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Mapping of low-nitrogen tolerance genes in Japonica rice at seedling stage by genome-wide association study

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Nitrogen is an essential nutrient, which plays an important role in plant growth and development process and increases crop production. However, excessive nitrogen application will lead to a series of problems such as water eutrophication and economic costs. Therefore, it is of great significance to explore rice low-nitrogen tolerance genes and breed new varieties with higher nitrogen utilization efficiency for improving the economic benefits and agricultural sustainability of agricultural production. In this study, 295 *japonica* rice varieties were used as materials to measure root dry weight, leaf dry weight and root-shoot ratio at seedling stage under low and high nitrogen conditions. By using Genome-wide association analysis and haplotype analysis of 587 genes among the 47 QTLs obtained, we finally identified significant phenotypic differences between the different haplotypes of the 96 genes. Based on the criteria of $|\log_2 FC| > 1$ and $p < 0.05$, 5 genes (*Os06g0538400*, *Os11g0195500*, *Os11g0213700*, *Os11g0213800*, *Os12g0472800*) were significantly different in the expression of Longjing 31 (low-nitrogen tolerant variety), but not in Songjing 10 (low-nitrogen sensitive variety), and they were named the more valuable candidate genes for low-nitrogen tolerance. *Os11g0213700* and *Os11g0213800*, as genes containing LRR structure, may regulate root development and low nitrogen stress response by interacting with *KAI2*. Mining low-nitrogen tolerance genes in rice is of great significance to rice growth and agricultural development. The results of this study provide an important molecular basis for identifying low-nitrogen tolerance genes and breeding low-nitrogen tolerant rice varieties.

Keywords Rice, Nitrogen, GWAS, RNA-seq

Rice (*Oryza sativa L.*), serving as the main carbohydrate source for a majority of the earth's population, stands as a cornerstone of food security and a vital link in chain of socioeconomic development and ecological balance, embodying its global significance across multiple dimensions of human survival, agricultural sustainability, and cultural diversity[1,2]. Nitrogen (N) is the most important factor affecting plant growth. Because of the great demand for N in rice growth and development, N is often used to increase rice yield in the process of planting rice[3]. However, the relationship between rice yield and N application rate is not always proportional, and excessive or absent N fertilizer limits the increase of rice yield. Excessive N fertilizer will also reduce rice quality[4], increase field lodging[5], and even lead to poor environment[6]. Consequently, developing rice varieties with enhanced low-N tolerance has become a key breeding priority.

Dry matter distribution, a core processes of plant growth and development, directly affects growth efficiency and yield formation by regulating the allocation of photosynthetic products among roots, stems and leaves[7]. Root dry weight (RW), leaf dry weight (LW) and root-shoot ratio (RSR) are key indicators reflecting plant resource allocation strategies[8]. RSR, defined as the ratio of RW to LW, represents the balance of resource distribution between belowground and aboveground plant parts[9]. Studies have shown that a higher RSR is positively correlated with low-N tolerance, as increased resource allocation to roots enhances N uptake

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capacity[10]. Therefore, investigating the relationship between dry matter partitioning and low-N tolerance is of great significance for elucidating the mechanisms underlying rice adaptation to low-N environments and for breeding N-efficient rice varieties.

If it is expected to increase the yield by applying reasonable N fertilizer, we should mainly improve the tolerance of crops to N[11]. Low-N tolerant rice varieties can still maintain high yield under N reduction conditions[12]. In addition, people have conducted extensive research on low-N tolerance related indicators, and successfully identified a series of low-N tolerance genes in recent years. Zhang et al.[13] found that overexpression of *TOND1* can improve tolerance to low-N stress and yield of rice. Liu et al.[14] successfully identified *OsTCP19* as a negative regulatory gene for the response of tiller number to N in rice. The expression of *OsAMT1.2* was also found to be enhanced under low N condition[15]. In addition, *GRF4*[16], *OsDof25*[17] and *NRT1.1B*[18] were also identified as low-N tolerance genes. The study of these genes not only screened the genotypes of low-N tolerant rice varieties, but also helped to understand the response mechanism of rice to N fertilizer, thereby contributing to sustainable agricultural development[19].

In recent years, with the development of molecular biology and genomics technology, Genome-Wide Association Studies (GWAS), RNA-seq and QTL mapping[20,21] etc. are widely used in the process of gene identification. At present, GWAS has successfully identified genes related to rice disease resistance[22], photoperiod sensitivity[23], rice quality[24] and yield-related traits[25]. Studies on the tolerance of rice to abiotic stresses (such as low N, drought) have been extensively conducted[26,27]. Yu et al. successfully identify *OsNLP4* as a N efficient gene in rice by GWAS[28]. RNA-seq is often used to study the response mechanism of plant abiotic stress[29] and the regulation mechanism of plant metabolic pathways (such as photosynthesis)[30]. With the strategy of combining GWAS and RNA-seq, Li et al. successfully identified heat-resistant candidate genes in rice[29]. With the strategy of combining QTL mapping and RNA-seq, Yuan et al. successfully revealed *OsSTL1* and *OsSTL2* as two causal salt tolerance genes[31]. These studies provide a solid theoretical foundation and genetic resources for molecular improvement of low-N tolerance and yield improvement of rice. However, low-N tolerance is an extremely complex physiological process, and the study of its molecular mechanism still needs to be further explored. These gene discoveries provide a solid theoretical foundation for understanding the mechanisms of low-N tolerance in rice and offer robust theoretical support for yield improvement. Although significant progress has been made in identifying low-N tolerance genes, most studies have primarily focused on mature stages[19]. In contrast, *japonica* rice at the seedling stage remains relatively understudied. The seedling stage is particularly critical for root architecture establishment, as early root system development under low-N stress directly determines nutrient acquisition capacity and profoundly impacts subsequent yield potential[32]. This study aims to integrate RNA-seq and GWAS approaches to identify SNP significantly associated with low-N stress responses and screen candidate genes for N tolerance. The findings are expected to establish a crucial molecular foundation for breeding low-N tolerant rice varieties and provide molecular targets for improving N use efficiency.

Results

Phenotypic data analysis

To assess phenotypic changes under low and high N conditions, LW, RW and RSR at seedling stage of 295 rice varieties were measured (Table S2). Data indices such as mean and coefficient of variation of LW, RW and RSR are shown in Table S2. Under low and high N conditions, the coefficients of variation ranged from 17.41 to 25.77% and 20.2–28.14% respectively. The coefficients of variation of LW, RW and RSR under high N condition were higher than those under low N condition. Among the relative traits, the coefficient of variation of RWR was the highest and that of LWR was the lowest. The highest coefficient of variation for relative RWR was observed under both N conditions. Each trait exhibited had abundant genetic variation and approximate normal distribution (Figs. 1, S1). Since the low-N tolerance is a quantitative trait, the Pearson correlation analysis was performed on these phenotypic traits. The results showed there were extremely significant positive correlations between RW and LW, and between RW and RSR under high, low and relative N conditions (Fig. 2). The correlation coefficients were 0.644 and 0.649 under low N condition, 0.707 and 0.502 under high N condition, and 0.556 and 0.649 under relative condition (Table S3).

QTLs identification

Based on previous studies[33], all 295 rice varieties (Table S4) studied showed no significant population stratification. This population could be divided into three subpopulations with low genetic relatedness among individuals, minimizing potential confounding effects in subsequent GWAS analysis. Forty-seven significant leading SNPs were identified by GWAS (Fig. 3, Table S5). A total of 587 genes were identified within these 47 QTL intervals, of which 572 genes were associated with one trait and 15 genes were associated with two traits (Table S6). These significant leading SNPs were important for seedling response to N (LW, RW and RSR). The number of QTLs and leading SNPs associated with LLW, HLW, LWR, LRW, HRW, RWR, LRSR, HRSR and RSRR were 9, 4, 1, 7, 2, 8, 9, 1 and 6, respectively. They were distributed on all 12 linkage groups of rice and could explain phenotypic variation from 7.94 to 12.37%, respectively. These QTLs and significant leading SNPs are likely to have potential application value in further exploration of rice varieties with low-N tolerance in the future.

Candidate gene mining

Among these 587 genes, a total of 160 genes exhibited distinct haplotype variations, with 133, 24 and 3 genes having 2, 3 and 4 haplotypes, respectively (Table S6). Based on phenotypic data analysis of 295 *japonica* rice varieties, we identified that 96 out of these 160 genes exhibited significant phenotypic differences among their haplotypes (Table S7, Fig. S2). Therefore, we hypothesized that these 96 genes were candidate genes for low-N tolerance associated with LW, RW and RSR. The pathway with the highest number of enriched genes among these

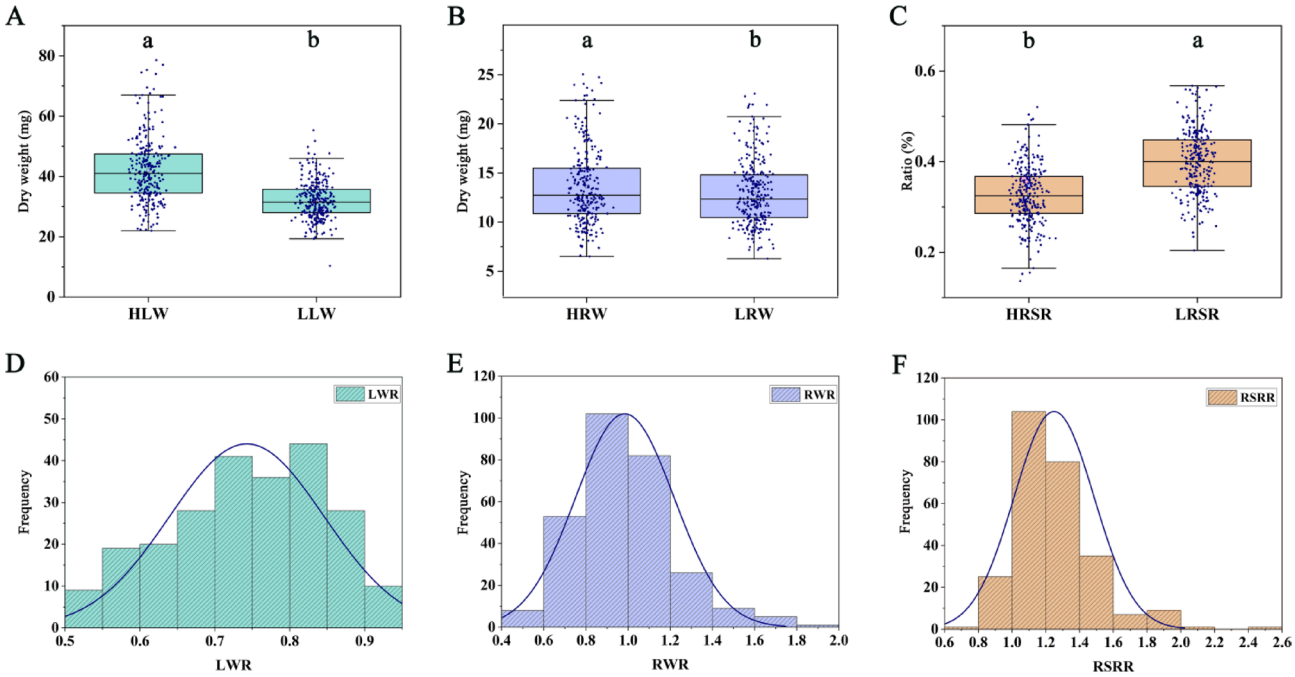

Fig. 1. Distribution of phenotypic data of 295 rice varieties under high and low N conditions. (**A**) HLW: Leaf dry weight under high N condition. LLW: Leaf dry weight under low N condition, (**B**) HRW: Root dry weight under high N condition. LRW: Root dry weight under low N condition, (**C**) HRSR: Root-shoot ratio under high N condition. LRSR: Root-shoot ratio under low N condition, (**D**) LWR: Relative value of leaf dry weight under low and high N conditions, (**E**) RWR: Relative value of root dry weight under low and high N conditions, (**F**) RSRR: Relative value of root-shoot ratio under low and high N conditions. Lowercase letters represented the significance between low and high N conditions at the 0.05 level. The significant difference analysis was performed using the One-way ANOVA.

96 genes was the metabolic pathway, with a total of 4 genes. The number of genes enriched in endocytosis and ribosome pathway were 2, respectively. A total of 27 subcategories were annotated for these 96 genes, including 17 biological process categories, 2 cellular component categories and 8 molecular function categories (Fig. 4). Based on previous research data, we established the following criteria: significant differential expression was defined as $|\log_2 FC| > 1$ with $p < 0.05$ in Longjing 31 (low-N tolerant variety), while no significant differences were observed in Songjing 10 (low-N sensitive variety). From the initially screened 96 genes, we identified 5 genes that showed significant differential expression specifically in Longjing 31[34]. These five genes (*Os06g0538400*, *Os11g0195500*, *Os11g0213700*, *Os11g0213800* and *Os12g0472800*) were recognized as more valuable candidate genes (Table 1). qRT-PCR analysis revealed that in Longjing 31, three of these genes were up-regulated while two were down-regulated. No significant differential expression was observed for these genes in Songjing 10 (Fig. 5). The qRT-PCR results for these five candidate genes showed consistent expression trends with the RNA-seq data.

Dominant haplotype analysis of candidate genes

The linkage disequilibrium (LD) of these five candidate genes are shown in Figs. 6A and D and 7A and D. Two different haplotypes were formed by a nonsynonymous SNP in the CDS region of two genes, *Os06g0538400*, *Os12g0472800* (Figs. 6B and 7E). Two different haplotypes of *Os06g0538400* showed significant phenotypic differences in LLW and LRW. The Hap1 of *Os06g0538400* was the dominant haplotype for LLW and LRW (Fig. 6C). There were significant phenotypic differences between two haplotypes of *Os12g0472800* in LWR and RSRR. Hap1 of *Os12g0472800* significantly enhanced LWR compared to other haplotypes (Fig. 7E). Two SNPs in the CDS region of *Os11g0195500* formed 3 haplotypes which exhibited significant phenotypic differences in LLW, HRW and LRSR. The Hap2 and Hap3 of *Os11g0195500* was the dominant haplotype for LLW and LRSR, and the Hap2 was the dominant haplotype for HRW (Fig. 6E, F). *Os11g0213700* and *Os11g0213800* were located in the same QTL interval, and both of these two genes possessed three haplotypes which were defined by 5 and 2 SNPs in the CDS region, respectively (Fig. 7A–C). The Hap3 of *Os11g0213700* was the dominant haplotype for LLW, and the Hap1 was the dominant haplotype for HRSR (Fig. 7C). No nonsynonymous SNPs were identified in the promoter region of any of these 5 genes. There were significant phenotypic differences in RWR and RSRR among different haplotypes of *Os11g0213800* (Fig. 7B). Haplotypes of *Os11g0213700* exhibited significant phenotypic differences in LLW, RWR and HRSR (Fig. 7C).

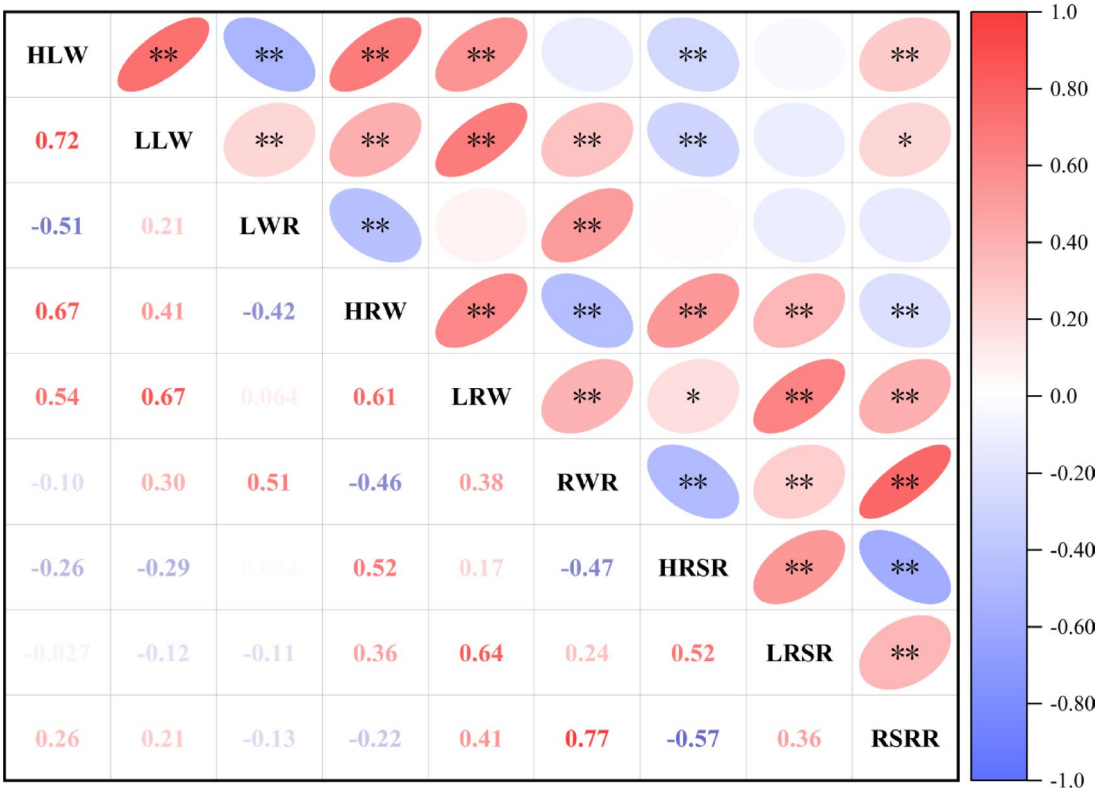

Fig. 2. Pearson correlation analysis of nine N tolerance-related traits (HLW, LLW, LWR, HRW, LRW, RWR, HRSR, LRSR, RSRR). The upper part of the figure is a significant test label by One-way ANOVA test. *, the difference significance at the 0.05 level. **, the difference significance at the 0.01 level. The lower part is the correlation coefficient between each trait.

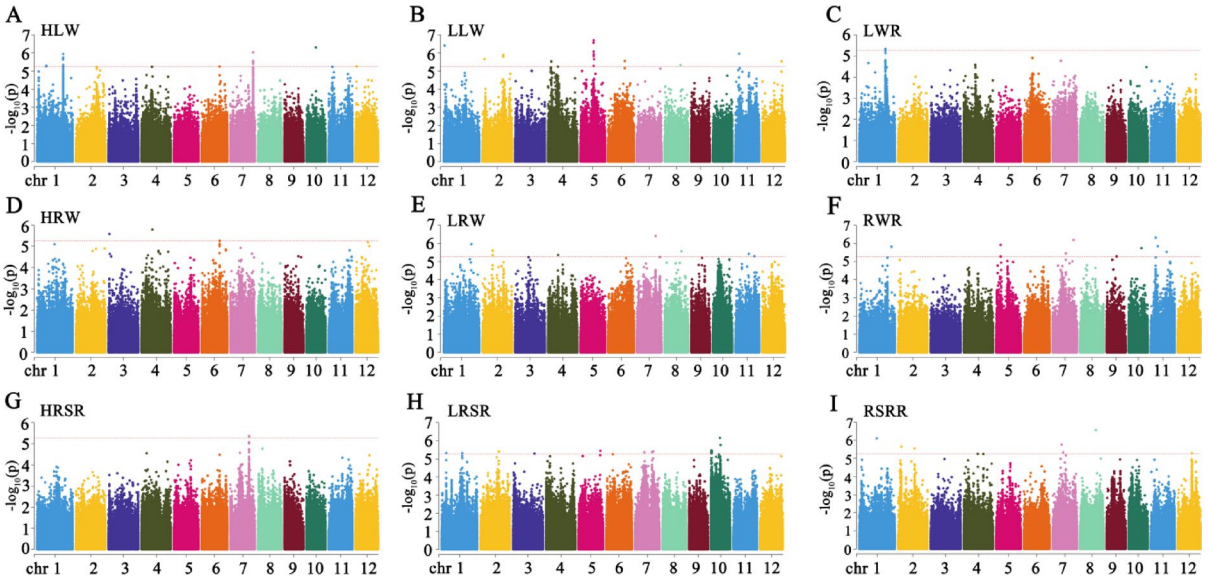

Fig. 3. Manhattan plots of LW, RW and RSR. (**A**) Manhattan plots of LW under high- N conditions. (**B**) Manhattan plots of LW under low-N condition. (**C**) Manhattan plots of relative LW under low and high-N conditions. (**D**) Manhattan plots of RW under high-N condition. (**E**) Manhattan plots of RW under low-N condition. (**F**) Manhattan plots of relative RW under low and high-N conditions. (**G**) Manhattan plots of RSR under high-N condition. (**H**) Manhattan plots of RSR under low-N condition. (**I**) Manhattan plots of relative RSR under low and high-N conditions.

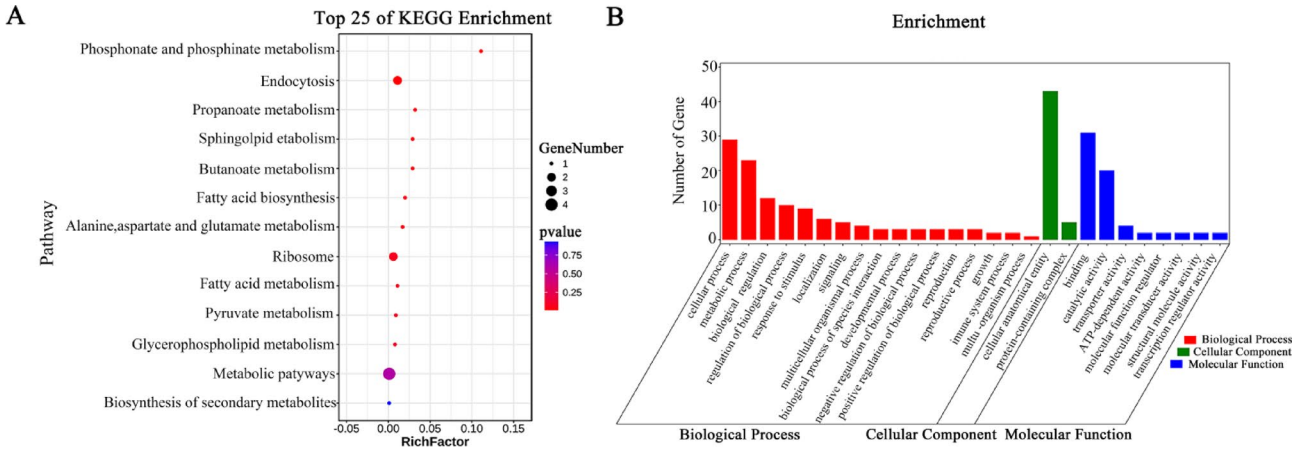

Fig. 4. Enrichment analysis of 96 genes. (A) KEGG functional classification and biological pathway enrichment of 96 genes. (B) Graphene oxide classification of 96 genes.

Candidate genes	Regulated	QTLs	Annotation
Os06g0538400	Down	qLLW6	Conserved hypothetical protein
Os11g0195500	Down	qLLW11	Phytoalexin
Os11g0213700	Down	qRWR11-1	Similar to NBS-LRR disease resistance protein homologue (Fragment)
Os11g0213800	Down	qRWR11-1	Similar to NBS-LRR disease resistance protein homologue (Fragment)
Os12g0472800	Up	qRSRR12	B repeat unit of collagen binding surface protein (cna) containing protein

Table 1. Differentially expressed candidate genes under the low-N condition.

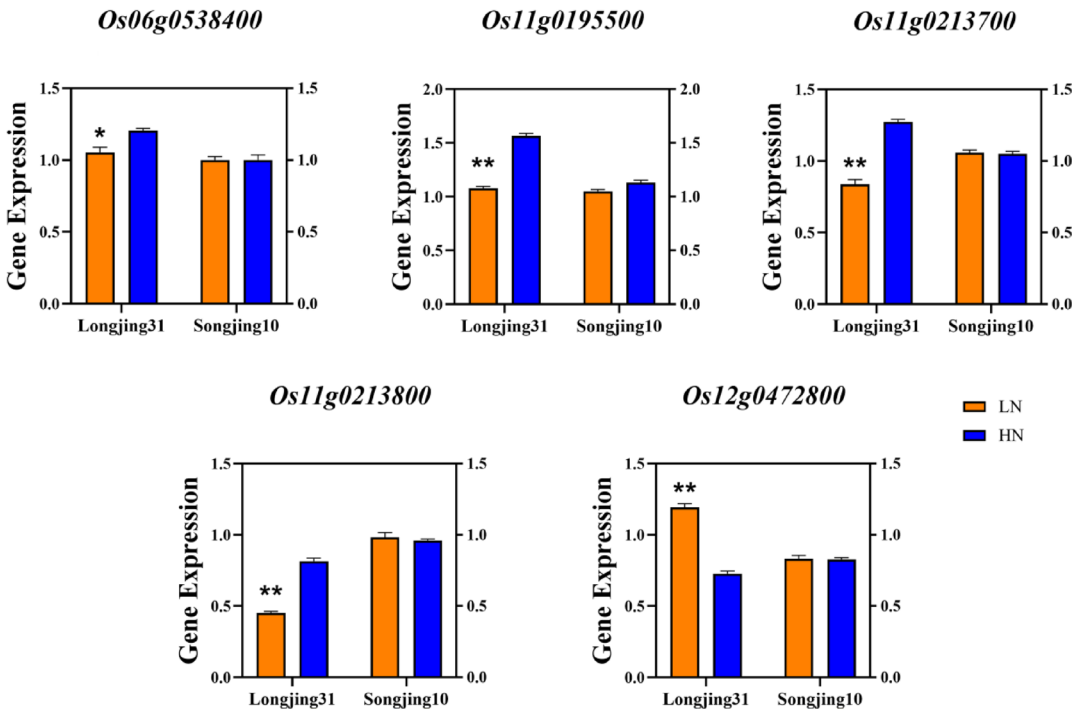

Fig. 5. Comparative expression profiles of five candidate genes between Longjing 31 and Songjing 10 under differential N conditions. * For the difference significance at the 0.05 level, ** for the difference significance at the 0.01 level. LN: low N treatment; HN: high N treatment.

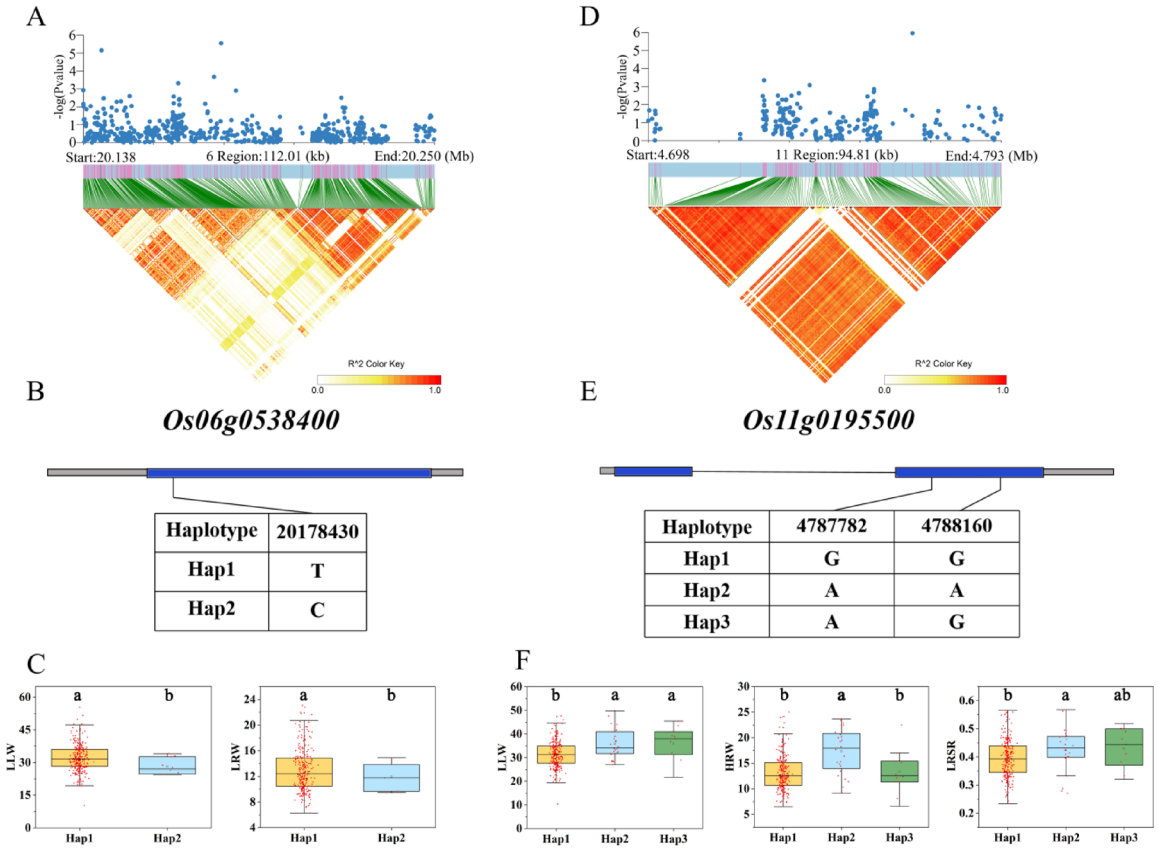

Fig. 6. Analysis of gene architecture and haplotypes of *Os06g0538400* and *Os11g0195500*. (A) Regional Manhattan plot and linkage disequilibrium (LD) heat map of *Os06g0538400*. (B) Gene structure of *Os06g0538400*. (C) Haplotype analysis of *Os06g0538400*. (D) Regional Manhattan plot and LD heat map of *Os11g0195500*. (E) Gene structure of *Os11g0195500*. (F) Haplotype analysis of *Os11g0195500*. Lowercase letters indicated significance between treatments at the $P = 0.05$ level. The significant difference analysis was performed using the One-way ANOVA.

Discussion

N fertilizer is very important for agricultural development[35]. If N fertilizer is applied excessively, it may result in a series of environmental and ecological problems[36]. Dry matter allocation is one of the important strategies for plants to adapt to low-N environment[37]. Under low-N condition, plants maximize dry matter accumulation by optimizing dry matter distribution[38]. Under low-N condition, low-N tolerant rice varieties typically show higher RW and lower LW[39]. Although lower LW may lead to a temporary decrease in photosynthetic efficiency, it improves plant N acquisition efficiency from the soil[40]. In addition, higher RSR is also closely related to the improvement of N use efficiency, because the regulation of dry matter partitioning involves complex genetic and molecular mechanisms[41]. Key genes in the N signaling pathway (such as NRTs and GS) are also involved in the regulation of dry matter distribution, thus affecting the low-N tolerance of plants[42,43]. These findings provide an important molecular basis for elucidating the relationship between dry matter distribution and low-N tolerance. Therefore, this study identified low-N tolerance genes by measuring RW, LW and RSR under high and low-N conditions.

At present, it has been studied that plants optimize resource acquisition by adjusting biomass allocation under nutrient stress conditions[44]. The results of this research demonstrated that under high and low-N conditions, the average LW were 42.07 and 32.14 mg, the RW were 13.61 and 12.94 mg, and the RSR were 0.33 and 0.40, respectively (Table S2). This study found that the rice varieties showed higher RSR under low-N condition (Fig. 1). This distribution pattern indicated that low-N tolerant varieties can allocate more photosynthates to roots to enhance their ability to obtain N from soil. This result is consistent with the research of Poorter[7]. In addition, higher RSR is also closely related to adaptive changes in root morphology, which further enhances the competitiveness of plants in low-N environment[45].

Conventional QTL mapping only utilizes the genetic differences between parents, and GWAS can identify a large number of variations existed in natural populations[46]. Multiple QTLs related to plant weight, panicle length and other panicle traits were identified through GWAS[47]. By GWAS, Chen et al.[48] located 75 QTLs and identified 5 candidate genes using root morphological traits including diameter and length. In this study, 295 *japonica* rice varieties were adopted to measure LW, RW and RSR at seedling stage under low and high-N conditions. A total of 587 genes (Table S6) were identified within the 47 QTL intervals in this research, among which 96 genes

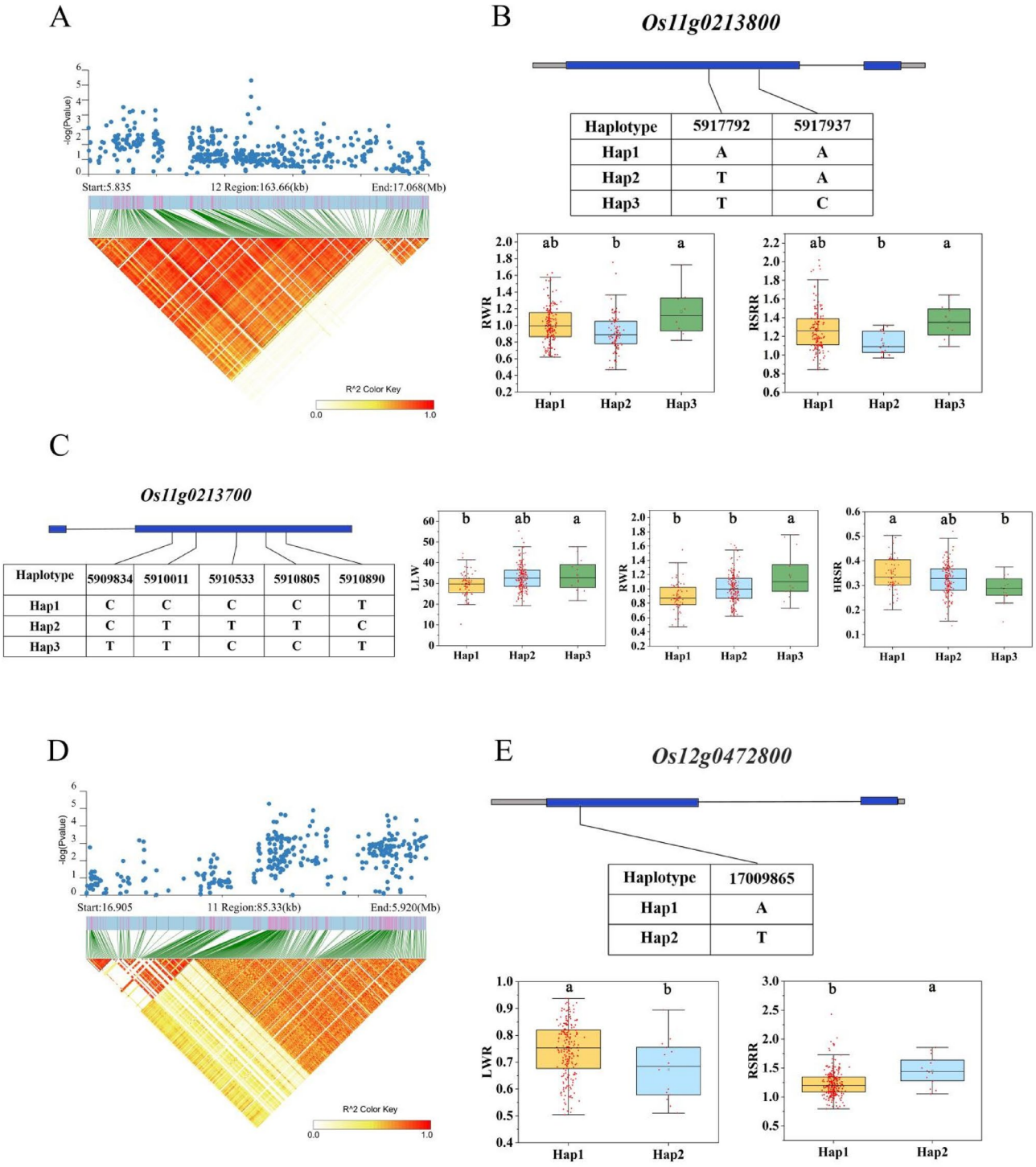

Fig. 7. Analysis of gene architecture and haplotypes of *Os11g0213700*, *Os11g0213800* and *Os12g0472800*. (A) Regional Manhattan plot and linkage disequilibrium (LD) heat map of *Os11g0213700* and *Os11g0213800*. (B) Gene structure and haplotype analysis of *Os11g0213700*. (C) Gene structure and haplotype analysis of *Os11g0213800*. (D) Regional Manhattan plot and LD heat map of *Os11g0213700* and *Os12g0472800*. (E) Gene structure and haplotype analysis of *Os12g0472800*. Lowercase letters indicated significance between treatments at the 0.05 level. The significant difference analysis was performed using the One-way ANOVA.

with different haplotypes will cause significant differences in different traits of 295 rice varieties. Therefore, we hypothesized that these 96 genes are low-N tolerance candidate genes related to LW, RW and RSR.

Low-N tolerance is a complex trait. Many QTLs and genes for low-N tolerance genes have been studied, such as *OsMYB305*[49], *OsGS1;1*[50] and *OsPTR9*[51]. During the last few years, the combined application of GWAS and RNA-seq has made it possible to study the genetics of complex traits[52,53]. For example, the N utilization-

related gene *OsNAC68* was successfully identified through combining GWAS and RNA-seq analyses[54]. In this study, RNA-seq was performed to clarify the expression differences of these 96 genes. RNA-seq was performed on them. Among the 96 haplotype variant genes, 5 genes showed significant differences in the expression of Longjing 31, and were referred to as more value candidate genes. However, there have been no reports of N related on these 5 candidate genes. Studies had shown that some low-N tolerance genes were involved in the regulation of root development and hormone signaling pathways, such as auxin and cytokinin, which play an important role in regulating RSR and root morphology[55]. These findings provide an important theoretical basis for molecular breeding of low-N tolerant rice. Therefore, we can continue to use the combined application of GWAS and RNA-seq to identify excellent genes associated with N utilization and acquisition.

In this study, five candidate genes were screened, among which *Os11g0195500* (*OsPAD4*) is a known phytoalexin gene belonging to the lipase-like protein family[56]. This family plays a crucial role in plant immune signaling, particularly in effector-triggered immunity (ETI) and pattern-triggered immunity (PTI) pathways[57]. Notably, intracellular nucleotide-binding NLR receptors cooperate with helper LRR proteins from the *ADR1* and *NRG1* families, as well as lipase-like proteins such as *PAD4*, to induce ETI immune responses[58]. Functional annotation analysis revealed that *Os11g0213700* and *Os11g0213800* belong to the NBS-LRR gene family, which is typically associated with disease resistance. *MAX2*, a ubiquitously expressed protein in plants, contains a characteristic LRR domain and has been demonstrated to interact with *KAI2* to regulate plant growth, development, and stress responses. Notably, *KAI2*-mediated signaling serves as a master regulator of root system architecture[59]. During the seedling stage, root development directly determines N acquisition efficiency. Under N-deficient conditions, plants employ adaptive strategies including enhanced lateral root formation, increased root hair density, and architectural remodeling of root systems to optimize N uptake and improve low-N tolerance[60]. These information supports these five candidate genes as low-N tolerance genes, so we will further verify the function. These genes showed different haplotypes, and there were significant differences among different haplotypes. The introduction of favorable alleles in rice varieties can improve N-related traits. For example, the LLW and LRW dominant haplotypes of *Os06g0538400* gene showed significant advantages in 'Liaoyan 16' and 'common upland rice', respectively. The LLW and HRW dominant haplotypes of *Os11g0195500* gene showed significant advantages in 'Liaoyan 16' and 'Liaoxing 6', respectively. Therefore, combining GWAS and RNA-seq is a validated strategy to identify favorable alleles and breed excellent varieties. This not only provides a new perspective for understanding the molecular mechanism of N uptake and utilization in rice, but also provides an important theoretical basis and technical support for the breeding rice varieties with low-N tolerance.

Methods

Plant materials

A collection of 295 *japonica* rice varieties, including both domestic (China) and international accessions, was analyzed in this study. Firstly, seed dormancy was broken for all 295 varieties, followed by surface sterilization and thorough removal of disinfectant residues. This study adopted a completely randomized two-factor design, comprising 295 varieties and 2 N concentrations, with each treatment combination replicated 3 times biologically. Seeds of each variety were subjected to hydroponic germination in a constant-temperature incubator at 31 °C for 48 h. For each variety, 64 uniformly germinated seeds with synchronized shoot lengths were selected and equally divided into two experimental groups. The germinated seeds were individually transplanted into two 96-well plates (one seedling per well), with each plate placed in a 1 L hydroponic container before being transferred to growth chambers. One group was assigned to the low-N treatment while the other received the high-nitrogen treatment. Each treatment group was established with three biological replicates, with each replicate cultured in a separate 96-well plate. Seedlings were cultivated in climate-controlled greenhouses with strict regulation of temperature and hydration: daytime temperature maintained at 23.8 °C for 10 h and nighttime temperature at 22.4 °C for 14 h, under a constant light intensity of 300 μmol/m²/s. Urea was employed as the N source. During the initial growth phase, all seedlings were grown under high-N conditions (40 ppm) for 5 days to ensure normal growth. Subsequently, the seedlings were divided and maintained under either high-N (40 ppm) or low-N (8 ppm) conditions for an additional 21 days. The nutrient solution was refreshed every 7 days throughout the experimental period[34].

Phenotypic data

After 21 days of low and high N cultivation, the leaves and roots of seedings were sampled and cleaned with ultrapure water, killed out in an oven at 105 °C for 30 min, and dried to constant weight at 80 °C. The dry weights of leaves and roots were measured, and the root-shoot ratio was calculated. The relative value of each trait was represented by dividing the phenotypic value under low N condition by that under high N condition. RSR is the ratio of RW to LW.

GWAS

In this study, DNA was extracted from 295 *japonica* rice varieties, and Illumina HiSeq 2000 was used to perform high-throughput sequencing of qualified sample DNA. 788,396 SNPs with the lowest allele frequency above 0.05 and the deletion rate below 20% were identified and were selected for later analysis. GWAS was performed using the MLM method of Tassel 5.0 software[61]. The threshold for SNPs significantly associated with traits was set as $P < 5.46 \times 10^{-6}$, and the Manhattan plot and Q-Q plot were drawn by CMplot software in R language[62]. To determine the lead SNP (most significant variant), we first removed correlated SNPs within specified physical intervals. Gene annotations within candidate QTL regions were subsequently extracted from the Ensembl Plants database (https://plants.ensembl.org/). The LDBlockShow software was used to calculate the pairwise linkage disequilibrium (LD) of the leading SNPs. The R2 value was calculated within the intervals of ±2 Mb

for the lead SNP. The average value was calculated based on the top 10% R2 value within the range of 1.5-2 Mb, and was recorded as the background value of LD attenuation. The LD attenuation interval of the leading SNPs was represented by the background value of LD attenuation plus 0.2[63]. If more than two significant SNPs were located in the same LD interval, these SNPs were designated as the same QTL, and the SNP with the smallest P value was regarded as the lead SNP.

Haplotype analysis

The location of each gene was acquired through China Rice Data Center (https://www.ricedata.cn/). Using RiceSNPSeek (https://snp-seek.irri.org/), we retrieved all coding SNPs resulting in amino acid alterations (non-s ynonymous) within gene exons. The nonsynonymous SNPs located in the exon region and the SNPs located in the 2 kb interval of promoter before ATG were adopted for haplotype analysis.

Candidate gene prediction

Candidate genes were predicted for all genes within the LD attenuation interval of each lead SNPs based on the phenotypic data of 295 *japonica* rice varieties. RNA-seq data of Longjing 31 (low-N tolerant variety) and Songjing 10 (low-N sensitive variety) were adopted[34]. Combined with transcriptome data in the low-N tolerant variety Longjing 31, these differential expressed genes which presented phenotypic differences among different haplotypes were defined as more valuable candidate genes. The threshold for determining differential expressed genes is $|\log_2 FC| > 1$ and $p < 0.05$.

Quantitative real-Time PCR

The TranZol Up RNA kit (TransGen Biotech) was used to extract total RNA from the samples, and the PrimeScript TM RT Master Mix (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China) was used to synthesize cDNA according to the instructions. The BlazeTaqTM SYBR Green qPCR Master Mix 2.0 (GeneCopoeio, Guangzhou, China) was used for qRT-PCR on the RocheLightCycler96 system. Each sample had 3 biological and technical replicates. The CT values were recorded, and the expression levels of the 5 candidate genes were calculated using the $2^{-\Delta\Delta CT}$ method. *OsActin1* was used as an internal control, and primers design by Primer5.0 software were listed in Table S1.

Data analysis

The mean values, ranges, and coefficients of variation of phenotypic data were recorded and organized using Microsoft Excel. Statistical correlations were analyzed with IBM SPSS Statistics 26.0 (SPSS Inc., Chicago, IL, USA). Origin 2021 was adopted to complete the drawing. KEGG and GO analyses were performed to mining enriched pathways among N-specific DEGs through the online platform (https://cloud.metware.cn/ # / tools / tool-list). Pathways showing statistical significance ($p < 0.05$) were regarded as significantly enriched.

Conclusions

In this study, among 295 *japonica* rice varieties, seedling responses to N were assessed through LW, RW, and RSR measurements. GWAS identified 47 QTL intervals, and 587 candidate genes. The results of haplotype analysis showed that 160 of the 587 genes presented different haplotype in the association panel. Among the 96 genes with significant differences among different haplotypes, 5 genes had significant differences in the expression of Longjing 31, and were referred to as more valuable candidate genes. These results provided a theoretical basis for screening low-N tolerance genes and breeding new varieties with low-N tolerant. These results provided a theoretical basis for identifying low-N tolerance genes and developing improved varieties, laying the foundation for future breeding efforts utilizing molecular marker-assisted selection or genome-editing approaches to breed novel low-N tolerant varieties.

Data availability

The RNA-seq data for this study can be found in the National Center for Biotechnology Information under the accession number PRJNA835804 (https://www.ncbi.nlm. nih.gov/, accessed on 10 June 2023).

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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