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ORIGINAL ARTICLE

Genome-wide association study and BSR-seq identify nitrate reductase–related genes in rice landraces (*Oryza sativa* L.)

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Abstract

Nitrogen (N) is an essential nutrient for rice (*Oryza sativa* L.) growth and development. However, the lower nitrogen use efficiency (NUE) results in an N fertilizer surplus, which causes many environmental problems. In this study, genome-wide association studies were used to detect nitrate reductase (NR)-related loci in 419 rice landraces. Using the general linear model (GLM), mixed linear model (MLM), linear model (LM), and linear mixed model (LMM), we found six, nine, seven, and six significant single-nucleotide polymorphisms (SNPs) associated ($p < 1 \times 10^{-5}$) for three traits. Moreover, 98 significant SNPs were associated (logarithm of odds ≥ 3) with three traits through 3 V multi-locus random-SNP-effect mixed linear model. Interestingly, we found that Chr1_15896481 was significantly associated in the GLM, MLM, LM, and LMM models. Meanwhile, this significant locus overlapped with a candidate region in bulked segregant RNA sequencing. Through integrated analysis, we identified a most likely candidate genomic region 15,627,420–16,084,761 bp on chromosome 1. By performing functional annotation, RNA sequencing, and real-time quantitative polymerase chain reaction (RT-qPCR) analysis for the genes within this interval, we identified five candidate genes that may

Abbreviations: BL, bud length; BSR-seq, bulked segregant RNA sequencing; DEGs, differentially expressed genes; GLM, general linear model; GO, gene ontology; GOGAT, ferredoxin-glutamate synthase; GS, glutamine synthetase; GWAS, genome-wide association study; InDel, insertion-deletion; KEGG, Kyoto encyclopedia of genes and genomes; LD, linkage disequilibrium; LM, linear model; LMM, linear mixed model; MLM, mixed linear model; MRL, main root length; NIR, nitrite reductase; NR, nitrate reductase; NUE, nitrogen use efficiency; QTLs, quantitative trait loci; RN, root number; RNA-seq, RNA sequencing; RT-qPCR, real-time quantitative polymerase chain reaction; SNPs, single-nucleotide polymorphisms.

affect NR activity. *Os01g0378400* exhibits a gene expression pattern highly similar to that of *OsNR1.2*. It belongs to the NAC transcription factor family, which is involved in plant N metabolism. *Os01g0377700* is homologous to an ammonium transporter gene (*Cre06g293051*). *Os01g0383700* encodes a WD40 domain protein, *Os01g0379400* encodes an F-box protein, and *Os01g0382800* encodes a DYW-type PPR domain protein. These findings will provide valuable genetic resources for NUE genetic improvement in rice breeding.

Plain Language Summary

Chlorate and nitrate have similar structures and are competitive inhibitors of nitrate reductase (NR), inhibiting the nitrate reduction process. Most of the rice lacking NR activity is chlorate-resistant, so screening natural populations of rice with chlorate can help to identify key NR-related genes from them. In this study, we identified the sensitivity of 419 rice landraces to $KClO_3$ and found that they could be categorized into five sensitivity classes. Using genome-wide association studies and bulked segregant RNA sequencing analysis, we identified an overlapping candidate interval. By analyzing the genes on this candidate interval, we found five candidate genes that are most likely to be involved in nitrate reduction. This study identified some candidate genes that may be related to NR activity. These findings provide valuable genetic resources for breeding new N-efficient varieties.

1 | INTRODUCTION

Nitrogen (N) is an essential nutrient for plant growth and development (Guan, 2017; Jiang et al., 2018). In the past few decades, the widespread application of N fertilizer has significantly advanced agricultural production and increased grain yield (Hsieh et al., 2018). However, nitrogen use efficiency (NUE) remains low, which averages only around 30% in most crops (Jiang et al., 2018). Consequently, excessive use of N fertilizers not only wastes resources but also contributes to numerous environmental issues, including elevated greenhouse gas emissions, water eutrophication, and soil acidification (Galloway et al., 2008; Guo et al., 2010; J. Wang et al., 2014; C. Q. Yu et al., 2019). Therefore, enhancing crop NUE and reducing N fertilizer inputs are essential for sustainable agricultural development.

Plants mainly rely on inorganic N in the forms of nitrate and ammonium to support their growth and development (Y. H. Gao et al., 2020; Hu et al., 2015; Q. Wang, Nian, et al., 2018). Rice (*Oryza sativa* L.) possesses aerenchyma tissue that enables oxygen transport from the shoots to the roots and releases oxygen into the rhizosphere. This oxidation of the rhizosphere promotes nitrification by soil bacteria, converting 15%–40% ammonium to nitrate (Z. Gao et al., 2019; Hu et al., 2015; Y. L. Li et al., 2007; Y. J. Yang et al., 2016). Therefore, the uptake and assimilation of nitrate have become significant factors affecting NUE in rice. Nitrate is absorbed through specific root uptake transporters and is subsequently reduced to nitrite by nitrate reductase (NR) (Z. Gao et al., 2019; Okamoto et al., 2003). Nitrite is further reduced to ammonium by nitrite reductase (NIR) (Lam et al., 1996; Liu et al., 2022). NR serves as a key regulatory step in N assimilation (Alejandro et al., 2017; Mauceri et al., 2020). Overexpression of NR or NIR frequently results in increased N uptake. Recent studies in rice have demonstrated that overexpressing the *Indica OsNR2* gene in *Japonica* rice can boost tiller numbers, grain yield, and NUE under high N supply (Z. Gao et al., 2019). Ammonium, either produced from nitrate assimilation or absorbed directly from the soil via members of the NH_4^+ transporter/methylamine permease/rhesus (AMT/MEP/RH) family, is assimilated into glutamine and glutamic acid through the action of the glutamine synthetase/ferredoxin-glutamate synthase (GS/GOGAT) (Celine et al., 2010; Liu et al., 2022).

Chlorate is the chlorine analog of nitrate and serves as a substrate for NR similar to nitrate (Kabange et al., 2021). NR reduces chlorate to toxic chlorite, which can cause yellowing and inhibit plant growth (Z. Gao et al., 2019; Ou et al., 2024). Therefore, $KClO_3$ can be used to study N metabolism in higher plants by monitoring the activity of NR and the plants' response to chlorate, which is related to the process of nitrate assimilation (Zhang & Chu, 2020). Studies have identified quantitative trait loci (QTLs) related to chlorate resistance in RILs of 9311 and Nipponbare, revealing

differences in nitrate assimilation between *indica* rice and *japonica* rice, largely attributed to allele variation in *OsNR2* (Z. Gao et al., 2019). Additionally, a double haploid population of 127 lines was developed using the F_1 generation of ZYQ8/JX17, where $KClO_3$ resistance analysis identified three QTLs linked to chlorate resistance (Teng et al., 2006). Hu et al. (2015) further found that a mutation in *NRT1.1B* is a significant factor contributing to the $KClO_3$ sensitivity and nitrate utilization difference between *indica* and *japonica* rice. The $KClO_3$ sensitivity test has been widely used to identify plant varieties with low N tolerance, offering a distinct phenotype that makes the $KClO_3$ sensitivity test a valuable tool for evaluating crop NUE.

In this study, genome-wide association studies (GWAS) were used to detect $KClO_3$ sensitivity association loci in 419 rice landraces. Meanwhile, we selected 60 very high/low-sensitivity varieties for bulked segregant RNA sequencing (BSR-seq) and RNA sequencing (RNA-seq) analysis, respectively, based on bud length (BL) sensitivity to chlorate. We found that a significant locus from GWAS overlapped with a candidate region in BSR-seq. Through performing functional annotation, RNA-seq, and real-time quantitative polymerase chain reaction (RT-qPCR) analysis on the genes within this interval, we identified five candidate genes that are most likely related to $KClO_3$ sensitivity. Our research provides valuable genetic resources for improving N fertilizer utilization and supports the genetic breeding of rice.

Core Ideas

- Extreme phenotypic differences were observed after $KClO_3$ treatment of 419 rice landraces.
- An overlapping candidate genomic region was identified by genome-wide association study and bulked segregant RNA sequencing.
- Five candidate genes potentially associated with nitrate reductase activity were identified.

2 | MATERIALS AND METHODS

2.1 | Materials

A total of 419 rice landraces from Guangxi were used, comprising 330 *indica* rice varieties, 78 *japonica* rice varieties, and 11 other varieties. Sequencing of this natural population was carried out using the specific locus amplified fragment sequencing technology. A total of 208,993 single-nucleotide polymorphisms (SNPs) were obtained based on a minor allele frequency > 0.05 and a deletion rate <0.5. The phylogenetic tree and population structure analysis were performed for all 419 rice landraces, and the whole panel mainly separated into six subpopulations based on population structure analysis (X. H. Yang et al., 2018). Certain achievements have been made in this population in exploring genes related to NUE (Liao et al., 2023), pericarp color, etc. (X. H. Yang et al., 2018).

2.2 | Identification of $KClO_3$ sensitivity of rice seedlings

Approximately 50 seeds were soaked for 24–48 h. After removing excess water, the seeds were placed in a 37°C artificial climate chamber for 12–24 h until germination, specifically until the white radicles emerged. The germinated seeds were then transferred to Petri dishes lined with filter paper and cultured with distilled water in an artificial climate chamber set at 28°C, with 75% relative humidity, and a light-dark cycle of 14 h/10 h. Each group included one control and three experimental treatments, with eight seeds per group. When rice BL reached approximately 2 cm, distilled water in the experimental group was replaced with a 0.02% $KClO_3$ solution (Z. Gao et al., 2019), following the method described by Teng et al. (2006) to assess rice sensitivity to chlorate. To maintain a consistent $KClO_3$ concentration, the solution in each petri dish was replaced daily. On the third day of treatment, BL and taproot length of rice seedlings were measured, and the number of roots per seedling was recorded. The sensitivity of chlorate can be calculated as follows: Chlorate sensitivity = ([Water treatment average value − Chlorate treatment average value]/Water treatment average value) (Teng et al., 2006).

2.3 | GWAS

GWAS were performed on the $KClO_3$ sensitivity of 419 rice landraces using 208,993 SNPs (X. H. Yang et al., 2018). In this study, the general linear model (GLM) and mixed linear model (MLM) of TASSEL software, the linear model (LM) and linear mixed model (LMM) of GEMMA software, and the 3 V multi-locus random-SNP-effect mixed linear model (3VmrMLM) (M. Li et al., 2022) were used for GWAS. Sites with $p < 1 \times 10^{-5}$ (X. Gao et al., 2008; Gong et al., 2017) are significantly correlated with GLM, MLM, LM, and LMM. Sites with loragrithm of odds ≥ 3 selected by 3VmrMLM are regarded as significant association sites. We selected the Nipponbare (version IRGSP-1.0) reference genome. Then, based on the linkage disequilibrium (LD) attenuation of rice, we selected a 150 kb interval covering the upstream and downstream of the SNP with significant correlation as the candidate interval.

2.4 | Cultivation of materials with extreme traits for BSR-seq and RNA-seq

In 419 rice landraces, we selected 60 very high-/low-sensitivity varieties for BSR-seq and RNA-seq analysis, respectively, based on BL sensitivity to chlorate. For each variety, 50 plump seeds were selected for germination. 20 germinated seeds with consistent sprouting status were selected and evenly sown in two plastic Petri dishes. After the buds grew to about 4 cm, we selected the buds of 10 seedlings in one of the Petri dishes for each part, put them into a 15-mL centrifuge tube, froze them in liquid N for 30–60 min, and took them out, and then stored them in an ultra-low temperature freezer at −80°C. At the same time, the seedlings in another Petri dish were treated with $KClO_3$ solution, and samples were taken and stored by the same method as above on the second day of treatment.

2.5 | RNA extraction, library construction, and sequencing

Total RNA was extracted from the tissue using TRIzol Reagent according to the manufacturer's instructions. Then RNA quality was determined by 5300 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). The qualified RNAs were mixed in equal amounts to construct the pre-treatment low-sensitivity pool (CL), the pre-treatment high-sensitivity pool (CH), the post-treatment low-sensitivity pool (TL), and the post-treatment high-sensitivity pool (TH). Library construction and sequencing were performed by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. on the Illumina NovaSeq 6000 sequencing platform in the strand-specific 2 × 150 bp paired-end mode. The RNA-seq transcriptome library was prepared following Illumina Stranded mRNA Prep, Ligation from Illumina using 1 μg of total RNA. Sequencing reads were checked for quality and filtered using Fastp (version 0.23.2) with the default settings of a 4-nt sliding window and an average base quality of Q20 (S. F. Chen et al., 2018). Reads were aligned against the reference genome of Nipponbare (version IRGSP-1.0) using HISAT2 (version 2.2.1) (Kim et al., 2019). The comparison results were processed using GATK's Best Practices process (Mckenna et al., 2010). SNP and insertion-deletion (InDel) were detected and filtered using GATK's Haplotyper method. SnpEff (version 5.1d) combined with *Oryza sativa* genome was used to functionally annotate the detected SNPs and InDels (Cingolani et al., 2014). Euclidean distance (ED) (Hill et al., 2013) and index-slid (Takagi et al., 2013) statistical methods were employed to analyze the BSR association of SNPs and InDels. The sliding window method (1Mb window length, 50 kb step size) was used to denoise the counting values. We took 0.995 quantile as the threshold and selected the region that at least contains 10 variant loci on the threshold line as the candidate region.

2.6 | DEG analysis

The expression level of each gene was measured using the number of fragments per million reads per kilobase (FPKM). DESeq2 was used to analyze differences in transcription levels of genes. Genes were considered differentially expressed with the following criteria: $|\log_2 (\text{fold change})| \geq 1$ and $p < 0.05$. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses were performed for differentially expressed genes (DEGs).

2.7 | Validation of candidate genes using RT-qPCR

To further validate the most likely candidate genes, we selected 10 very high/low- sensitivity varieties for RT-qPCR, based on BL sensitivity to chlorate. We referred to the method of sample culture and collection in BSR-seq, with the difference that three biological replicates were set up for each treatment separately. RT-qPCR was performed using green qPCR Master Mix in a 10 μL format on a BIO-RAD CFX Opus 384 Real-Time PCR System. The cycling program is as follows: 95°C for 2 min; 95°C for 15 s; 60°C for 15 s; 72°C for 30 s; 39 cycles; 95°C for 15 s; 60°C for 1 min; 95°C for 15 s; 60°C for 15 s. *Actin* (Liao et al., 2023) was used as an internal control, and gene expression level was calculated using the $2^{-\triangle\triangle Ct}$ method (Schmittgen & Livak, 2008). The primers used in the RT-qPCR experiments are listed in Table S1.

3 | RESULTS

3.1 | Phenotypic analysis of $KClO_3$ sensitivity in 419 rice landraces

After treatment with $KClO_3$ solution, sensitive rice seedlings displayed yellowing due to chlorate toxicity, along with significant inhibition in root number (RN), main root length (MRL), and BL (Figure 1A–C). In contrast, insensitive seedlings showed no significant changes in RN, MRL, and BL (Figure 1D–F). Phenotypic data indicated that the maximum sensitivity ratios for RN, MRL, and BL to $KClO_3$ were 0.8365, 0.6613, and 0.4687, respectively, while the minimum values were −0.0590, 0.0160, and −0.0420, respectively (Table 1 and Table S2). The sensitivity of the three traits to $KClO_3$ can be classified into five categories using

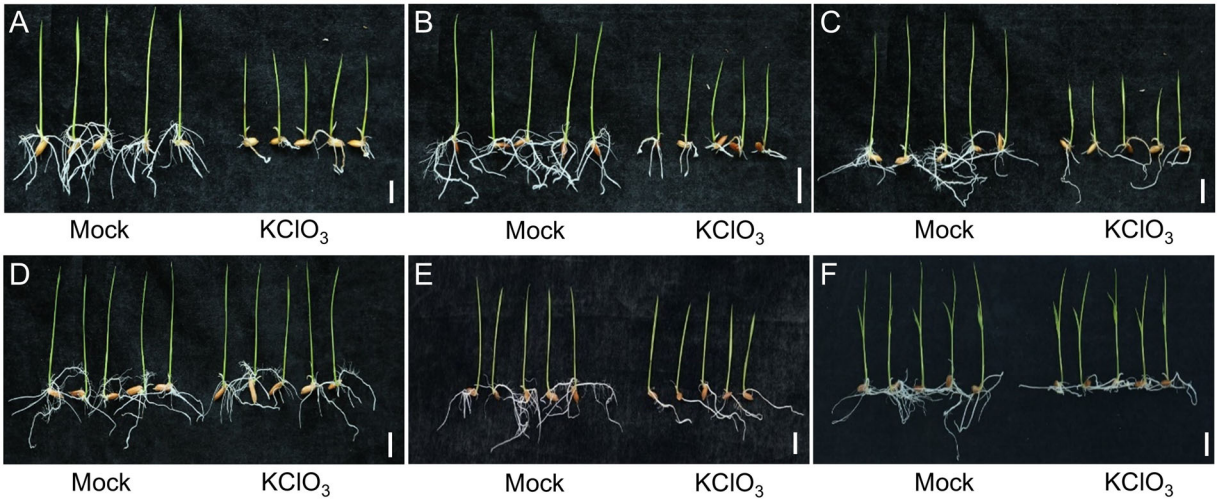

FIGURE 1 Phenotypic changes in rice seedlings treated with $KClO_3$ solution. (A) Rice variety with the largest sensitivity ratios for root number after $KClO_3$ treatment (C338). (B) Rice variety with the largest sensitivity ratios for main root length after $KClO_3$ treatment (C106). (C) Rice variety with the largest sensitivity ratios for bud length after $KClO_3$ treatment (C39). (D) Rice variety with the smallest sensitivity ratios for root number after $KClO_3$ treatment (C379). (E) Rice variety with the smallest sensitivity ratios for main root length after $KClO_3$ treatment (C337). (F) Rice variety with the smallest sensitivity ratios for bud length after $KClO_3$ treatment (C249). Bar: 1 cm.

TABLE 1 Phenotypic data analysis.

Traits	Maximum (material)	Minimum (material)	Average value	Standard deviation	Variable coefficient
RN	0.8365 (C338)	−0.0590 (C379)	0.5645	0.1654	0.2929
MRL	0.6613 (C106)	0.0160 (C337)	0.3667	0.1153	0.3063
BL	0.4687 (C39)	−0.0420 (C249)	0.2358	0.1081	0.4588

Abbreviations: BL, bud length; MRL, main root length; RN, root number.

a mean-standard deviation classification (Figure 2A–C). The study found 60–70 varieties with high sensitivity to chlorate, 60–80 varieties with low sensitivity, while the largest group had medium sensitivity. Among the 419 rice varieties treated with $KClO_3$, significant phenotypic differences were observed, fulfilling the requirements for constructing extreme pools. Moreover, we found that most sensitive varieties were *indica*, while most tolerant varieties were *japonica*. According to the sensitivity grades of BL to chlorate, we selected 60 rice varieties with very high sensitivity and 60 rice varieties with very low sensitivity respectively, and analyzed multiple agronomic traits of these varieties. We found that the plant height and panicle length of the tolerant varieties were significantly higher compared with those of the sensitive varieties. The effective panicle number, the number of grains per panicle, and the yield per plant of the sensitive varieties were significantly higher than those of the tolerant varieties. However, there were no obvious differences in the flowering stage and the seed setting rate (Figure S1). We conducted Pearson correlation analysis on three traits and found that the traits were significantly and positively correlated with each other (Figure 2D).

3.2 | GWAS of SNP markers related to chlorate sensitivity in rice

In this study, the GLM, MLM, LM, LMM, and 3VmrMLM models were used to conduct GWAS by correlating the sensitive phenotypic values of RN, MRL, and BL of the 419 tested materials, respectively, with 208,993 SNPs. A total of six significant SNPs associated with RN, MRL, and BL were detected by the GLM model, and they were distributed on chromosomes 1, 4, and 11. Among them, Chr1_15896481 was significantly associated with both RN and BL; Chr1_15896626 was significantly associated with both MRL and BL (Figure 3A–C; Table 2). The MLM model detected a total of nine significant SNPs associated with RN, MRL, and BL, which were distributed on chromosomes 1, 2, 4, 6, and 11. Among them, Chr11_28171917 was significantly associated with both RN and MRL (Figure 3D–F; Table 2). A total of seven significant SNPs associated with RN, MRL, and BL were detected by the LM model, and they were distributed on chromosomes 1, 4, 6, and 11. Among them, Chr1_15896422 and Chr1_15896481 were significantly associated with all three traits (Figure 3G–I; Table 2). The LMM

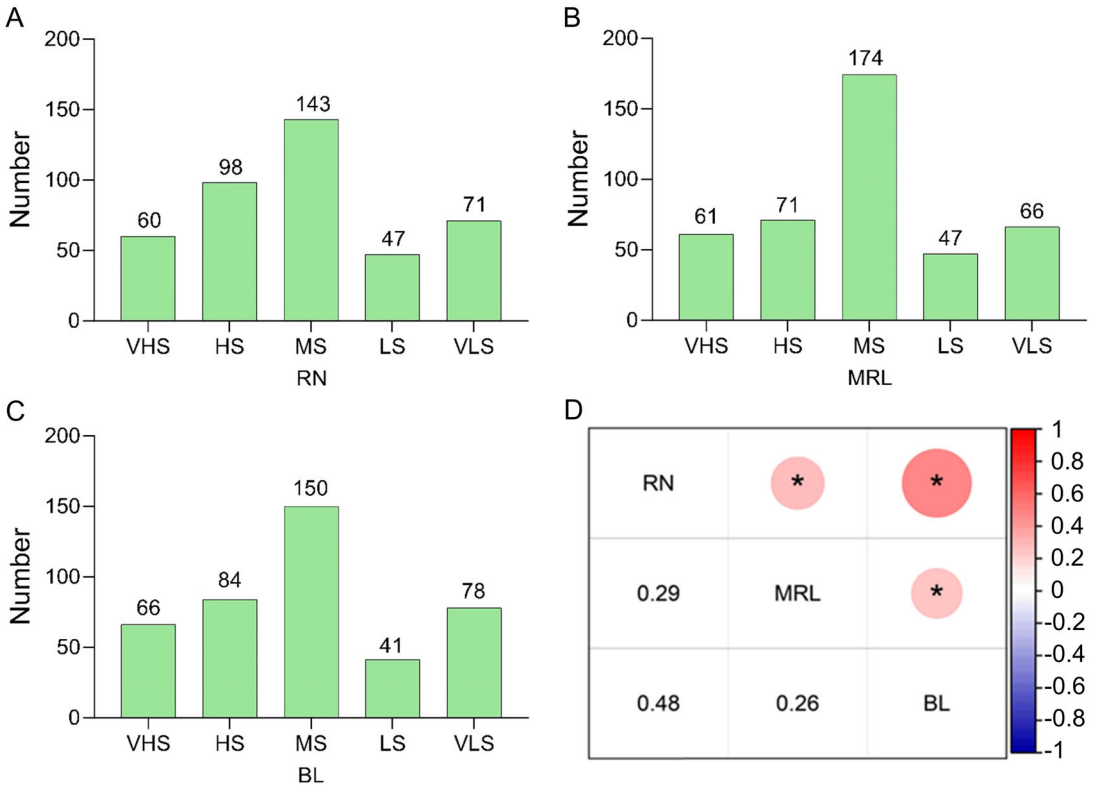

FIGURE 2 Evaluation result of chlorate sensitivity. (A–C) Sensitivity categories: VHS, very high sensitivity; HS, high sensitivity; MS, medium sensitivity; LS, low sensitivity; VLS, very low sensitivity. (D) Pearson correlation coefficient plot for three rice traits, with the color scale in the upper right indicating correlation strength. $*p \leq 0.05$. BL, bud length; MRL, main root length; RN, root numbers.

model detected a total of six significant SNPs associated with RN, MRL, and BL, which were distributed on chromosomes 1, 4, and 6. Among them, Chr1_15896422 and Chr1_15896481 were significantly associated with all three traits (Figure 3J–L; Table 2). We found some significant SNPs were associated with multiple models and traits. For example, Chr1_15896481 is a significant SNP associated with all three traits (RN, MRL, and BL) in the LM, LMM, and GLM models, and it is also associated with BL in the MLM model (Figure 3A–C,F–L). Therefore, this SNP is most likely related to $KClO_3$ treatment. Moreover, 98 significant SNPs were associated with three traits through 3VmrMLM. They were distributed on 12 chromosomes. Among these, Chr8_2870731, Chr10_3456904, and Chr12_24599311 were significantly associated with RN and BL. Within the linkage intervals of significant SNPs, four known and cloned NUE genes (*OsGS1;2*, *OsAMT5*, *OsNR2*, and *OsNPF3.1*) were identified (Figure 3M–O; Table S3).

3.3 | BSR-seq of candidate genomic regions related to chlorate sensitivity in rice

After filtering out low-quality reads and adaptors, we obtained a total of 45.54 Gb of clean data: 12.01 Gb from CL, 11.66 Gb from CH, 11.53 Gb from TL, and 10.34 Gb from TH. The percentage of bases with a quality score of Q30 was over 94.01%, and the GC content ranged from 47.43% to 49.06%. The clean reads were individually aligned to the Nipponbare reference genome, achieving alignment rates of 99.30% to 99.60%. The results of the above comparisons were processed using the Best Practices process of the GATK software, and SNP and InDel detection was performed using the Haplotyper method of GATK. Following this, low-grade SNPs and InDels were filtered out. A total of 1,186,370 SNPs and 386,429 InDels were derived from four mixed pools. High-quality SNPs and InDels were analyzed using ED and index-slid algorithms.

According to the correlation threshold, we used the ED arithmetic to identify eight candidate regions associated with CL versus CH, covering 1.44 Mb and containing a total of 246 genes, and five regions associated with TL versus TH, spanning 1.7 Mb and containing 324 genes. Among these, seven candidate regions were specific to CL versus CH, and four were specific to TL versus TH (Figure 4A). Additionally, index-slid analysis identified seven candidate regions associated with CL versus CH, covering 1.07 Mb with 187 genes, and three regions from TL versus TH, spanning 1.87 Mb and containing 362 genes. Among these, five candidate regions were specific to CL versus CH, and one was

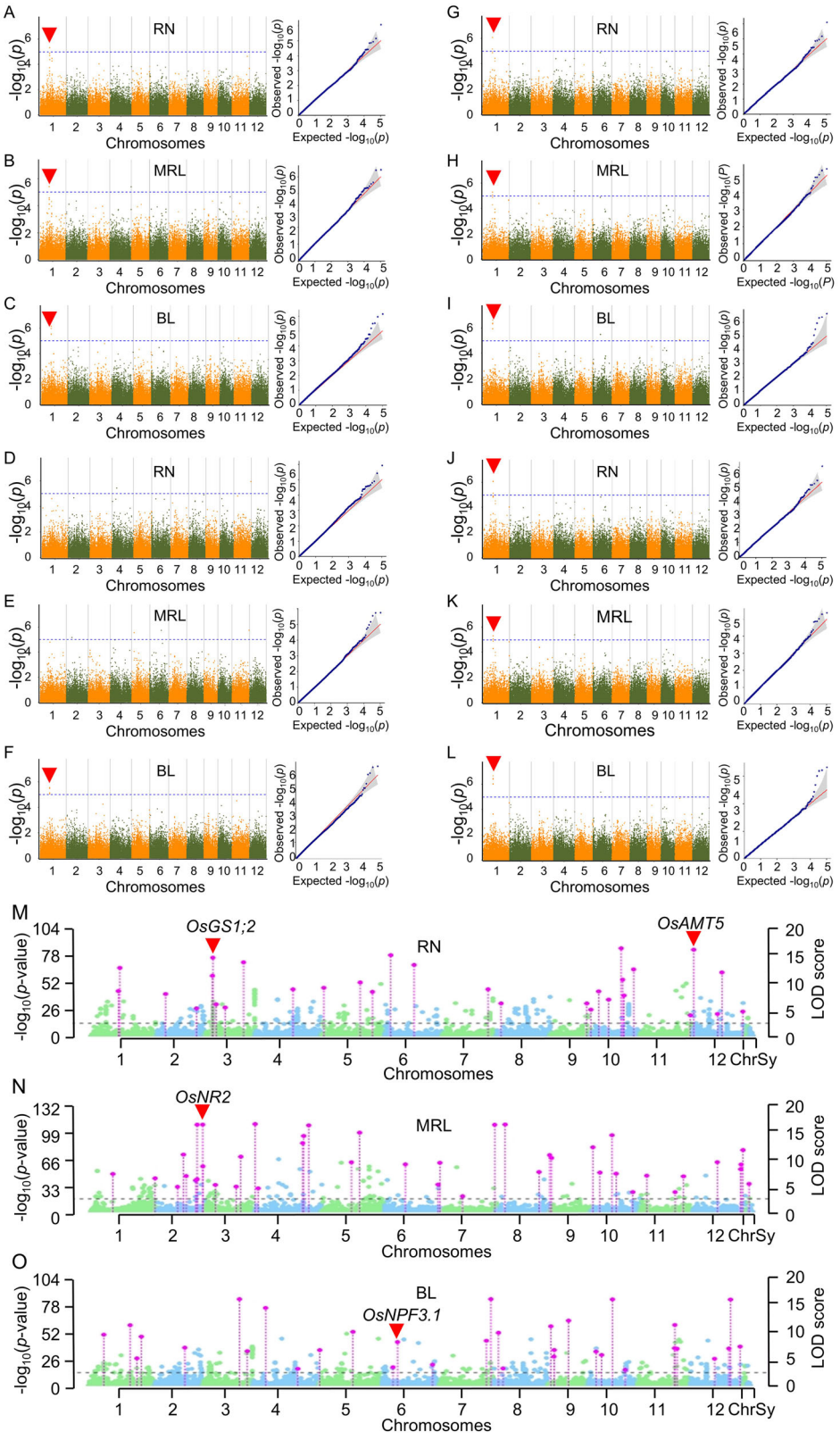

FIGURE 3 Manhattan plots and Q–Q plots of genome-wide association study for three rice traits. (A–C) Manhattan and Q–Q plots for general linear model (GLM). (D–F) Manhattan and Q–Q plots for mixed linear model (MLM). (G–I) Manhattan and Q–Q plots for linear model (LM). (J–L) Manhattan and Q–Q plots for linear mixed model (LMM). (A–L) The blue dotted line represents the threshold line, $p = 1 \times 10^{-5}$. (A–C) and (F–L) Red arrows indicate significant locus that can be further investigated; (M–O) Manhattan plots for 3VmrMLM. Red arrows indicate significant loci associated with cloned N efficiency genes; The black dotted line represents the threshold line, logarithm of odds ≥ 3. BL, bud length; MRL, main root length; RN, root numbers.

TABLE 2 Genome-wide association study (GWAS) of significant single-nucleotide polymorphisms (SNPs) statistics.

Traits	Chromosome	Position (bp)	*p*-value	Model
RN	1	15,896,481	4.67E-06	GLM
MRL	1	15,896,626	3.82E-06	GLM
MRL	4	35,196,583	3.97E-06	GLM
BL	1	15,896,626	7.12E-07	GLM
BL	1	15,896,422	7.12E-07	GLM
BL	1	15,896,481	2.94E-06	GLM
BL	1	15,896,457	3.13E-06	GLM
BL	11	7,844,698	6.58E-06	GLM
RN	4	7,791,301	3.79E-06	MLM
RN	11	28,171,917	1.17E-06	MLM
MRL	2	9,171,281	6.89E-06	MLM
MRL	5	4,009,946	2.82E-06	MLM
MRL	6	18,808,478	1.84E-06	MLM
MRL	11	28,171,917	1.79E-06	MLM
BL	1	15,896,626	2.85E-06	MLM
BL	1	15,896,422	3.26E-06	MLM
BL	1	15,896,481	7.29E-06	MLM
BL	1	15,896,457	8.86E-06	MLM
RN	1	15,896,481	8.29E-07	LM
RN	1	15,896,422	6.92E-06	LM
MRL	1	15,896,481	5.18E-06	LM
MRL	1	15,896,422	9.11E-06	LM
MRL	4	35,196,583	4.16E-06	LM
BL	1	15,896,422	2.43E-07	LM
BL	1	15,896,457	4.27E-07	LM
BL	1	15,896,481	4.86E-07	LM
BL	1	15,896,626	1.18E-06	LM
BL	6	12,554,764	3.24E-06	LM
BL	11	7,844,698	8.84E-06	LM
RN	1	15,896,481	8.66E-07	LMM
RN	1	15,896,422	7.46E-06	LMM
MRL	1	15,896,481	4.98E-06	LMM
MRL	1	15,896,422	9.13E-06	LMM
MRL	4	35,196,583	4.07E-06	LMM
BL	1	15,896,422	2.15E-07	LMM
BL	1	15,896,481	3.54E-07	LMM
BL	1	15,896,457	4.00E-07	LMM
BL	1	15,896,626	9.48E-07	LMM
BL	6	12,554,764	4.20E-06	LMM

Abbreviations: BL, bud length; GLM, general linear model; LM, linear model; LMM, linear mixed model; MLM, mixed linear model; MRL, main root length; RN, root numbers.

specific to TL versus TH (Figure 4B). By combining the results from both the ED algorithm and index-slid analysis, we found that regions 15,777,420–15,934,761 bp on chromosome 1, 27,709,426–27,998,586 bp on chromosome 2, and 17,550,020–17,648,444 bp and 17,780,857–17,813,535 bp on chromosome 5 were identified by both methods (Table 3). These candidate regions were only detected in the comparison of CL versus CH. Therefore, these results indicate a high likelihood that these candidate regions are associated with $KClO_3$ treatment.

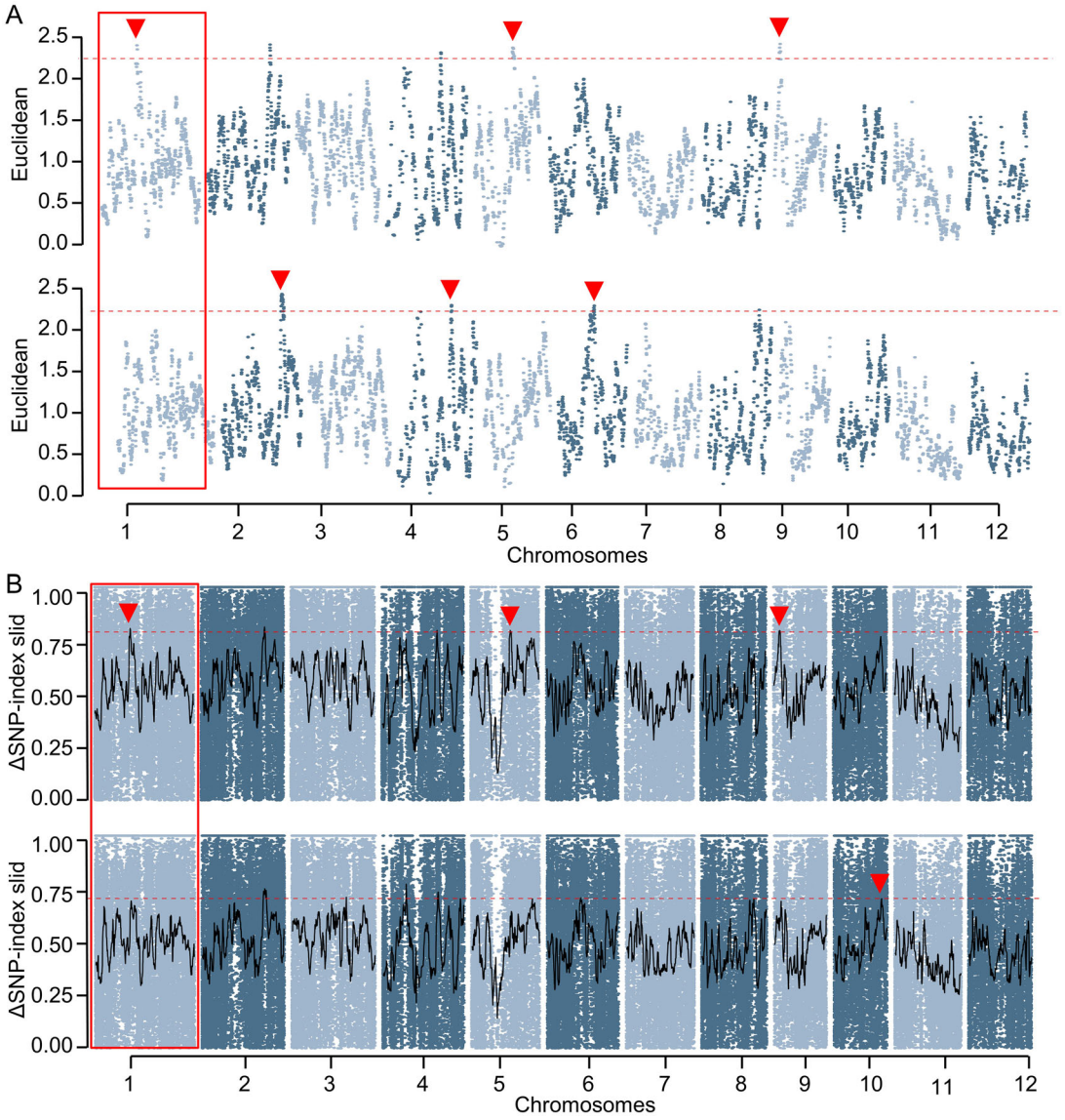

FIGURE 4 Bulked segregant RNA sequencing (BSR-seq) analysis result. (A) The Euclidean distance (ED) algorithm analysis result. (B) The index-slid analysis result. The *x*-axis represents chromosome names, and the *y*-axis shows *p*-value; The red dotted line marks the significant threshold, and red arrows indicate candidate region specific to CL versus CH and TL versus TH.

3.4 | Integrated identification of candidate genomic regions by GWAS and BSR-seq

One significant locus (Chr1_15896481) overlapped with a candidate region (Chr1: 15777420–15934761 bp) in BSR-seq. Both association analysis methods implicate the same candidate regions, highlighting this interval for further study. According to the LD decay value, there were 70 candidate genes in the upstream and downstream 150 kb centered on this candidate region. These candidate genes included 32 transposon genes, 17 expressed genes, and 21 putative functional genes. The transposon genes and duplicate genes were removed, leaving 37 genes as possible candidate genes. In these possible candidate genes, we found that *Os01g0379400* harbored 90 SNPs and 14 InDels. This gene encodes a protein containing an F-box domain. *Os01g0382000* harbored one SNP. Previous studies have reported that the expression of this gene is affected by plant hormones (Agrawal et al., 2000). In addition, we found that *Os01g0378400* belongs to the NAC transcription factors. Previous research indicates that OsNAC42, a protein with a NAC domain, can bind to the OsNPF6.1 promoter under low N conditions, activating *OsNPF6.1* expression and enhancing NO_3^- uptake in rice (Tang et al., 2019). Within the NAC transcription factors family, several genes have been identified that affect aspects of plant N metabolism, photosynthesis, tillering and yield, etc. Therefore, *Os01g0378400* may have a similar function. We also found *Os01g0377700*, homologous to *Cre06g293051*, a

TABLE 3 Candidate region analysis for bulked segregant RNA sequencing (BSR-seq).

Method	CL vs. CH	TL vs. TH
ED	Chr1: 15,777,420–15,934,761 bp	Chr2: 26,853,140–27,841,182 bp
	Chr2: 27,802,035–27,998,586 bp	Chr2: 28,080,553–28,124,878 bp
	Chr4: 24,254,662–24,343,780 bp	Chr4: 104,52,751–10,618,565 bp
	Chr5: 17,014,786–17,247,756 bp	Chr4: 24,233,530–24,435,934 bp
	Chr5: 17,306,005–17,449,986 bp	Chr6: 16,240,998–16,542,244 bp
	Chr5: 17,550,020–17,648,444 bp	
	Chr5: 17,780,857–17,813,535 bp	
	Chr9: 2,604,498–3,091,487 bp	
Index-slid	Chr1: 15,777,420–15,934,761 bp	Chr2: 26,853,140–28,247,511 bp
	Chr2: 27,709,426–27,998,586 bp	Chr4: 24,051,080–24,484,225 bp
	Chr4: 24,254,662–24,343,780 bp	Chr10: 20,851,257–20,894,778 bp
	Chr5: 17,433,197–17,449,986 bp	
	Chr5: 17,550,020–17,648,444 bp	
	Chr5: 17,780,857–17,813,535 bp	
	Chr9: 2,705,335–3,091,487 bp	

Abbreviations: CH, pre-treatment high-sensitivity pool; CL, pre-treatment low-sensitivity pool; ED, Euclidean distance; TH, post-treatment high-sensitivity pool; TL, post-treatment low-sensitivity pool.

known ammonia N transporter in *Chlamydomonas reinhardtii* (*Chlamydomonas reinhardtii* K.), suggesting *Os01g0377700* may have a similar function. These candidate genes show strong potential for impacting NUE and warrant further investigation.

3.5 | Mining N-related genes by RNA-seq

In order to further analyze the regulatory network of nitrogen metabolism, we conducted RNA-seq analysis on the materials used in BSR-seq. To minimize experimental operation errors, we evenly divided the total amount of each pooled sample in BSR-seq into three equal parts. RNA-seq of the 12 rice samples produced 102.61 Gb clean data, with high-quality bases (Q30) exceeding 93.99%. Clean reads from each sample were sequentially aligned to the Nipponbare reference genome, achieving alignment rates between 92.68% and 96.55%. Cluster analysis of biological replicates, based on transcriptomic data, showed clear separation between different treatment conditions and tight clustering within the same treatment conditions, confirming the reliability of the data (Figure 5C).

After RNA-seq and quality control, we identified a total of 41,806 genes, including 40,046 known genes and 1760 newly discovered ones. Based on the quantitative expression data, differential expression analysis was conducted using DESeq2 software, with threshold set at $|\log_2 (\text{fold change})| \geq 1$ and $p < 0.05$, resulting in 10,731 DEGs. Between pre-treatment samples (CL vs. CH), we identified 6699 DEGs, consisting of 2824 significantly upregulated and 3875 significantly downregulated genes (Figure 5A). In post-treatment samples (TL vs. TH), we found 7951 DEGs, with 2840 genes significantly upregulated and 5111 significantly downregulated (Figure 5B). Hierarchical clustering of these DEGs revealed distinct expression patterns: most genes upregulated in CL versus CH were downregulated in TL versus TH, and vice versa, indicating that these DEGs may be strongly associated with chlorate sensitivity in rice (Figure 5C). After excluding duplicate transcripts, a total of 3919 DEGs were shared between CL versus CH and TL versus TH. Notably, more DEGs were uniquely observed in TL versus TH (4032) than in CL versus CH (2780) (Figure 5D). Here, we found that three of the 37 possible candidate genes were significantly differentially expressed: *Os01g0383700, Os01g0382400*, and *Os01g0382900*. Among them, *Os01g0383700*, a gene belonging to the WD40 family, caught our attention. This gene may play an important role in the differences in chlorate sensitivity of rice and deserves focused attention.

3.6 | GO and KEGG pathway enrichment analyses of DEGs

To investigate molecular changes in rice before and after $KClO_3$ treatment, DEGs were analyzed through GO and KEGG pathway enrichment analyses. Unique DEGs from the CL versus CH and TL versus TH were analyzed through GO. We found that the "Nutrient reservoir activity" term was enriched only in TL versus TH (Figure 6A,B), which includes *OsNR1.2*, the gene linked to nitrate assimilation. We found that enrichment analysis for KEGG pathway

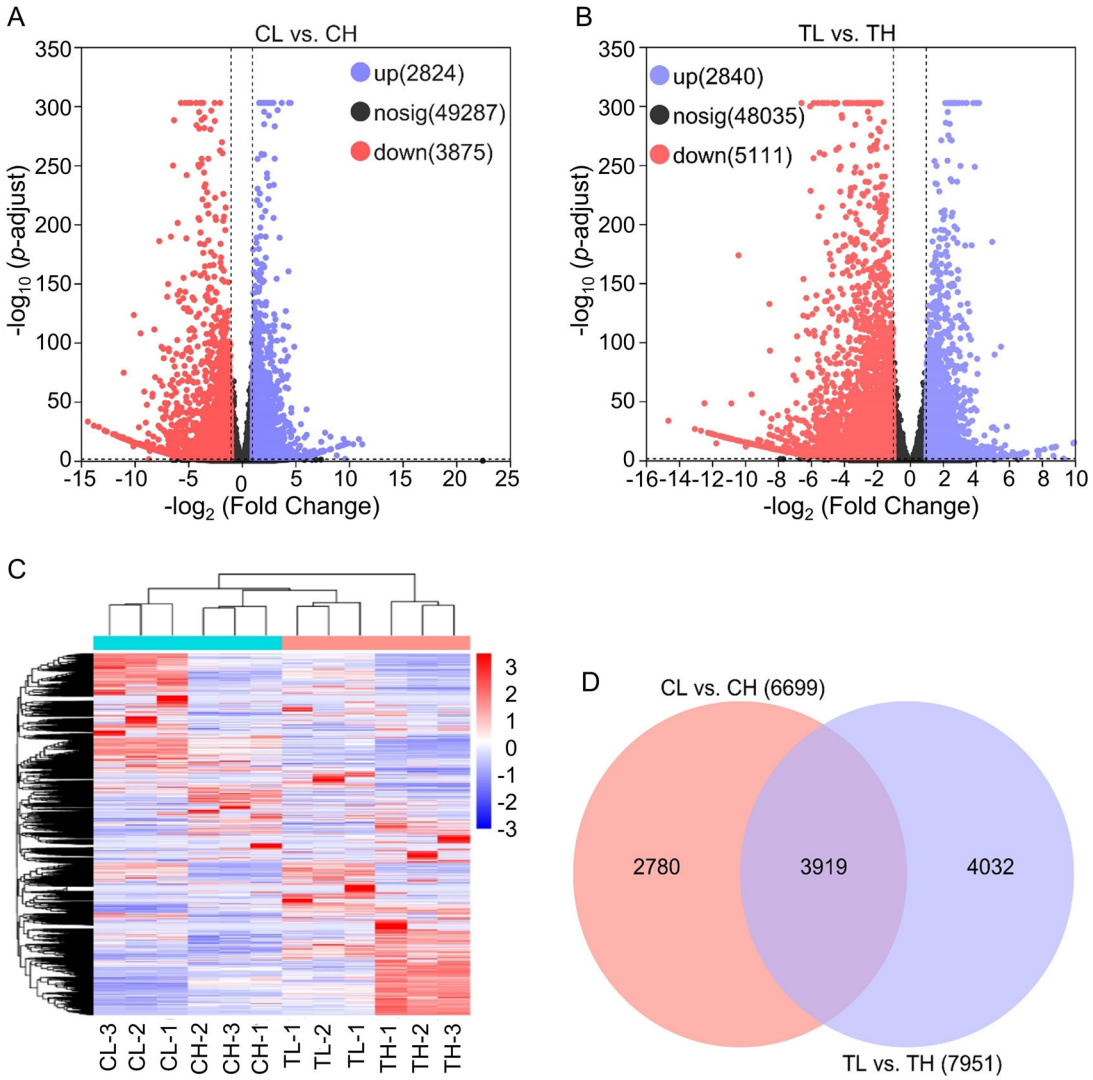

FIGURE 5 Differentially expressed genes between low-sensitivity and high-sensitivity rice lines before and after $KClO_3$ treatment. (A, B) Volcano plot displaying differentially expressed genes (DEGs) in the pairwise comparisons: (A) CL versus CH (low sensitivity vs. high sensitivity before treatment) and (B) TL versus TH (low sensitivity vs. high sensitivity after treatment). (C) Hierarchical clustering analysis of DEGs for CL versus CH and TL versus TH comparisons, with downregulated genes shown in blue and upregulated genes in red, highlighting distinct expression patterns between conditions. (D) Venn map analysis showing DEGs shared and unique to the CL versus CH and TL versus TH comparisons.

revealed pathways related to N and energy metabolism, such as "N metabolism," "Photosynthesis," "Photosynthesis-antenna proteins," and "Plant hormone signal transduction." These pathways included N-related genes, with a greater number of DEGs in the TL versus TH (Figure 6C,D). Notably, known N-related genes were enriched only in the "N metabolism" pathway of TL versus TH. They are *OsNR1.2*, *OsNRT2.4*, *OsGS1;3*, *OsGS2*, and *Fd-GOGAT1*. This indicates that $KClO_3$ treatment has a significant impact on the expression of N-related genes, potentially disrupting the normal N metabolism process in rice and warranting further investigation into the underlying molecular mechanisms.

3.7 | Expression validation of candidate genes

The 37 candidate genes on chromosome 1 and some known N-related genes, *OsNR2*, *OsGS1;2*, and *OsNR1.2*, were verified by RT-qPCR in 20 different rice varieties, including very low-sensitive and very high-sensitive varieties. Then we statistically analyzed the percentage of genes expressed with a significant difference between CL versus CH and TL versus TH. We found 13 genes with significant differences in expression levels between CL and CH and TL and TH in more than half of the materials (Figure S2; Table S4). We carried out functional annotation, expression level analysis,

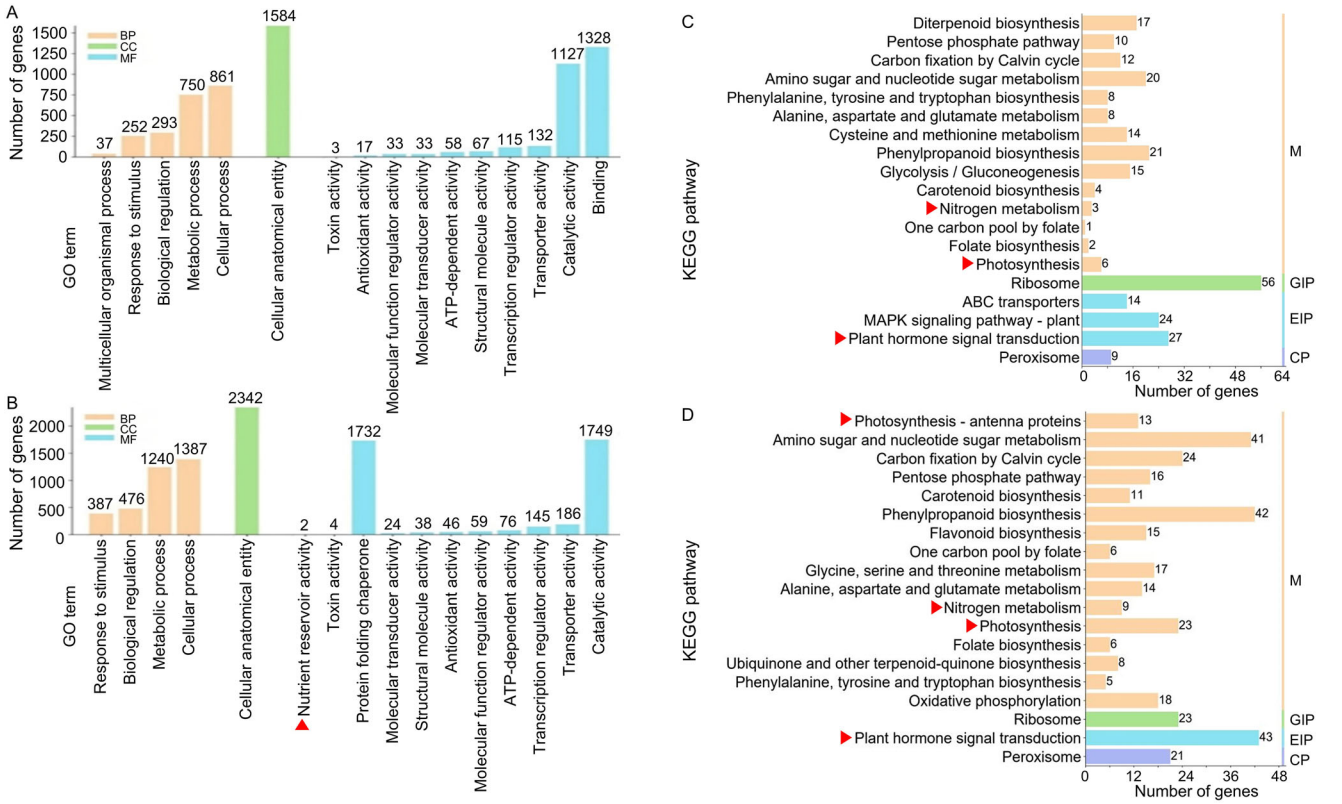

FIGURE 6 Gene ontology (GO) term and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses of differentially expressed genes (DEGs). (A) GO analysis for CL versus CH unique DEGs. (B) GO analysis for TL versus TH unique DEGs. (C) KEGG pathways analysis for CL versus CH unique DEGs. (D) KEGG pathways analysis for TL versus TH unique DEGs. BP, biological process; CC, cellular component; MF, molecular function; M, metabolism; GIP, genetic information processing; EIP, environmental information processing; CP, cellular processes. (B) Red arrow indicate TL versus TH-specific DEGs enriched in pathways that may be related to N metabolism. (C, D) Red arrows indicate pathways that may contain genes related to N.

sequence alignment, and structural analysis on these 13 genes. Finally, we determined that *Os01g0377700*, *Os01g0382800*, *Os01g0383700*, *Os01g0379400*, and *Os01g0378400* were the most likely candidate genes (Figure 7A–E). *Os01g0377700* is homologous to *Cre06g293051*. Cre06g293051 is an ammonium transporter protein that participates in the transport of NH_4^+. We speculate that *Os01g0377700* may have similar functions to *Cre06g293051* and participate in N transport. *Os01g0382800* is a DYW-type PPR protein. Studies have shown that PPR proteins with DYW domains play an important role in plant photosynthesis. It has an 80% difference in expression levels between CL versus CH and TL versus TH (Figure 7B). *Os01g0383700* encodes a WD protein. We found that there was a significant difference in the expression of this gene in RNA-seq. Through RT-qPCR, we discovered that there was a 60% difference in its expression level when comparing CL versus CH and TL versus TH (Figure 7C). *Os01g0379400* encodes a protein containing an F-box domain. We found that there were SNPs and InDels variations in this gene during the BSR-seq analysis. Here, we also discovered that the expression level of this gene differed before and after chlorate treatment in multiple groups of materials. Therefore, this gene can be regarded as one of the most likely candidate genes for further research. The expression patterns of *Os01g0378400* in sensitive and tolerant rice varieties were similar to those of known N-related genes (Figure 7E,F). *Os01g0378400* belongs to the genes of the NAC transcription factor family. Multiple genes in this family have been confirmed to be associated with N metabolism, photosynthesis, the number of tillers, the yield per plant, and so on. Therefore, *Os01g0378400* deserves further investigation.

4 | DISCUSSION

4.1 | Determination of candidate intervals

GWAS is widely used to identify genes associated with complex traits (C. J. Li et al., 2022; Visscher et al., 2012; Yoshida et al., 2022; Y. Yu et al., 2022). BSR-seq, the newer approach, enhances mapping efficiency by integrating bulked segregant analysis with the convenience of RNA-seq, allowing for precise interval identification (Wu et al., 2018; F. Q. Yu et al., 2016). In this study, we found one significant locus from GWAS overlapping with a candidate region in BSR-seq. We

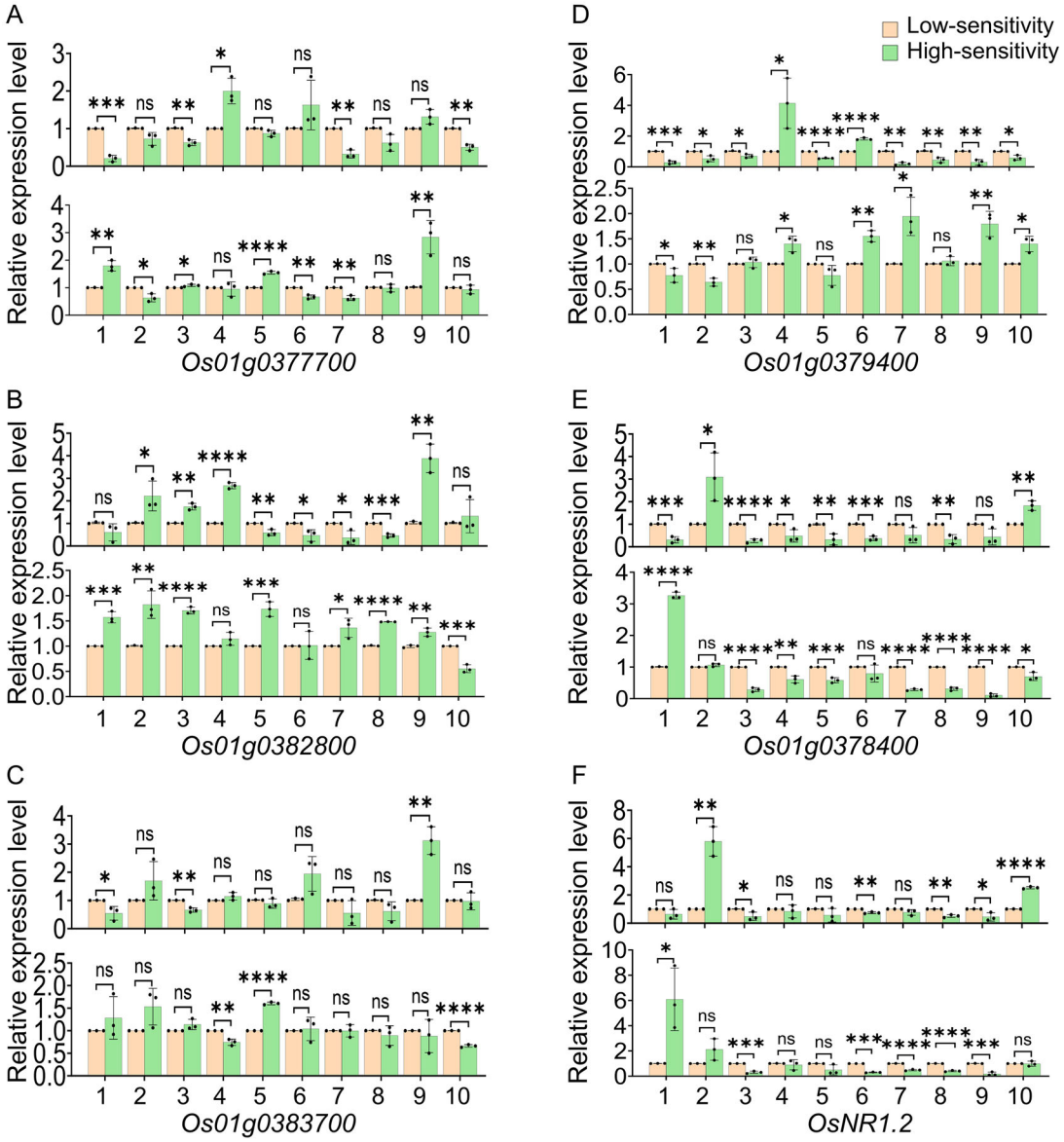

FIGURE 7 Expression analysis of key candidate genes and known N-related genes. (A–E) The expression levels of the most likely candidate genes (*Os01g0377700*, *Os01g0382800*, *Os01g0383700*, *Os01g0379400*, and *Os01g0378400*). (F) The expression levels for one known N-related genes (*OsNR1.2*). In each figure, the top half denotes CL versus CH, and the bottom half denotes TL versus TH. The expression levels were measured in 10 low-sensitivity varieties (orange) and 10 high-sensitivity varieties (green), with values normalized to the expression of the rice *Actin* gene. The *y*-axis indicates the level of gene expression. The *x*-axis represents the material used to detect the amount of gene expression (1: C197 and C7; 2: C198 and C15; 3: C208 and C32; 4: C242 and C39; 5: C247 and C83; 6: C248 and C143; 7: C249 and C152; 8: C309 and C164; 9: C315 and C338; 10: C316 and C383). Since the expressions in CL versus CH and TL versus TH were opposite, we determined that the expression of this gene in this variety was significantly different before and after the $KClO_3$ treatment. ns indicates no significant difference, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

utilized GWAS and BSR-seq analyses to identify the relevant candidate intervals that contribute to rice chlorate sensitivity differences, which improved the accuracy of the mapping.

4.2 | Analysis of known N-related genes

In this study, we identified several known N-related genes, including *OsNR2* (Z. Gao et al., 2019), *OsNR1.2* (Han et al., 2022), *OsGS1;2* (Teng et al., 2006), *OsGS1;3* (Tabuch et al., 2005), *OsGS2* (Cai et al., 2010), *Fd-GOGAT1* (H. L. Chen et al., 2016), *OsNRT1.1B* (Hu et al., 2015), and *OsNRT2.4* (Wei et al., 2018). In sensitive varieties treated with $KClO_3$, expression levels of *OsNR1.2*, *OsGS2*, *Fd-GOGAT1*, *OsNRT2.4*, and *OsNRT1.1B* were significantly lower than in tolerant varieties. This suggests that chlorate, when taken up by the plant, is mistaken for nitrate when it is taken up by the plant, occupies nitrate assimilation channels, inhibits normal functions of N assimilation pathways, and leads to reduced gene expression (Kabange et al., 2020). There is feedforward

amplification interaction between *OsNR2* and *OsNRT1.1B*, each of which can enhance the expression of the other (Z. Gao et al., 2019). Our study found that the expression levels of *OsNR1.2* and *OsNRT1.1B* were significantly downregulated in TL versus TH. Therefore, there may also be a feedforward interaction amplification relationship between *OsNR1.2* and *OsNRT1.1B*. When rice seedlings were treated with $KClO_3$, chlorate competed for nitrate assimilation channels, and the expression of *OsNR1.2* was downregulated. When there is a feedforward interaction with an amplification effect between *OsNR1.2* and *OsNRT1.1B*, the expression of *OsNRT1.1B* will also be downregulated. Moreover, we found that the expression level of *OsNRT2.4* was downregulated in TL versus TH. *OsNRT2.4* plays a crucial role in the transport of nitrate, which facilitates its movement from the underground parts of the plant to the above-ground parts (Kant, 2018). Additionally, it is involved in the redistribution of nitrate from older leaves to new leaves and roots (Wei et al., 2018). It is shown that *NRT2.4* expression is repressed when N demand is met, creating a negative feedback regulation imposed by high N levels (either at whole plant levels or from external high nitrate) (Vidal et al., 2020). With $KClO_3$ treatment, the nitrate assimilation process is disrupted, leading to reduced expression of *OsNRT2.4*. We found that the expression levels of *OsNRT2.4* in the sensitive varieties were significantly lower than those in the tolerant varieties. Thus, in sensitive varieties, chlorate may occupy the nitrate assimilation pathway, causing plants to misjudge the N levels, which in turn leads to the reduced expression of *OsNRT2.4*. Previous studies have shown that certain transcription factors regulate genes involved in nitrate transport and reduction processes. For instance, NLP7 activates the transcription of many nitrate-responsive genes, such as NR-related genes, *NRT2.1*, and *NRT2.2* (Castaing et al., 2009; R. C. Wang et al., 2009). Therefore, the decrease in *OsNR1.2* expression could lead to reduction of the activity of its regulatory transcription factor, affecting expression of related nitrate transport genes, potentially including *OsNRT2.4*.

4.3 | Five candidate genes were identified as potentially associated with NUE in rice

We selected the 37 candidate genes for RT-qPCR analysis. We found that the expression pattern of *Os01g0378400* is similar to that of some known N-related genes (Figure 7E,F). *Os01g0378400* belongs to the NAC transcription factors, which are involved in plant N metabolism. For instance, the OsNAC42 protein binds to the OsNPF6.1 promoter, activates its expression, and enhances NO_3^- uptake in rice (Tang et al., 2019). Additionally, the transcription factor *OsSNAC1* is known to positively regulate the expression of NO_3^- transporters like OsNRTs, which improves N uptake efficiency in rice (Qi et al., 2023). Another NAC-domain transcription factor, *ANAC055*, plays a role in N deficiency-induced leaf yellowing, and its expression pattern under N-deficient conditions mirrors that of the typical N deficiency-inducible gene *NRT2.5* (Sakuraba et al., 2023). Thus, we hypothesize that *Os01g0378400* is a strong candidate gene that is likely to play an important role in NO_3^- assimilation. *Os01g0383700* encodes a protein with a WD40-repeat domain. We found that it had significantly different expression levels by RNA-seq, as well as a 60% difference in expression levels between CL versus CH and TL versus TH by RT-qPCR (Figure 7C). In BSR-seq, we also found a gene *OsTTG1* with a WD40-repeat domain, which we previously studied. *OsTTG1* is a previously reported gene related to anthocyanins (X. H. Yang et al., 2021), was also found in earlier studies to interact with multiple N-related genes, which was indicated by RNA-seq analyses of wild-type, *osttg1*, and *OsTTG1*-OE materials (Zhu et al., 2024). Therefore, we conjectured that *Os01g0383700* and *OsTTG1* may be related to nitrate assimilation. At the same time, other genes with significant expression differences between CL versus CH and TL versus TH in over half of the varieties examined are also noteworthy. The *Os01g0377700*, homologous to *Cre06g293051*, encodes a transporter-like protein associated with ammonia N transport, suggesting that *Os01g0377700* may have a similar function. *Os01g0382800* is a DYW-type PPR protein. Studies have shown that PPR proteins with DYW domains play an important role in plant photosynthesis. It has an 80% difference in expression levels between CL versus CH and TL versus TH (Figure 7B). The *Os01g0379400* has an F-box domain. We found that there were SNPs and InDels in this gene during the BSR-seq analysis. It has a 60% difference in expression levels between CL versus CH and TL versus TH (Figure 7D). So, this gene is likely related to N metabolism. We know that chlorate and nitrate have similar structures and are competitive inhibitors of NR, inhibiting the nitrate reduction process and hindering the utilization of nitrate. Most of the rice lacking NR activity is chlorate-resistant, so screening natural populations of rice with chlorate can help to identify key NR-related genes from them. In this study, we identified five candidate genes that were most likely to be related to NR. These candidate genes, particularly *Os01g0378400*, *Os01g0379400*, and *Os01g0383700*, warrant further investigation to clarify their potential roles in N assimilation.

5 | CONCLUSION

In this study, GWAS were used to detect chlorate sensitivity-related loci in 419 rice landraces. Under the GLM, MLM, LM, and LMM, we found six, nine, seven, and six significant SNPs associated with three traits. Moreover, 98 significant SNPs were associated with three traits through 3VmrMLM. Additionally, a total of 18 candidate regions were identified using BSR-seq. An overlapping candidate interval was

obtained by GWAS and BSR-seq analysis. According to the LD decay value, there were 70 candidate genes within 150 kb upstream and downstream centered on this candidate interval. Through RNA-seq analysis, we found that after being treated with chlorate, the expression levels of multiple genes related to nitrate assimilation in sensitive rice varieties were significantly lower than those in tolerant rice varieties. Therefore, the transcriptional levels of genes related to nitrate assimilation may change after being treated with chlorate. We carried out functional annotation, expression level analysis, sequence alignment, and structural analysis on these candidate genes. Finally, we determined that *Os01g0377700*, *Os01g0382800*, *Os01g0383700*, *Os01g0379400*, and *Os01g0378400* were the most likely candidate genes, among which *Os01g0378400*, *Os01g0379400*, and *Os01g0383700* warrant further investigation to clarify their potential roles in N assimilation. This study provides a theoretical foundation for genetic improvement in NUE in rice.

AUTHOR CONTRIBUTIONS

Shuangshuang Luo: Conceptualization; visualization; writing—original draft; writing—review and editing. **Zuyu Liao**: Investigation; validation. **Shilv Huang**: Supervision; writing—review and editing. **Xiuzhong Xia**: Data curation; software. **Zongqiong Zhang**: Investigation; resources. **Baoxuan Nong**: Funding acquisition. **Tongping Luo**: Formal analysis. **Chenli Zhu**: Methodology; validation. **Can Chen**: Project administration. **Hui Guo**: Project administration. **Rui Feng**: Project administration. **Yinghua Pan**: Supervision. **Shuhui Liang**: Data curation; project administration. **Yongcheng Li**: Validation. **Jianhui Liu**: Validation; writing—original draft. **Yongfu Qiu**: Supervision. **Danting Li**: Project administration; resources; writing—review and editing. **Xinghai Yang**: Funding acquisition; methodology; supervision; writing—review and editing.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed in this study are included in this published article and its supplementary information files. The raw sequences generated in this study were deposited at the NCBI under accession number.

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