in roots) were found to be commonly differentially expressed between roots and shoots under diverse conditions with low level of N (i.e., LN, LNP, LNK, LNPK), but for LP and LK conditions, the proportion of common DEGs between roots and shoots was much lower (Fig. S3). Most of the common DEGs in both roots and shoots showed similar expression patterns under diverse low nutrient stresses (Table S7). Heat map analyses of the union of 7519 DEGs in roots under diverse nutrient stresses indicated that their expression patterns under LN, LNK, LNP, and LNPK were similar, while DEGs exhibited similar patterns under LK and LPK (Fig. 2C). Similarly, among the union of 8914 DEGs in shoots, DEGs under LN, LNK, LNP, and LNPK showed similar expression patterns, while the expression patterns of DEGs under LK and LPK were similar (Fig. 2D).

3.3. Functional enrichments of DEGs under individual and multiple N, P, and K starvation

By gene ontology (GO) enrichment analysis, 238 to 746 GO terms were found to be significantly enriched ($P < 0.05$) in each group of DEGs under single or multiple N, P, and K starvation conditions in roots and shoots (Table S8). Interestingly, 25 GO terms of biological processes, including carbohydrate metabolic process, response to hormone, and ROS metabolic process, were commonly significantly enriched in DEGs of shoots under all of these low nutrient stresses (Fig. 2E). Similarly, 66 GO terms of biological processes, including organic acid metabolic process, oxidation-reduction process, metal ion transport, and lipid metabolic process, were commonly significantly enriched in root DEGs under all these low nutrient stresses (Fig. S4). In addition, KEGG pathway enrichment analysis showed that 10 to 37 pathways were significantly enriched ($P < 0.05$) in DEGs of roots and shoots under single or multiple N, P, and K starvations (Table S9). Phenylpropanoid



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LN	LP	LK	LNP	LNK	LPK	LNPK	Enriched GO term
1631	204	291	1550	1710	644	1396	Single-organism process
970	119	159	944	998	348	823	Single-organism metabolic process
447	58	86	433	471	190	374	Oxidation-reduction process
1196	147	214	1144	1263	463	1027	Single-organism cellular process
745	95	143	680	790	338	625	Response to stimulus
261	31	45	290	271	112	249	Carbohydrate metabolic process
138	21	29	138	146	74	130	Single-organism catabolic process
222	34	50	207	239	102	187	Transmembrane transport
204	35	43	170	211	98	165	Response to endogenous stimulus
11	4	4	9	9	4	11	Regulation of organ growth
130	28	28	139	144	60	131	Cell wall organization or biogenesis
230	38	47	199	238	107	191	Response to organic substance
192	32	39	164	194	86	157	Response to hormone
329	48	65	292	328	145	271	Response to chemical
92	17	28	100	95	49	94	Response to oxidative stress
11	4	4	9	9	4	11	Organ growth
38	10	16	42	44	30	48	Hydrogen peroxide catabolic process
168	28	35	150	177	82	131	Response to oxygen-containing compound
85	21	22	92	94	42	96	Cell wall organization
51	11	17	55	59	33	60	Reactive oxygen species metabolic process
39	10	16	44	47	30	51	Hydrogen peroxide metabolic process
88	21	22	95	98	43	99	External encapsulating structure organization
4	2	3	4	4	2	4	Syncytium formation
317	44	64	297	336	142	273	Single-organism transport
319	45	65	298	339	143	276	Single-organism localization

Fig. 2. Overview of the differentially expressed genes (DEGs) in rice roots and shoots under individual and combined nutrient starvations (LN, LP, LK, LNP, LNK, LPK and LNPK). (A, B) The numbers of up- and down-regulated DEGs in roots and shoots under individual and combined nutrient starvation treatments for 7 days. (C, D) Heat maps showing the expression patterns and clustering of the differentially expressed genes (DEGs) under the seven kinds of nutrient stresses in rice roots and shoots. Genes differentially expressed (log2 fold change$\geq$1 or $\leq$ -1, P-value$\leq$0.05) at least under one kind of nutrient stress were selected for analysis. Log2 fold change (treatment/control) was used for the hierarchical clustering analysis based on Euclidian distance. (E) GO terms of biological processes that are commonly enriched ($P \leq 0.05$) in the DEGs of shoots under individual and combined nutrient stresses. The intensity of the red color represents the P-value of the enriched GO terms (the higher intensity means lower P-value), and the number of DEGs in each GO term are shown in the box.

biosynthesis pathway (map00940) was commonly significantly enriched in both shoots and roots under all of these low nutrient treatments (Fig. S5).

By GO enrichment analysis of the union of 13,000 DEGs under all of these low nutrient stresses, 547 GO terms of biological process group, 287 GO terms of molecular function group, and 69 GO terms of cellular component group were significantly enriched (Table S10). Nitrate transport, nitrate assimilation, response to nitrate, ammonium transmembrane transporter activity, ammonium ion metabolic process, phosphate transportation, cellular response to phosphate starvation, transcription and hormone metabolic process were included in the GO terms of biological process group (Tables S10–14). Among all these DEGs, at least 122 transcription factor genes were commonly responsive to two of these low nutrient stresses in both roots and shoots (Fig. 3; Table S11). These transcription factors belong to a diverse range of families, such as NAC, WRKY, bZIP, ERF, and NF-YA, and most of them showed similar expression pattern under diverse nutrient starvations in both roots and shoots (Fig. 3). Genes associated with nitrate and ammonium transport also showed different expression patterns under diverse low N/P/K stresses (Fig. 4; Table S12). Under N starvation, a group of genes associated with N (nitrate/ammonium) transport were induced, but some N transporter genes were repressed. Several genes potentially involved in N transport and metabolism were changed by LP, LK, and LPK. For example, *LOC_Os01g65100* and *LOC_Os02g40710* encoding a peptide transporter and an ammonium transporter OsAMT1; 3, respectively, were all repressed by LP, LK and LPK. The expression of a number of genes associated with P and K transport and homeostasis were also altered by diverse low nutrient stresses; many of them were induced by LP, LK and LPK (Fig. 4; Table S12). It is notable that a group of genes associated with P and K transport and homeostasis were induced by N starvation in shoots but were repressed by N starvation in roots (Fig. 4). In addition, many genes associated with metabolisms of hormones like auxin, gibberellin, cytokinin, brassinosteroid, and polyamine were altered by diverse low N/P/K stresses; most of these genes showed similar expression patterns in roots and shoots (Fig. S6; Table S13). It is interesting that numerous genes that are potentially involved in jasmonic acid (JA)-mediated signaling pathway, salicylic acid-mediated signaling pathway, and plant responses to defense hormones like salicylic acid (SA) and JA were differentially expressed under various low nutrient stresses (Fig. S7; Table S14).

3.4. Common DEGs under single and multiple N, P, and K starvation

A total of 66 and 174 common DEGs was shared by single and multiple N, P and K starvation in roots and shoots, respectively (Figs. S8A and B; Tables S15–16). Most of these genes exhibited similar expression pattern under different low nutrient stresses in roots and shoots (Figs. S8E and F), suggesting they were commonly regulated under N, P, and K starvations. Three genes (LOC_Os01g41240, LOC_Os02g52040 (*OsPHI-1*), and LOC_Os12g12390) were repressed by all the single and multiple low nutrient stresses in both roots and shoots (Tables S15–16). Whereas some genes showed distinct expression patterns. For example, ammonium transporter gene *OsAMT1;3* (LOC_Os02g40710) and urea active transporter gene *OsDUR3* (LOC_Os10g42960) were repressed by LP, LK, and LPK, but were induced by LN, LNP, LNK, and LNPK in roots (Fig. S8E; Table S15). By GO and KEGG enrichment analyses, the common DEGs under different low nutrient stresses were found to be functionally enriched in a series of biological processes, such as carbohydrate metabolism, cell wall organization and plant hormone signal transduction (Tables S22–23). In addition, 139 and 201 DEGs were commonly responsive to single LN, LP, and LK stress in roots and shoots, respectively (Fig. S11; Tables S17–18). Nearly all these common DEGs showed similar expression patterns, but a group of common DEGs exhibited distinct expression patterns, especially for the DEGs of roots under LK (Fig. S11). For multiple nutrient stress (i.e., LNP, LNK, LPK, and LNPK), 530 and 1111 genes were commonly differentially expressed in roots and shoots, respectively (Fig. S12; Tables S19–21). Among them, 72 DEGs were commonly found in both roots and shoots, and a proportion of them showed differential expression patterns in roots and shoots (Fig. S12). For example, ethylene responsive transcription factor *OsERF922* (LOC_Os01g54890) and *LOC_Os04g48290* (mate efflux family protein) were up-regulated in shoots but were repressed in roots; 19 genes including two peroxidase precursor related genes (LOC_Os07g48060 and LOC_Os06g16350) and three LTP family protein precursor related genes (*LTPL71* (LOC_Os03g46180), *LTPL5* (LOC_Os06g34840), and *LTPL72* (LOC_Os03g46150)) were up-regulated in roots but were repressed in shoots (Fig. S13B; Table S21).

3.5. Unique DEGs under single and multiple N, P, and K starvation

A number of DEGs (e.g., 77 in LP shoot, 540 in LN root, 878 in LNP root) was unique to each of the low N/P/K stress (Table S24; Figs. S8A and B). The percentage of DEGs that are unique to each nutrient stress ranged from 6.6% in LNPK shoot to 23.5% in LNP root (Figs. S8C and D). It is notable that the proportion of unique DEGs in LNP root was much higher than that of LN or LP, suggesting that the combined LNP stress may have a unique effect on transcriptome reprogramming. The proportion of unique DEGs in LPK shoot was also higher than that of LP and LK, suggesting the combined LPK stress may have a specific effect on global gene expression. We also compared the unique DEGs under single and multiple N/P/K starvations with previous reported unique DEGs in Arabidopsis roots (Kellermeier et al., 2014), by using the best-hit Arabidopsis homologous genes of unique DEGs in rice. The best-hit homologous genes of four to seven rice unique genes were found to be unique to LNP, LNK, or LNPK stress in Arabidopsis (Fig. S9), suggesting their similar and specific responses to these combined nutrient stresses. But for LN, LP, LK, and LPK treatments, there was no common unique DEGs between rice and Arabidopsis (Fig. S9). The few number of common unique genes between rice and Arabidopsis may result from that the number of unique genes in Arabidopsis was very low, that only the best-hit homologous gene was used for comparison, and that the different growth conditions were used for rice and Arabidopsis, i.e., hydroponic culture in rice and agar-plate culture in Arabidopsis.

GO enrichment analysis showed that a group of GO terms were enriched for the unique DEGs under each low nutrient stress (Table S25). For example, regulation of phosphate transport (GO:0010966), and terpenoid catabolic process (GO:0016115) were significantly enriched in the unique DEGs of LN root; oxidation-reduction process (GO:0055114), lipid biosynthetic process (GO:0008610) and iron ion binding (GO:0005506) were significantly enriched in the unique DEGs of LP root; protein phosphorylation (GO:0006468), phosphorus metabolic process (GO:0006793), and defense response (GO:0006952) were significantly enriched in the unique DEGs of LNP root (Table S25). KEGG enrichment analysis showed that a group of metabolic pathways were significantly enriched for the unique DEGs under individual or combined nutrient starvation (Table S26). For example, glycerophospholipid metabolism (map00564) and alpha-Linolenic acid metabolism (map00592) were enriched in LP shoot; tryptophan metabolism (map00380) and limonene and pinene degradation (map00903) were enriched in LK shoot; while beta-Alanine metabolism (map00410) and phenylpropanoid biosynthesis (map00940) were enriched in LPK shoot (Table S26).

3.6. Core DEGs shared under individual and combined N, P and K starvation

Considerable overlap was found between diverse single and/or multiple low nutrient stresses (Fig. 5A and B). For example, around 60% of LP-responsive genes were also responsive to LN, LNP, LNK, and LNPK in roots and shoots, and more than 56% of LK-responsive genes were also responsive to LN, LNP, LNK, LPK, and LNPK in shoots (Fig. 5C and D). As

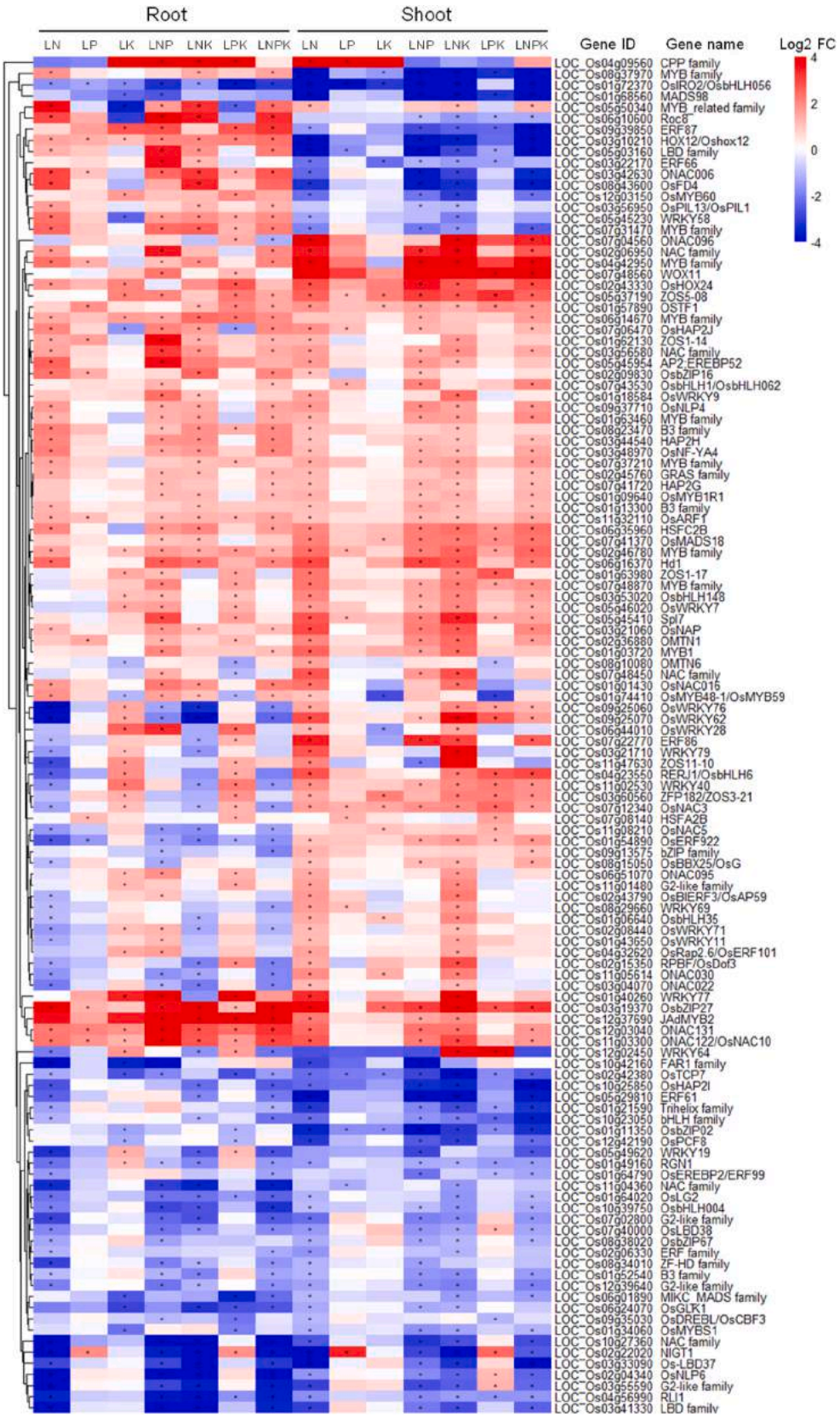

Fig. 3. Expression patterns of a group of transcription factor genes in roots and shoots of rice seedlings under single and multiple N, P, and K starvation. Here, only the genes differentially expressed in both roots and shoots, and responsive to at least two kinds of nutrient stress were shown. The asterisks indicate significant changes (an absolute fold change $\geq$2 and P-value $\leq$0.05).

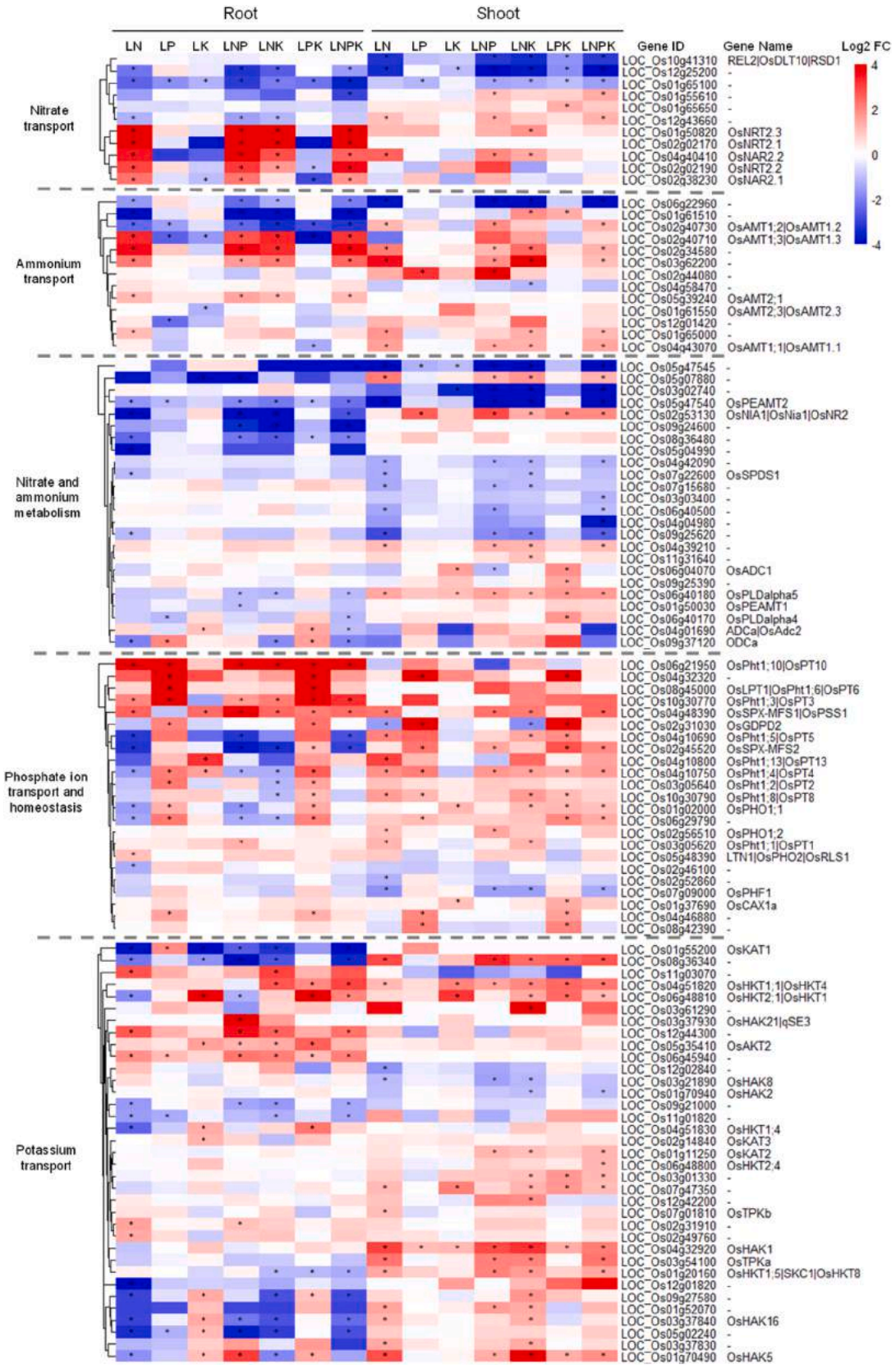

Fig. 4. Expression patterns of DEGs associated with N, P, and K transport and/or metabolism in roots and shoots of rice seedlings under single and multiple N, P, and K starvation. The asterisks indicate significantly differentially expressed miRNAs with an absolute fold change $\geq$2 and P-value $\leq$0.05. Red color indicates induction, and blue color indicates repression.

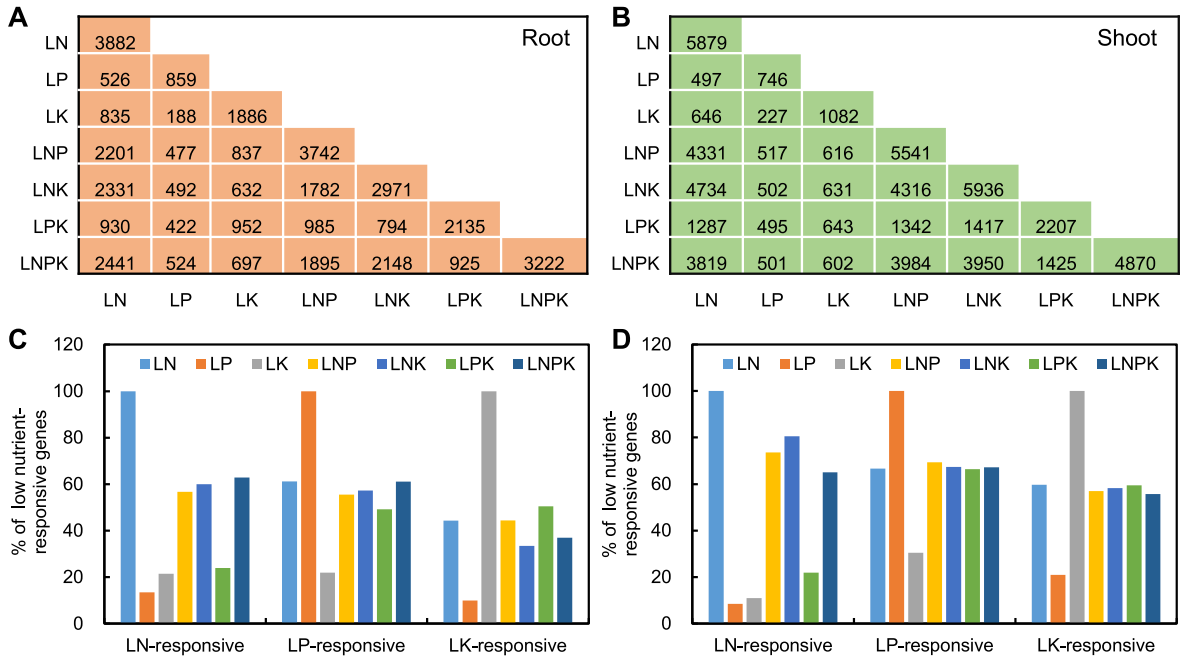

Fig. 5. Overview of the overlapped DEGs shared by individual and combined nutrient stresses. (A, B) Number of overlapped DEGs in roots (A) and shoots (B) of rice seedlings under individual and combined nutrient starvation. (C, D) Representation of the percentages of DEGs under individual nutrient stress (LN, LP, and LK) that are shared by other individual or combined nutrient stresses in rice roots (C) and shoots (D).

shown in Fig. S10A and B, 120 and 195genes were found to be commonly differentially expressed under single LN, LP, LK, and combined LNPK stresses in roots and shoots, respectively (Table S27). GO and KEGG enrichment analysis showed that a group of GO terms including trehalose biosynthetic process (GO:0005992), cell wall organization (GO:0071555) and iron ion homeostasis (GO:0055072), and a group of KEGG terms including carbon fixation in photosynthetic organisms (map00710) and plant hormone signal transduction (map04075) were significantly enriched in the common DEGs under diverse low N/P/K stress in roots or shoots (Tables S28–29).

Most of the common DEGs showed similar expression patterns in shoots under individual and combined N/P/K starvation, but nearly half of the common DEGs showed differential expression patterns in LK roots (Figs. S10C and D). Twenty-three genes that were up-regulated by LK were repressed by LN, LP and LNPK in roots, including two aminotransferase genes (LOC_Os03g08530 and LOC_Os05g15530) (Table S27). By clustering of the common DEGs under LN, LP, LK and LNPK stresses, 7 and 8 clusters were found in shoots and roots, respectively (Figs. S14–15; Tables S30–31). DEGs within the same cluster showed similar expression patterns. The overrepresented GO terms of biological processes were distinct in each cluster. For example, regulation of transcription and biosynthetic process were enriched in cluster 2 (C2) of roots, where all DEGs were up-regulated; single-organism process and polysaccharide metabolic process were enriched in the C4 cluster of roots, where all DEGs were down-regulated under diverse low nutrient stresses (4–15).

3.7. Core DEGs shared under individual and combined N and P starvation

As shown in Fig. 6A and B, a large number of DEGs were shared between LN, LP, and LNP stress in roots or shoots. About 60% and 80% of DEGs under LNP were shared with LN and/or LP in roots and shoots, respectively. A total of 406 and 448 genes were commonly differentially expressed under LN, LP, and LNP stresses in roots and shoots, respectively; twenty-eight of them were shared by roots and shoots (Table S32). Expression patterns of the common 28 genes were largely similar in roots and shoots (Fig. S16). Three of them were potentially involved in iron homeostasis, including *OsIRO2* (LOC_Os01g72370), *OsNRAMP1* (LOC_Os07g15460), and *OsVIT2* (LOC_Os09g23300), suggesting the interaction between iron homeostasis and LN/LP responses. GO and KEGG enrichment analyses in the common DEGs shared by LN/LP/LNP showed that many terms were overrepresented, including cell wall organization, lipid biosynthetic process, and photosynthesis in the common DEGs of shoots, and ion homeostasis, monocarboxylic acid metabolic process and flavonoid biosynthetic process in common DEGs of roots (Table S33).

The common DEGs exhibited similar expression patterns under individual and combined LN/LP/LNP stresses in roots and shoots (Fig. 6C and D). Only a small set of genes showed distinct expression patterns. For example, four genes induced by LP were repressed by LN and LNP in both roots and shoots, including *NIGT1* (LOC_Os02g22020) encoding a MYB transcription factor (Fig. S16). The common DEGs of LN, LP, and LNP were classified into 8 and 10 clusters in shoots and roots, respectively, according to their expression directions (Fig. 6E, Fig. S17; Tables S35–36). In each cluster, the DEGs were enriched in different biological functions. For example, in the C2 cluster of shoots, 8 genes potentially involved in photosynthesis were up-regulated by LN and LP, and their expression levels under combined LNP were much higher than that under individual LN or LP, suggesting an additive effect of LN and LP on their expression. In the C5 cluster of shoots, 10 genes potentially involved in cell wall organization or biogenesis were down-regulated by LN, LP, and LNP. In the C1 cluster of roots, 7 genes potentially involved in secondary metabolic process were all down-regulated, and three of them (LOC_Os01g63540, LOC_Os02g32770, and LOC_Os10g08319) encode cytochrome P450 (Fig. S17; Table S36). In the C4 cluster of roots, three genes involved in phosphate transport (*OsPHO1;1*, *OsPHO1;3*, *OsPht1;4*) were up-regulated by LP, but repressed by LN and LNP.

3.8. Core DEGs shared under individual and combined N and K starvation

A large proportion of DEGs was shared between LN, LK and LNK (Fig. 7A and B). Around 60% and 81% of LN-responsive genes were found to be responsive to LNK in roots and shoots, respectively. Around

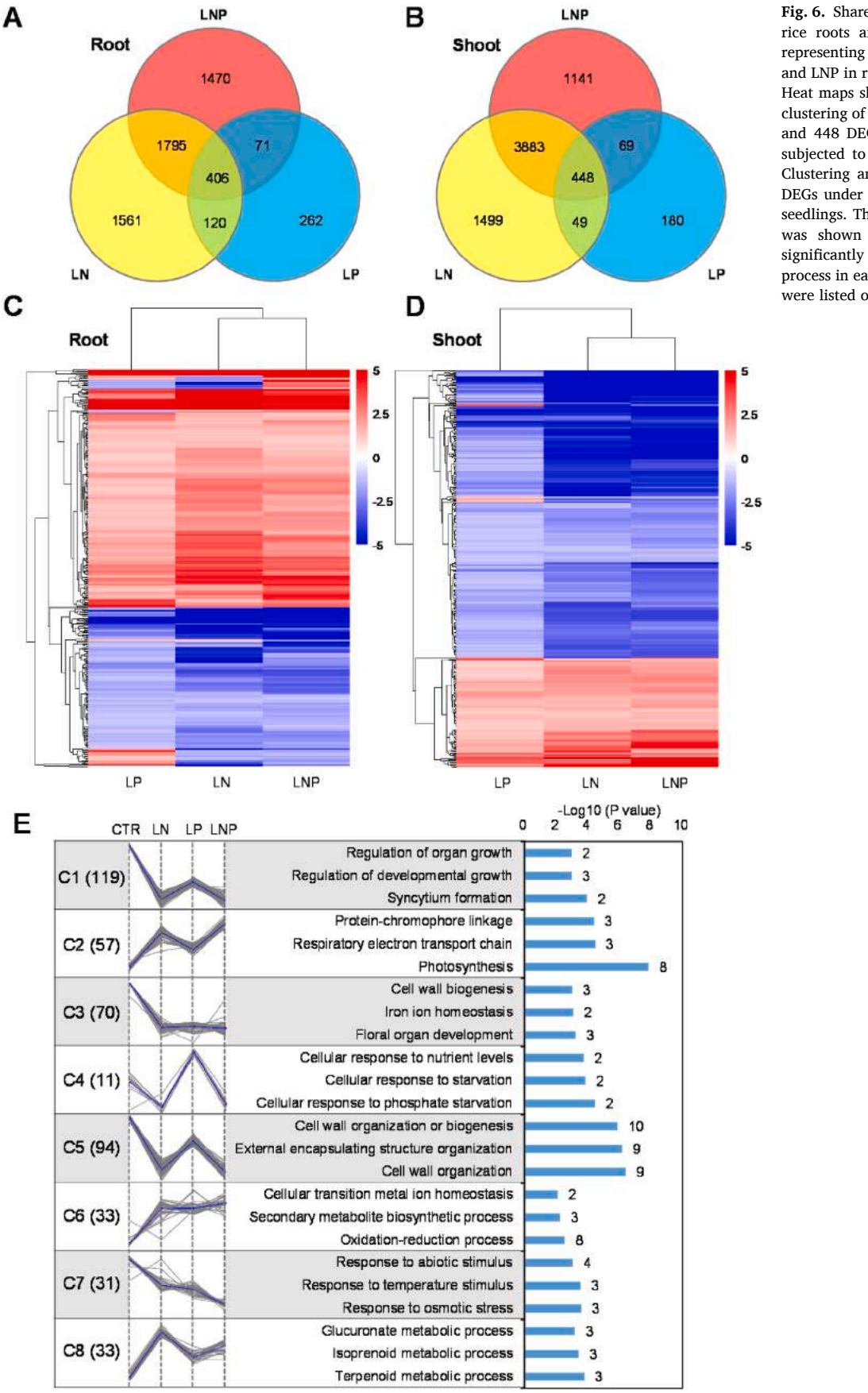

Fig. 6. Shared DEGs between LN, LP and LNP in rice roots and shoots. (A, B) Venn diagram representing the overlap DEGs under LN, LP, and LNP in rice roots (A) and shoots (B). (C, D) Heat maps showing the expression pattern and clustering of the common 406 DEGs in roots (C) and 448 DEGs in shoots (D) of rice seedlings subjected to LN, LP, and LNP treatments. (E) Clustering and expression profiles of common DEGs under LN, LP, and LNP in shoots of rice seedlings. The number of DEGs in each cluster was shown in the brackets. The top three significantly enriched GO terms of biological process in each cluster and the number of DEGs were listed on the right of the panel.

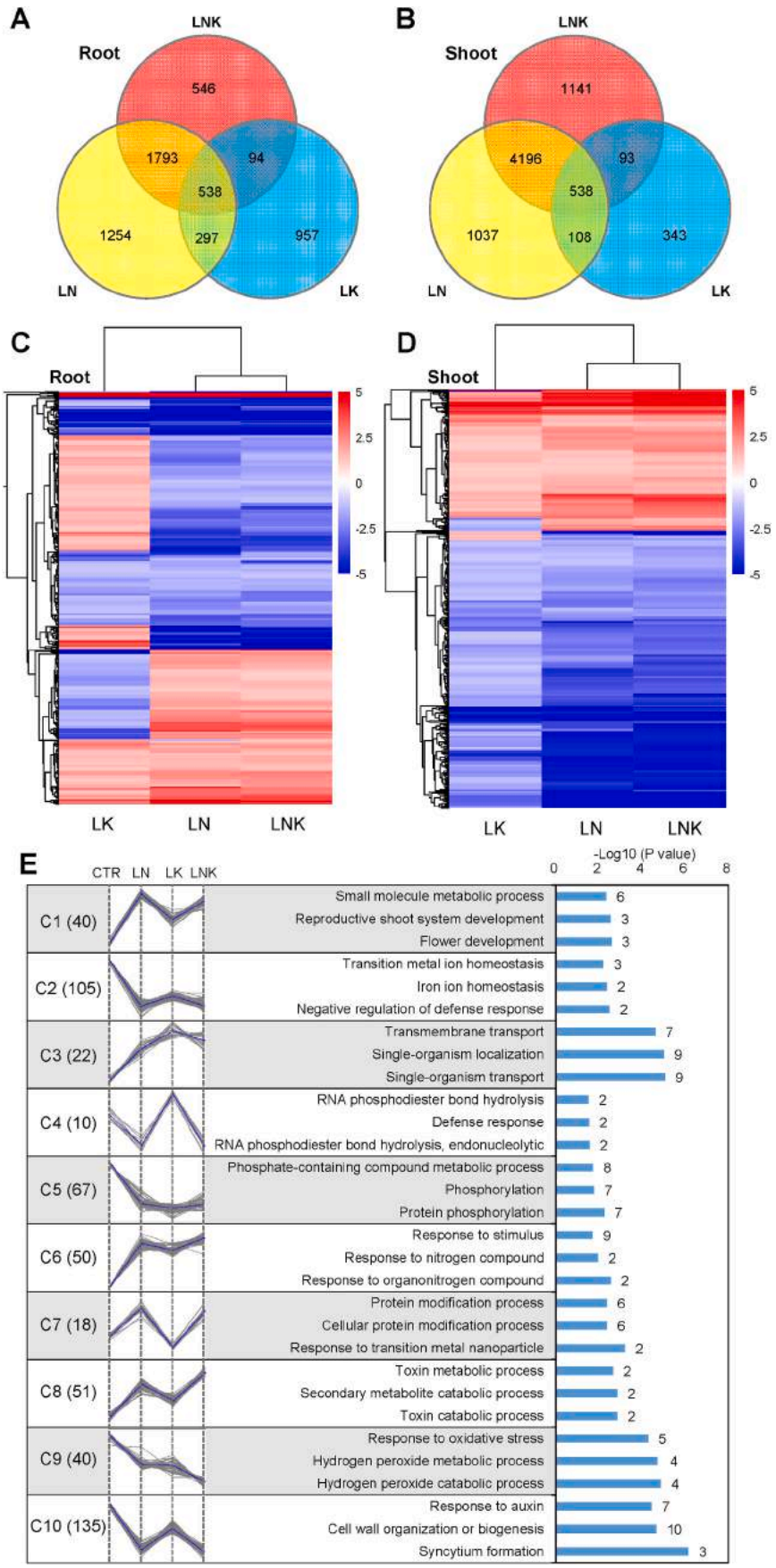

Fig. 7. Shared DEGs between LN, LK and LNK in rice roots and shoots. (A, B) Venn diagram representing the overlap DEGs under LN, LK, and LNK in rice roots (A) and shoots (B). (C, D) Heat maps showing the expression pattern and clustering of the common 538 DEGs in roots (C) and 538 DEGs in shoots (D) of rice seedlings subjected to LN, LK and LNK treatments. (E) Clustering and expression profiles of common DEGs under LN, LK, and LNK in shoots of rice seedlings. The number of DEGs in each cluster was shown in the brackets. The top three significantly enriched GO terms of biological process in each cluster and the number of DEGs were listed on the right of the panel.

44% and 34% of LK-responsive genes were also responsive to LN and LNK in roots and shoots, respectively. The number of DEGs that were commonly responsive to LN, LK, and LNK was 538 in both roots and shoots (Fig. 7A and B; Table S37). Twenty-eight genes were commonly responsive to LN/LK/LNK in both roots and shoots, including *OsCML15* (LOC_Os05g31620), a calmodulin-like protein-coding gene commonly induced by LN, LK, and LNK, and *OsIRO2* (LOC_Os01g72370), a bHLH transcription factor gene commonly repressed by LN, LK, and LNK (Fig. S18). By GO and KEGG enrichment analyses, a set of terms like organic acid metabolic process, phenylpropanoid metabolic process, chitin metabolic process, and defense response were enriched in the common DEGs of roots, and several terms like cell wall organization or biogenesis, ROS metabolic process, and iron ion homeostasis were enriched in the common DEGs of shoots (Tables S38–39).

The common DEGs showed a very similar expression pattern under LN and LNK in both roots and shoots (Fig. 7C and D). The expression pattern of common DEGs under LK was also similar to that under LN and LNK in shoots, but it was different from that under LN and LNK in roots. About half of the common DEGs under LK showed opposite expression compared with that under LN and LNK in roots (Fig. 7C and D; Table S37). The common DEGs in roots and shoots were both divided into 10 clusters according to their expression responses (Fig. 7E and Fig. S19). In the C1 cluster of roots, 116 genes were down-regulated under LK but were up-regulated under LN and LNK; genes encoding N transporters (e.g., OsNRT2.4 and OsAMT1; 3), transcription factors (e.g., OsMADS55, OsNF-YA5, OsMYB48, OsWRKY23, and OsWRKY58), and other proteins (e.g., OsSAUR11, and OsPrx30) were included in this cluster (Fig. S19; Table S40). In the C2 cluster of roots, 89 genes were up-regulated under LK but were down-regulated under LN and LNK; these genes included transporter genes like *OsHAK16* (LOC_Os03g37840), *OsHAK17* (LOC_Os09g27580), and *OsABCC9* (LOC_Os04g13210), and transcription factor genes like *OsRGN1* (LOC_Os01g49160), *OsWRKY19* (LOC_Os05g49620), *OsWRKY62* (LOC_Os09g25070), and *OsWRKY76* (LOC_Os09g25060) (Fig. S19; Table S40). In the C3 cluster of roots, 64 genes were gradually repressed by LN, LK, and LNK, including two K transporter genes *OsKAT1* (LOC_Os01g55200) and *OsHAK4* (LOC_Os08g36340) (Fig. S19; Table S40). Around 65% of the common DEGs were all repressed by LN, LK, and LNK in shoots, and these genes were classified into four clusters, i.e., C2, C5, C9 and C10. Some genes potentially involved in iron homeostasis, protein phosphorylation, response to oxidative stress, and cell wall organization were enriched in these clusters (Fig. 7E), suggesting a group of biological processes is shared in plant responses to LN and LK. On the other hand, genes potentially involved in flower development, single-organism transport, and response to stimulus were enriched in cluster C1, C3, and C6, respectively; in these clusters, genes were all induced by LN, LK, and LNK (Fig. 7E). It is interesting that genes potentially involved in flowering regulation, such as *Hd3a/FT* (LOC_Os06g06320), *RFT1/FT-L3* (LOC_Os06g06300), and *OsMADS18* (LOC_Os07g41370), were all induced by LN, LK and LNK in shoots (Table S41).

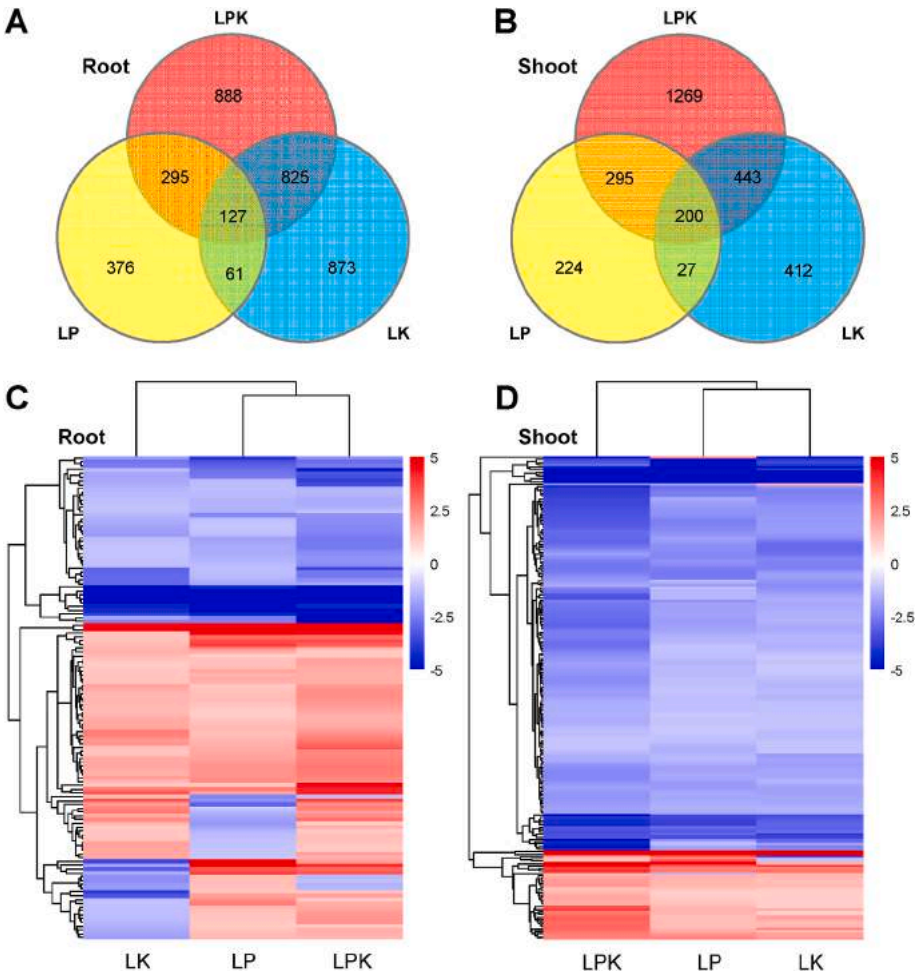

Fig. 8. Shared DEGs between LP, LK and LPK in rice roots and shoots. (A, B) Venn diagram representing the overlap DEGs under LP, LK, and LPK in rice roots (A) and shoots (B). (C, D) Heat maps showing the expression pattern and clustering of the common 127 DEGs in roots (C) and 200 DEGs in shoots (D) of rice seedlings subjected to LP, LK and LPK treatments.

3.9. Core DEGs shared under individual and combined P and K starvation

The number of DEGs that were commonly responsive to LP, LK, and LPK was 127 in roots and 200 in shoots (Fig. 8A and B; Table S42). Four genes including *LOC_Os02g52040* (*OsPHI-1*), *LOC_Os04g45520*, *LOC_Os01g41240*, and *LOC_Os12g12390*, were commonly responsive to LP, LK, and LPK in both roots and shoots (Fig. S20). The proportion of overlapped DEGs between LP and LK was relatively small; only 22% and 30% of LP-responsive genes were found to be responsive to LK in roots and shoots, respectively. But the proportion of DEGs under individual LP and LK that was overlapped with DEGs under combined LPK reached to about 50% in roots and 60% in shoots (Fig. 5C and D; Fig. 8A and B), suggesting an accumulative effect of LP and LK under the combined LPK stress. It is notable that the proportion of DEGs that are unique to LPK was extremely high; 41.6% (888 genes) and 57.5% (1269 genes) of LPK-responsive genes were unique, respectively, meaning that they were not responsive to LP or LK in roots and shoots (Fig. 8A and B). GO and KEGG enrichment analyses showed that cell wall organization, regulation of developmental growth, and plant hormone signal transduction were significantly overrepresented in the common DEGs of shoots (Tables S43–44).

Most of the common DEGs showed similar expression patterns under LP, LK, and LPK, especially in shoots (Fig. 8C and D). In shoots, most of the common DEGs were repressed by LP, LK and LPK, including some genes involved in hormone metabolism and signaling, such as *OsGA2ox9* (LOC_Os02g41954), *GID1L2* (LOC_Os07g41590), *OsACS5* (LOC_Os01g09700), *OsACO7* (LOC_Os01g39860), *OsIAA14* (LOC_Os03g58350), and *OsSAUR8* (LOC_Os02g24700) (Tables S42–43). Forty-five genes were commonly up-regulated by LP, LK and LPK in roots, including *OsPht1;4* (LOC_Os04g10750), HAD superfamily phosphatase (LOC_Os01g09540), *OsNAC10* (LOC_Os11g03300), *OsCML15* (LOC_Os05g31620), peptide transporter *PTR2* (LOC_Os01g67630), trehalose phosphatase (LOC_Os06g11840), and lipoxygenase *OsLOX9* (LOC_Os08g39840) (Table S42). A portion of common DEGs showed differential responses under LP or LK in roots (Fig. 8C). For example, abscisic acid-stress-ripening-inducible protein *OsASR2* (LOC_Os04g34600), chalcone synthase (LOC_Os11g35930), and plastocyanin-like domain-containing protein (LOC_Os04g46130) were repressed by LK but were induced by LP and LPK; calmodulin binding protein (LOC_Os08g27170), *OsNADP-ME3* (LOC_Os05g09440), *OsJAZ8* (LOC_Os09g26780), and ent-kaurene synthase *OsDTS2* (LOC_Os04g10060) were down-regulated by LP but were up-regulated by LK and LPK (Table S42). The common DEGs were divided into 7 clusters in shoots and 8 clusters in roots according to their expression responses (Figs. S21–22; Tables S45–46). In the C3 cluster of roots, where genes were gradually repressed, three genes potentially involved in N transport were overrepresented, including urea transporter *OsDUR3* (LOC_Os10g42960), peptide transporter (LOC_Os01g65100), and ammonium transporter *OsAMT1;3* (LOC_Os02g40710) (Fig. S21; Table S45). In the C1 cluster of shoots, 41 genes were gradually down-regulated by LP, LK and LPK, and four genes potentially involved in cell wall organization were overrepresented, including pectinesterase (LOC_Os02g54190), peroxidase (LOC_Os05g41990), and polygalacturonases (LOC_Os12g36810 and LOC_Os05g50960) (Fig. S22; Table S46).

3.10. Transcription factors potentially responsible for the regulation of common DEGs under individual and multiple nutrient starvations

To identify potential transcription factors (TFs) responsible for the low nutrient-responsive genes, we searched the plant regulatory data analysis platform PlantRegMap using the corresponding DEGs under single or multiple N/P/K stresses as a query. For DEGs in roots, 38 to 66 TFs were significantly overrepresented, and for the DEGs in shoots, 20 to 78 TFs were significantly overrepresented ($p < 0.05$) under diverse low nutrient stresses (Table S47). These TFs belong to a diverse range of families, such as WRKY, MYB, NAC, bZIP, CAMTA, bHLH, and G2-like. For the DEGs that were commonly responsive under all these single and multiple low nutrient stresses, 34 TFs and 17 TFs were significantly overrepresented in roots and shoots, respectively (Table S48). The top four overrepresented TFs for the 66 common DEGs of roots were all MYB family genes, while the most overrepresented TF for the 174 common DEGs in shoots was OsWUS/MOC3 (LOC_Os04g56780) encoding a homeobox domain containing protein (Table S48). For the other common DEGs (e.g., LN/LP/LNP, LN/LK/LNK, LP/LK/LPK, LN/LP/LK/LNPK), the number of TFs significantly overrepresented in roots (35–47) were also more than that in shoots (13–21) (Table S48). We then constructed coexpression regulatory networks of some most overrepresented TFs for the common DEGs under single and multiple low nutrient stresses in roots and shoots (Table S49; Fig. 9; Figs. S23–25). Of the 15 most overrepresented TFs of the common DEGs of LN, LP, and LNP in roots and shoots, OsGLK1 and three WRKY TFs, including OsWRKY23, OsWRKY67, and OsWRKY71 were included (Table S49; Fig. 9). It is notable that one of overrepresented TFs for the common DEGs of LN, LK, and LNK in both roots and shoots, LOC_Os02g46780 encoding a MYB TF, was induced by LN, LK and LNK in roots (Fig. S23).

3.11. Confirmation of DEGs in responses to diverse nutrient starvations

To confirm the RNA-seq results, we selected 21 genes that were responsive to single and/or multiple low N/P/K stresses and investigated their expression levels by qRT-PCR. The selected candidates included seven genes that are potentially involved in N transport (ammonium transporters and nitrate transporters), three genes that are possibly involved in Pi transport and signaling (*OsPHO1;3*, *OsPHT1;6*, and *OsSPX2*), two genes potentially involved in K transport (*OsHAK5* and *OsHKT2;1*), two genes potentially involved in iron homeostasis (*OsIRO2* and *OsNRAMP1*), four genes potentially involved in transcriptional regulation (*OsNAC10*, *OsNF-YB9*, *OsMYBR22* and *OsRLI1*), and three gene potentially involved in hormone biosynthesis and transport (*OsBISAMT1*, *OsCKX11*, and *OsPIN9*). The expression patterns of these genes were almost similar to the results of RNA-seq (Fig. S26). These results indicated that the RNA-seq data obtained in this study is reliable.

4. Discussion

N, P and K are the three most important macronutrients in plants. Deficiency of N, P and/or K in soil has a significant impact on plant growth and development and thus impairs crop yield and quality. Although much progress has been made in the physiological and molecular mechanisms associated with N, P, and K nutrition in plants, the interaction between N, P, and K is still at the emerging stage. In this study, the comparison of the morphological and physiological responses suggested the interaction between N, P, and K nutrient stresses in rice roots and shoots. The comparative transcriptome analyses indicated that the number of DEGs under multiple nutrient stress was not simply approximate to the summation of DEGs under single nutrient stress, suggesting the crosstalk between N, P, and K nutrients in rice plants. This study provided insights into the molecular mechanism underlying the crosstalk among these macronutrients in rice.

4.1. N is the predominant nutrient affecting the transcriptome under combined low nutrient starvations

Rice is a kind of NH_4^+-preferring grain crop (Zhu et al., 2009), and partial NO_3^- nutrition can improve N use efficiency and promote rice growth (Duan et al., 2007). Here, we used both NH_4^+ and NO_3^- as N resources, which is consistent with other studies of N nutrition in rice (Shin et al., 2018; Yang, W. et al., 2015b). Based on the comparisons of plant growth and transcriptome profiles under seven diverse single and multiple low nutrient conditions (LN, LP, LK, LNP, LNK, LPK, LNPK), LN (1/20 N) was observed to have the greatest effect on rice growth and

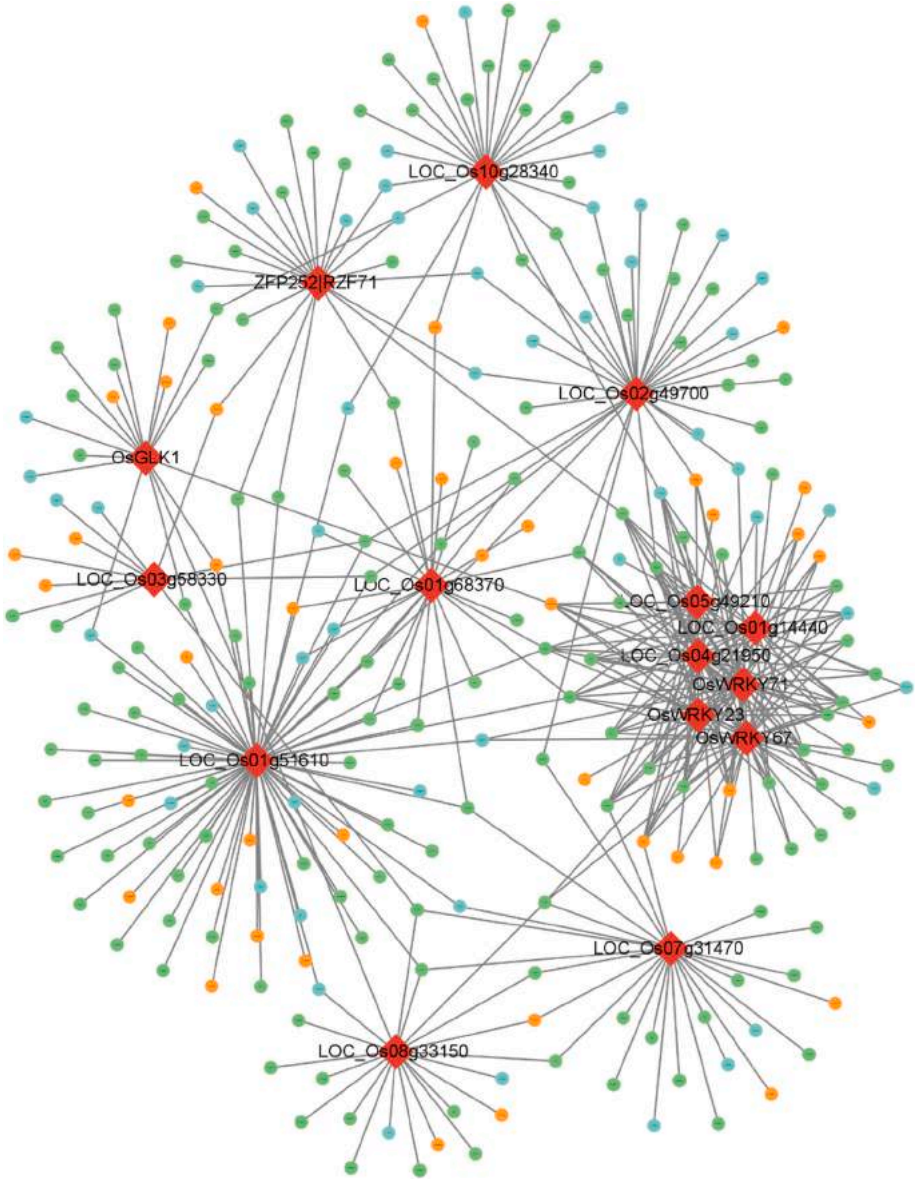

Fig. 9. Predicted transcription factors that potentially regulate the common DEGs under LN, LP and LNP conditions in rice roots and shoots. The red square represents enriched transcription factors. Orange circle represents up-regulated genes, blue circle represents down-regulated genes, and green circles represents genes up- or down-regulated under LN, LP, and LNP conditions.

transcriptome response, which was even greater than LNP, LNK, and LNPK combined stresses. These results suggest that individual low N/P/K stresses induce stronger molecular responses than combined LNP, LNK, and LNPK stresses. LN affected the expression of 3882 and 5879 genes in roots and shoots, accounting for 52% and 66% of the total DEGs under diverse low nutrient stresses in roots and shoots, respectively (Fig. 5A and B). While DEGs under LK (1/20 K) accounted for 25% and 13% of the total DEGs in roots and shoots, respectively; LP (1/20) accounted for 11% and 8% of the total DEGs in roots and shoots, respectively (Fig. 5A and B). Around 76% and 78% of DEGs under the combined LNPK stress were shared by LN in roots and shoots, respectively; whereas the percentage shared by LP or LK was only 10%–22% (Fig. 5A and B; Figs. S10A and B). Under the combined LNP and LNK stresses, around 60%–80% of DEGs were also shared by LN in roots and shoots (Fig. 5A and B; Fig. 8A and B; Fig. 7A and B). Therefore, it is obvious that N is the predominant nutrient to affect the transcriptome under the combined low nutrient stresses, such as LNK, LNP, and LNPK. This is consistent with previous studies showing that LN has the greatest impact on plant growth and development, which is followed by LP and LK in rice and other plants (Ma et al., 2020, 2022; Nezamivand-Chegini et al., 2023).

4.2. Genes associated with N uptake and utilization are influenced by P and K starvations

It has been known that the effects of multiple nutrient deficiencies are not necessarily additive, and that deficiency of one nutrient can impact other nutrients (Guo et al., 2021; Kellermeier et al., 2014; Medici et al., 2019). In agricultural practices, the co-application of N, P, and K fertilizers can improve nitrogen use efficiency (NUE) and grain yield in rice (Du et al., 2022; Duncan et al., 2018), suggesting the promotion of N utilization by P and K. In this study, the N concentration in rice roots were found to be suppressed by LP and LPK treatments (Fig. S2), suggesting these treatments may reduce the uptake of N. GO enrichment analyses also showed that GO terms associated with N transport and assimilation (e.g., ammonium transmembrane transport and

organonitrogen compound catabolic process) were enriched in DEGs of roots under LP, LK and/or LPK (Table S8). *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3*, which are three NH_4^+ transporter genes belonging to AMT1 family (Sonoda et al., 2003), were found to be repressed by LP, LK, and/or LPK in roots or shoots (Fig. 4), suggesting that NH_4^+ uptake is suppressed in rice roots under LP, LK, and LPK. The expression of *OsNAR2.1*, which encodes a partner protein of NO_3^- transporters, was induced by LN but repressed by LK and LPK in rice roots (Fig. 4). Knockdown of *OsNAR2.1* suppresses both high- and low-affinity NO_3^- transportations by repressing the expression of NO_3^- transporter genes like *OsNRT2.1*, *OsNRT2.2* and *OsNRT2.3a* (Yan et al., 2011). *OsNRT2.2* encoding a high-affinity NO_3^- transporter (Zhu et al., 2022), was also suppressed by LPK stress (Fig. 4). Thus, the uptakes of both NH_4^+ and NO_3^- may be repressed under LP, LK and/or LPK stresses by reducing the expression of NH_4^+ and/or NO_3^- transporter genes in rice roots. In Arabidopsis, the expression of *AtNRT1.5*, a gene encoding NO_3^- transporter responsible for NO_3^- loading into the xylem, and thus facilitates root-to-shoot NO_3^- transportation, was also found to be induced by K starvation (Lin et al., 2008). In addition, *OsDUR3*, encoding a high-affinity urea transporter (Wang et al., 2012), was induced by LN but repressed by LP, LK, and LPK in rice roots, suggesting the possible suppression of urea uptake under LP and LK.

OsNR2, a gene encoding a NADH/NADPH dependent NO_3^- reductase (Gao et al., 2019), was found to be increased by LP and LPK stresses in rice shoots, although it was repressed by LN in roots; these results suggest that the assimilation of NO_3^- could be enhanced to promote the efficient utilization of N under LP/LK conditions in rice shoots. Recently, it has been suggested that internal NO_3^- utilization positively modulates P uptake and utilization and is linked with P use efficiency (Ueda and Wissuwa, 2022). Under LP and LK conditions, the requirement of N should be decreased because the photosynthesis and growth is reduced in plants. The possible decrease of N uptake could be an adaptive strategy under low P/K stresses by cutting down the unnecessary consumption of energy associated with N transport and metabolism and avoiding the over-accumulation of NH_4^+, which is toxic to plants. This could also be supported by previous reports that the N and P concentrations in plant shoots are closely related (Ziadi et al., 2008), and that there are close relationship between K supply and N metabolism in plants (Armengaud et al., 2009). It has also been demonstrated that K deficiency symptoms in plant leaves under the application of high amount N fertilizer are more severe than that under the application of low amount N fertilizer (Xie et al., 2020).

4.3. P and K starvation responses are affected by N availability

It is notable that a large proportion of the LP-responsive genes were influenced by LN in roots and shoots (Fig. 5C and D). Around 47% and 60% of LP-responsive genes were commonly responsive to both LN and LNP in roots and shoots, respectively (Fig. 6). Therefore, it is reasonable to speculate that N availability extensively affects plant transcriptomic responses to LP in both roots and shoots. The interaction between N and P has been revealed at the physiological and molecular levels in plants (Hu et al., 2019; Nezamivand-Chegini et al., 2023; Wang et al., 2020). A group of genes induced by LP have been demonstrated to be repressed by N limitation in plants such as Arabidopsis and maize (Medici et al., 2019; Torres-Rodríguez et al., 2021). In this study, we also found a set of genes that showed different responses to LP and LN/LNP in rice roots or shoots (Fig. 4). For example, *OsPHO1;1*, *OsPHO1;3*, and *OsPHT1;4* that are potentially involved in Pi transport and translocation (Secco et al., 2010; Ye et al., 2015), were induced by LP but repressed by LN and LNP in rice roots (Fig. 4; Fig. S17; Table S36), suggest that N starvation could repress part of the molecular responses under LP stress. Some important regulators involved in the N–P interaction have been identified, including AtNIGT1s in Arabidopsis and OsNRT1.1b in rice. Arabidopsis NIGT1 transcription factors can modulate the expression of NO_3^--responsive genes and Pi-starvation responsive genes, and their level is regulated by both Pi and NO_3^- signals at different levels under the controls of PHR1 and NLP7, respectively (Kiba et al., 2018; Maeda et al., 2018; Wang et al., 2020). In rice, NO_3^- transporter OsNRT1.1b interacts with Pi sensor OsSPX4 under NO_3^--sufficient conditions to promote the degradation of OsSPX4, and thus activates the activities of OsNLP3 and OsPHR2, which are central transcription factors in NO_3^- response and Pi starvation response, respectively (Hu et al., 2019). Here, *OsNIGT1* (LOC_Os02g22020) was found to be induced by LP and LPK, and was repressed by LN/LNP/LNK/LNPK, suggesting that it may have a role similar to AtNIGT1 in regulating the interaction of N and P nutrition. In addition, *OsRLI1*, which encodes a MYB-transcription factor closely related to OsPHR2 (Ruan et al., 2018), was found to be repressed by nutrient stresses with LN (including LN, LNP, LNK, and LNPK) in both roots and shoots. Recently, OsRLI1 has been shown to be induced by NO_3^- nutrition (Zhang et al., 2021), which is consistent with its repression under LN conditions. OsRLI1 is involved in NO_3^--induced Pi starvation response by positively regulating the expression of Pi starvation-induced genes and enhancing the role of OsPHR2 in Pi starvation response by competing with OsPHR2 for SPX proteins, which are repressors of OsPHR2 (Zhang et al., 2021). It is also interesting that the expression of *OsRIL1* was repressed under the combined LPK stress in rice roots and shoots, but whether the repression of *OsRLI1* is associated with the possible suppression of N uptake and accumulation in plants roots under LPK (Fig. S2A) deserve further investigation.

In this study, a large group of LK-responsive genes (44% in roots and 60% in shoots) were found to be responsive to LN in roots and shoots (Fig. 5C and D). About 29% and 50% LK-responsive genes were also commonly responsive to both LN and LNK in roots and shoots, respectively (Fig. 7). These results suggest a close relationship between K and N nutrition and an impact of N availability on LK response. A large group of genes showed different expression pattern under LK and LN/LNK. For example, K^+ transporter *OsHAK5*, *OsHAK16* and *OsHAK17* that function in K^+ uptake and translocation (Feng et al., 2019; Yang et al., 2014), were induce by LK but repressed by LN and LNK in rice roots. *OsNRT2.4* and *OsAMT1;*3 that are involved in NO_3^- and NH_4^+ uptake respectively (Bao et al., 2015; Wei et al., 2018), were repressed by LK but induced by LN and LNK in rice roots, suggesting that they play differential roles in LK and LN responses. These results also suggest the possible antagonistic effect of LN on LK responses in plants. The antagonistic interaction between K^+ and NH_4^+, which are highly similar with respect to charge, size, and hydration energy, has been documented (Coskun et al., 2017). It has been shown that the induction of *AtHAK5* by K^+ starvation is repressed by the presence of low concentration of NH_4^+ in Arabidopsis roots (Rubio et al., 2008), and that removal of NH_4^+ from nutrient solutions increases K^+ uptake in roots of barley and Arabidopsis plants (Coskun et al., 2013). In rice plants, which are tolerant to excess NH_4^+, the effect of NH_4^+ on K^+ uptake is complicated; NH_4^+ inhibits high-affinity K^+ uptake but activates low-affinity K^+ uptake (Szczerba et al., 2008). In addition, many other genes (65% of the common DEGs) showed similar expression patterns under LN, LK, and LNK (Fig. 7). The combined LNK stress even exerted a cumulative effect on the expression of a group of genes in rice roots or shoots (Fig. 7E; Fig. S19). These results suggest the existence of synergistic interactions between N and K nutrition. *OsHAK1*, which is involved in K^+ acquisition and translocation (Chen et al., 2015), was commonly induced by LK, LN, and LNK, and its induction was highest under LNK in rice shoots (Table S41). It has been known that NO_3^- is transported from roots to shoots in the xylem with K^+ acting as a counter-ion (Coskun et al., 2017), and the modulation of transporters that load NO_3^- into the xylem can affect the translocation of K^+ from root to shoot (Lin et al., 2008; Xia et al., 2015). In agricultural practices, the combined use of N and K fertilizers has a synergistic effect on the promotion of grain yield as well as NUE in rice production (Hou et al., 2019).

4.4. Interaction between P and K nutrition

The P concentrations in roots and shoots under LK were significantly increased when compared with control (Fig. S2), suggesting the potential interaction between P and K nutrition. A proportion of LP-responsive genes (about 22% in roots and 30% in shoots) were found to be responsive to LK (Fig. 5C and D). Most of the common DEGs showed similar expression patterns under LP, LK and LPK in rice roots and shoots (Fig. 8C and D). These results also suggest the interaction between LP response and LK response. The expression profiling in roots and shoots under LPK was more similar to that under LK than that under LP (Fig. 2C and D), suggesting that K may be the predominant limiting factor affecting the transcriptome under combined LPK stress. There was a high proportion of DEGs that are exclusively responsive to LPK, but not to LP or LK (Fig. 8), suggesting that responses under the combined LPK is not the simple sum of LP and LK. The combined LPK may have a more severe effect on plant growth compared with the individual LP and LK. It has been revealed that there is a synergistic interaction between P and K in plant photosynthesis (Yan et al., 2022). A recent study indicated that external high K^+ concentrations inhibit Pi uptake in Arabidopsis, which further induces Pi starvation response in a PHR1/PHL1-dependent manner (Ródenas et al., 2019). Some studies suggested that the shortage of nutrients, such as P, S, and N, reduce the acquisition of K by down-regulating genes associated with K^+ uptake and translocation (Rodenas et al., 2017). Here, some nutrient transporter genes showed similar expression pattern under LP, LK and LPK, including *OsPHT1;4* and *OsHAK1* that were commonly induced, and *OsAMT1;3* and *OsDUR3* that were commonly repressed under LP, LK, and LPK in roots or shoots (Table S42). A previous study of genome-wide expression profiling also revealed that a number of genes including P and K transporter genes are commonly up-regulated by P and K starvations (Wang et al., 2002). The upregulation of Pi transporter may associated with the higher P concentration in roots under LK conditions. But whether and how K starvation induces Pi uptake is still unclear, and further investigations are required to enlarge the understanding of the molecular mechanism underlying P and K interaction.

4.5. Reprogramming of plant defense responses under N, P, and K starvations

Plant hormones, especially JA and SA, are involved in plant defense responses (Bari and Jones, 2009). Here, a number of genes associated with JA and SA responses were significantly changed under low nutrient conditions (Fig. S7), suggesting that plant defense response is reprogrammed under nutrient starvation stresses. JA is well-known to positively regulate plant defense against insect herbivory and necrotrophic pathogens (Wang et al., 2019). It has been reported that wound- and volicitin-induced JA levels are increased in maize plants grown under LN when compared with plants grown under medium N (Schmelz et al., 2003). The resistance of barley leaves to aphid is significantly enhanced under N deficiency conditions (Comadira et al., 2015), but whether it is associated with the accumulation of JA is unclear. In this study, a number of genes potentially involved in JA response were induced in rice shoots under conditions with low N availability. But whether and how the biosynthesis of JA is increased by LN stress is unclear and deserves further investigations.

Here, several JAZ transcription factor genes like *OsJAZ8/10/12*/13 that are potentially involved in JA signaling were up-regulated by LN in rice shoots (Fig. S7). Recently, it has been reported that overexpression of *OsJAZ9* can promote NH_4^+ absorption, sugar and amino acid accumulation, and root growth under low N stress in rice (Sun et al., 2020), and that overexpression of *OsJAZ9* can improve K deficiency tolerance by modulating JA levels and responses (Singh et al., 2020). Genes encoding JA biosynthetic enzymes like lipoxygenase, allene oxide synthase, and allene oxide cyclase have been found to be strongly up-regulated by K deficiency and quickly repressed by K resupply in Arabidopsis (Armengaud et al., 2004). The biosynthesis of oxylipins (including jasmonates) and glucosinolates that are involved in herbivore defenses are increased by K deficiency in Arabidopsis (Troufflard et al., 2010). In addition, JA biosynthesis has been reported to be increased by P deficiency in plants like Arabidopsis and cotton (Khan et al., 2016; Luo et al., 2021). The resistance of cotton to pathogen *Verticillium dahliae* is enhanced under P deficiency by activating JA biosynthesis and phenylpropanoid pathway (Luo et al., 2021). Nutrient deficiencies can also alter SA levels and SA-related signaling (Conesa et al., 2020). It has been demonstrated that Pi starvation response and SA/JA-dependent plant immunity are coordinated with PHR1/PHL1 functioning as central regulators (Castrillo et al., 2017). In addition, genes associated with biosynthesis and signaling of other hormones that are also involved in plant defense responses were significantly affected under diverse low nutrient stresses (Fig. S6). But further studies are required to dissect molecular mechanisms and the biological significance underlying the linkage between hormone-mediated defense responses and nutrient starvation responses.

4.6. Potential transcription factors that simultaneously regulate N, P, and K starvation responses

Among the common DEGs of LN, LP, and LK in roots, six transcription factor genes (such as *OsNAC10*, *ONAC131*, *OsHOX12*, *OsIRO2*, *OsBBX6*, and *OsbHLH133*) were simultaneously up-regulated or down-regulated (Table S17; Fig. 3). Also, at least 16 transcription factor genes, such as *OsNAC3*, *OsTCP4*, *OsbZIP02*, *OsHOX28*, *OsMYB26*, *OsBUL1*, *OsbZIP06*, *OsbHLH173*, and *OsIRO2*, were simultaneously up-regulated or down-regulated in shoots (Table S18; Fig. 3). These results suggest that these transcription factors are potentially involved in the simultaneous regulation of N, P, and K primary nutrient starvation responses in rice plants. *OsIRO2*, which is a key component positively regulating iron acquisition (Ogo et al., 2007), was found to be commonly down-regulated by LN, LP, and LK in both roots and shoots, suggesting that reducing iron uptake and translocation may facilitate plant tolerance to N, P, and K starvations. *OsMYB26*, which has been identified to be a negative regulator of drought stress response (Chen, Y. et al., 2021b), was commonly down-regulated by LN, LP, and LK in rice shoots, suggesting that it may also negatively regulate rice responses to low N/P/K nutrient stress. OsHOX28 has been known to positively regulate tiller angle by controlling the local distribution of auxin (Hu et al., 2020). OsBUL1 has been found to positively regulate leaf angles based on the evidence that knockout mutant *osbul1* produced erect leaves, whereas *OsBUL1*-overexpression increased lamina inclination (Jang et al., 2017). OsbHLH73 has also been suggested to positively regulate flag leaf angle (Dong et al., 2018). Interestingly, *OsHOX28*, *OsBUL1*, and *OsbHLH73* were all repressed by LN, LP, and LK in rice shoots. It is well known that nutrient starvations such as LN and LP reduce tiller number and leaf angles (Liu et al., 2021; Ruan et al., 2018; Shindo et al., 2020). Further studies are required to investigate whether these transcription factors are involved in the regulations of tillering, leaf angle development and even nutrient utilization of rice plants under N/P/K starvations.

In addition, the common DEGs under single and multiple nutrient stresses in roots and shoots were used to predict potential transcription factors that regulate the expression of LN, LP and LK responsive genes simultaneously. Four MYB transcription factors (OsMYB30, OsMYB58, OsMYB60, and OsMYB108) and ZFP252 were included in the predicted transcription factors (Fig. S25; Table S49). Some of these transcription factors have also been documented to be involved in the regulation of stress responses. For example, ZFP252 positively regulates drought and salt tolerance in rice (Xu et al., 2008); OsMYB30 positively regulates defense against *Magnaporthe oryzae* by enhancing lignification in rice leaves (Li et al., 2020); OsMYB60 is involved in drought tolerance by positively regulating cuticular wax biosynthesis in rice leaves (Jian et al., 2022). However, whether and how these transcription factors

could regulate N, P, and K starvation responses in rice plants deserve further studies.

5. Conclusion

This study comprehensively analyzed the transcriptome in responses to single and multiple N, P and K starvation stresses in the first attempt. The number of DEGs under multiple nutrient stress was not the simple summation of DEGs under single nutrient stress, suggesting the crosstalk between N, P, and K nutrients. N, P, and K starvations triggered some common biological processes, such as plant defense responses. N was showed to be a predominant factor in the reprogramming of transcriptome under the combined nutrient stress. The comparative transcriptome also revealed the interactions of N and P, N and K, and P and K nutrition. It is conceivable that the availability of one nutrient could influence the uptake, translocation, storage, redistribution, metabolism, and signaling of other nutrient/nutrients. In addition, a group of transcription factors were commonly responsive to N, P, and K starvations, and a group of transcription factors were predicted to be upstream regulators of the common DEGs under diverse single and combined nutrient stress. This study would enlarge our understanding of N, P, and K crosstalk, and provide a platform for functional characterizations of some potential genes in N, P, and K interactions. Further characterization of potential regulators that commonly regulate N, P, and K starvation responses would facilitate the development of rice cultivars with simultaneously improved N, P, and K utilization efficiency and/or nutrient starvation tolerance.

Author contributions

Houqing Zeng conceived the study. Senhuan Dai, Haicheng Wu, Huiying Chen, Zihui Wang, Xin Yu, and Houqing Zeng performed the experiments. Senhuan Dai, Long Wang, Xianqing Jia, Cheng Qin, Yiyong Zhu, Keke Yi, and Houqing Zeng analyzed the data. Senhuan Dai, Yiyong Zhu, Keke Yi, and Houqing Zeng wrote the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2023.107642.

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