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Genome-wide association mapping identifies stable quantitative trait loci and candidate genes associated with heat stress resilience in rice seedlings

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Abstract

Heat stress is an increasing threat to rice production, necessitating the identification of genomic regions and candidate genes underlying heat stress resilience for crop improvement. In this study, we investigated the genetic basis of post-heat stress survival at the seedling stage using a genome-wide association study (GWAS) across two independent experiments. A total of nine quantitative trait loci (QTLs) associated with heat stress resilience were identified using a significance threshold of −log10(P) ≥ 3.0. Among these, *qSR3, qSR6*, and *qSR8* were consistently detected in both trials, highlighting their stability and potential value for molecular breeding. Several QTL regions co-localized with previously characterized stress-responsive genes, including *OsPTR1, OsCGT*, and *OsDSR-1*, suggesting the involvement of conserved stress adaptation pathways. Candidate gene analysis within the stable QTL regions identified 82 putative genes, from which *LOC_Os03g04370* was prioritized as a leading candidate based on its localization within the stable qSR3 locus, annotation as a protein-methionine-S-oxide reductase, conserved NAD(P)-binding domains, and potential role in oxidative stress regulation. Quantitative real-time PCR confirmed significant heat-induced upregulation of *LOC_Os03g04370* in rice varieties BNP162 and BNP206. Furthermore, NBT and DAB staining revealed differential accumulation of superoxide and hydrogen peroxide between heat-tolerant and heat-sensitive varieties, supporting the involvement of *LOC_Os03g04370* in maintaining redox homeostasis under heat stress. Collectively, this study identifies stable genomic regions and prioritizes candidate genes underlying seedling heat stress resilience in rice, providing valuable genetic resources for future functional validation and the development of heat-resilient rice cultivars under changing climatic conditions.

Introduction

Rising global temperatures due to climate change pose a significant threat to agricultural crop productivity worldwide. High temperatures, which are defined as temperatures > 30°C affect crop yields by direct and indirect effects (Schauberger et al., 2017). Research Indicates that for every 1°C increase in the global average temperature, there is a 3.2% reduction in global rice yields (Zhao et al., 2017). Furthermore, studies have demonstrated a decrease in the rate of yield increase for major cereal crops, which has negative implications for food security as the world population continues to grow (Wang et al., 2018). With an estimated 70% increase in food production needed to support a projected population of 9 billion people by 2050, there is a pressing need to prioritize the development of heat-tolerant crop varieties to mitigate the effects of climate change and enhance crop production.

Rice (*Oryza sativa* L.) is the main food crop for almost half of the global population (Geng et al., 2024; Priya et al., 2019; Rohman et al., 2014; Sarma et al., 2023; Xu et al., 2021). The ideal average temperature for rice plants is 28°C during the day and 22°C at night. While rice can tolerate temperatures up to 32°C without a significant reduction in grain yield, temperatures above 30°C have a negative impact on all stages of rice plant growth and development (Fahad et al., 2019). High temperatures adversely affect physiological processes and grain yield (Neupane et al., 2022; Xu et al., 2021). To meet the growing demand for rice, it is necessary to develop stable cultivars with high yield potential and resistance to

high-temperature stresses. High temperatures during the seedling stage can result in reduced germination, increased seedling mortality, stunted seedlings, and leaf yellowing or wilting, ultimately leading to lower yields (Samtani et al., 2022; Xu et al., 2021). Therefore, improving heat stress tolerance at the seedling stage is crucial for enhancing rice yield and increasing production.

Research on heat tolerance in rice has predominantly concentrated on the reproductive stage, a pivotal phase directly influencing grain yield. Various high-temperature-associated Quantitative Trait Loci (QTLs) have been unearthed across different rice populations (Huang et al., 2023; Xu et al., 2021),(Ps et al., 2017). Notably, *qSF1, qSF2, qSF3.2, qSSIPSS12.1, qSTIPSS9.1, qSTIY3.1*, and *qSTIY5.1*, were identified during flowering through sequencing-assisted approaches (Xu et al., 2021). Additionally, QTLs like *qWB8*, governing white-back and basal-white grains, have been linked to enhancing rice grain quality under heat stress (Wada et al., 2015). Furthermore, QTLs tied to spikelet fertility in response to heat stress at flowering, such as *qHTSF1.2, qHTSF2.1, qHTSF3.1*, and *qHTSF4.1*, have been identified (Ye et al., 2015). However, the genetic regulation of heat stress tolerance at other developmental stages remains elusive, with scant QTLs identified at the seedling stage. Notably, *OsHTAS*, a dominant major QTL on chromosome 9, has been cloned for conferring tolerance to 48°C temperatures (Liu et al., 2016), alongside the identification of numerous QTLs for seedling growth under heat stress, including *RLHT5.1* for root length (Kilasi et al., 2018). Thus, there exists a critical gap in understanding heat stress tolerance at the seedling stage.

Heat shock factors (HSFs) are key regulators of the heat stress response in plants. These transcription factors play a crucial role in controlling the expression of heat shock proteins (HSPs), which protect cells from the damaging effects of high temperatures by stabilizing denatured proteins and promoting cellular recovery (Bakery et al., 2024; Bunch & Calderwood, 2023). The role of HSFs in thermotolerance has been widely studied, with recent studies highlighting their potential for improving heat tolerance in crops. For instance, Sharma et al (Sharma et al., 2024) explored the molecular dialogue between light and temperature signalling in plants, revealing how HSFs integrate both pathways to enhance thermotolerance. Similarly, Samtani et al (Samtani et al., 2023) demonstrated that the ectopic overexpression of TaHsfA5 promotes thermomorphogenesis in Arabidopsis thaliana and enhances thermotolerance in rice (Oryza sativa), showing the promise of HSF manipulation for improving heat stress resistance in crops. These findings underscore the potential of targeting HSFs to improve heat tolerance across different plant species, including rice.

In the quest for novel genes associated with both biotic and abiotic stresses, the exploration of QTLs through Genome-Wide Association Studies (GWAS) within natural populations has emerged as a promising avenue. GWAS eliminates the necessity of constructing mapping populations and facilitates the simultaneous analysis of multiple alleles, capitalizing on recombination information derived from the extensive evolutionary history of natural populations (Li et al., 2021). Despite these advantages, the application of GWAS to investigate heat tolerance in rice at the seedling stage remains limited to date.

Unraveling the genetic basis of heat tolerance in rice seedlings is crucial. In our study, we analyzed a diverse population of 208 rice accessions, mainly Indica and Japonica varieties, with high genetic variability for GWAS. By subjecting the plants to high-temperature stress (45°C) and subsequent recovery at normal temperature, we identified nine candidate genes associated with *qSR3, qSR6*, and *qSR8* through GWAS. These genes offer potential for enhancing heat tolerance in rice breeding programs. Our research provides valuable insights into utilizing genetic diversity to develop heat stress-tolerant rice varieties, laying a strong foundation for future breeding efforts.

Materials and Methods

Plant Materials

In this investigation, we utilized a diverse population of 208 rice cultivars (Table S1) to evaluate rice heat tolerance during the seedling stage (Nyasulu et al., 2024). These cultivars were sourced from a variety of locations, primarily spanning 15 provinces across China, with additional samples obtained from the Philippines and Japan. The geographical distribution of these regions encompasses a broad range from 15° to 48° north latitude, covering temperate, tropical, and subtropical climates. Among the cultivars, 86 were classified as *Japonica* rice, while 124 were identified as *Indica* rice varieties and one as Intermediate variety (Table S1). The collection of rice material adhered to local laws and was carried out without any conflicts of interest. This diverse population was developed through multiple generations of cultivation in experimental fields located at Jiangxi Agricultural University in Nanchang, Jiangxi Province, and Linwang, Hainan Province.

Phenotyping

To ensure consistency, all rice seeds used in this experiment were harvested at physiological maturity, ensuring uniform seed development. Harvesting was performed at the same developmental stage to minimize variability in seed maturity. After collection, seeds were dried to a uniform moisture content of 12–14% using a shade-drying method with continuous air circulation to prevent heat damage. The dried seeds were then stored in airtight containers at 4°C to maintain viability and prevent deterioration before use.

To break dormancy, seeds underwent a 48-hour incubation period at 45°C in an oven. Post-dormancy treatment, seeds were surface-sterilized with sodium hypochlorite solution, followed by thorough rinsing with sterile water (Li et al., 2021). The seeds were then placed in Petri dishes and allowed to germinate under controlled conditions at 30°C for five days. After germination, twenty well-germinated seeds per rice cultivar were carefully transferred to new Petri dishes. This process was replicated three times to ensure the robustness and reliability of the results.

Heat stress tolerance at the rice seedling stage was quantified by assessing the survival rate of seedlings following heat stress exposure. The survival rate was determined by counting the number of

seedlings that exhibited visible signs of recovery, such as the emergence of new leaves and retention of green leaf tissue, 5 days after exposure to a heat stress treatment at 45°C for 52 hours (Wei et al., 2021). Seedlings that failed to show any growth or remained severely wilted were considered non-survivors. Seedling survival rates (SR) were assessed after the recovery period and served as a key indicator of heat stress tolerance at the seedling stage.

Quality control measures were rigorously implemented throughout the experiment to ensure accuracy and reliability of the results. These measures included regular calibration of equipment, thorough maintenance of steady environmental conditions (temperature, humidity, and light intensity), and periodic checks for contamination. These precautions were essential to minimize bias and uphold the integrity of the experimental outcomes. Survival rate was calculated using the following formula:

Total number of seedlings in a Petri dish (20)

According to Li et al. (2021), the survival rate was categorized into five classes: extremely sensitive ($0 \leq X < 20$), sensitive ($20 \leq X < 40$), slightly sensitive ($40 \leq X < 60$), tolerant ($60 \leq X < 80$), and extremely tolerant ($80 \leq X \leq 100$).

Detection of Reactive Oxygen Species (ROS) Accumulation in rice

Two rice varieties, representing heat-sensitive and heat-tolerant genotypes, were selected for further analysis from the 208 varieties studied. This focused comparison allows for a targeted investigation into how heat stress induces oxidative damage in rice plants with contrasting genetic backgrounds. To assess the accumulation of reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) were measured in both varieties. H_2O_2 accumulation in rice leaves was assessed by staining with 3,3-diaminobenzidine (DAB), while O_2^- levels were detected using nitro blue tetrazolium (NBT). Stressed and non-stressed rice leaves were immersed in 0.1% DAB solution and 0.2% NBT solution, then incubated at room temperature overnight. After staining, the seedlings were cleaned using double distilled water and then visualized under a microscope (Vergara et al., 2019).

Population structure

Population structure within the association panel was assessed using STRUCTURE v2.3.4 based on genome-wide SNP markers. The analysis was performed with assumed population numbers (K) ranging from 1 to 10, using a burn-in period of 50,000 iterations followed by 10,000 Markov Chain Monte Carlo (MCMC) iterations. For each K value, two independent runs were conducted to evaluate the consistency of clustering results. The optimal number of subpopulations was determined based on the ΔK method implemented **in** Structure Harvester, together with the consistency of clustering patterns across runs. Pairwise fixation index (FST) values among inferred subpopulations were calculated and visualized to evaluate genetic differentiation among groups.

QTL and Gene Mapping for Heat Tolerance during the Seedling Stage in Rice

Tassel 5.0 software was used to conduct a Genome-Wide Association Study (GWAS) on the heat stress tolerance of rice at the seedling stage (Bradbury et al., 2007). The analysis included principal component analysis (PCA) and kinship analysis on a dataset consisting of 2,858,438 SNP genotypes. Following data filtering, the standardized phenotypic data, SNP data, and principal component data were combined, and GWAS was carried out with kinship data as covariates using a mixed linear model. The R package R_MVP was used for plotting (L. Yin et al., 2021). QTLs were identified in regions where multiple SNPs (at least two) exceeded the significance threshold value of -log10 (P) ≥ 3.0.

Genomic DNA extraction, sequencing and genotyping

DNA was extracted from approximately 100 mg of fresh young leaves using the CTAB method, after which the quantity and quality of the DNA were measured. In addition, purity was determined by electrophoresis of DNA for 1 hour at 60 V in a 1% agarose gel. The 50K rice gene SNP microarray 'OsSNPNKs' was used for genotyping. SNPs on the microarrays were evenly distributed across the genome, with an average distance of < 1 kb between SNPs. Genotyping was performed with an Affymetrix AXIOM 2.0, and the target prep protocol QRC (P/N 702990) kit was used for DNA amplification, DNA fragmentation, microarray hybridization, DNA-binding single-base extension, and signal amplification. Staining and scanning were performed using a Gene Titan multichannel instrument(Li et al., 2021).

Quantitative real-time PCR

A total of 2.5 µl of RNA was reverse-transcribed into cDNA using the HiScript® III RT SuperMix Kit. For quantitative real-time PCR, 1 µg of total RNA was reverse-transcribed into cDNA using the FastKing gDNA Dispelling RT SuperMix Kit (Tiangen; KR118- 02). qRT—PCR analyses were also conducted using the SuperReal PreMix Plus (SYBR Green) Kit (Tiangen; FP205-2) with three biological replicates. Throughout the study, OsActin was used as a reference gene. The relative expression level was calculated following the method described by Livak & Schmittgen (Livak & Schmittgen, 2001).

Statistical analysis

In the data analysis, we used various software tools to analyze the collected data comprehensively. GraphPad Prism 10.0.3 was utilized to determine the correlation between two trials under heat stress and generate box plots and bar graphs. Structure 2.3.4 software was used to analyze population structure, while R Studio 4.2.2 with the scatterplot3d package was used for plotting PCA and kinship heatmap. Statistical analysis was conducted in R Studio 4.2.2 to compare gene expression levels between rice varieties BNP162 and BNP206 for each target gene. Raw qRT-PCR data underwent thorough processing, including normalization to internal controls and calculation of relative expression

levels using Excel functions. Direct comparisons of relative expression values between BNP162 and BNP206 were made to assess differences in gene expression levels. A Wilcox.test was performed for each target gene in R Studio 4.2.2 to determine statistical significance. P-values were calculated based on the Wilcox test results, with a significance threshold of $p < 0.1$ to identify statistically significant differences in gene expression between the two rice varieties. This comprehensive data analysis approach enabled a robust evaluation of the significance of observed differences in gene expression levels, offering valuable insights into their potential roles in heat tolerance mechanisms in rice seedlings.

Results

Variations in heat stress tolerance

A comprehensive evaluation of 208 rice accessions was conducted under rigorous heat stress conditions to assess heat stress tolerance, with the survival rate as the key metric. The survival rate showed significant variation in response to the imposed 45°C heat stress condition, highlighting diverse responses within the rice population (Fig. 1). A positive correlation was observed between survival rates in the first and second trials, Indicating consistency and reproducibility across experiments (Fig. 2a). There were no discernible differences in mean survival rate values between the successive trials, emphasizing the stability of experimental conditions and the resilience of rice accessions to environmental fluctuations (Fig. 2b).

The observed positive correlation between trials suggests the influence of genetic diversity on variations in survival rates across the rice population, emphasizing the genetic predispositions governing heat stress tolerance. To provide a detailed understanding of heat stress responses within the rice accessions, survival rates were categorized into five classes: extremely sensitive ($0 \le X < 20$), sensitive ($20 \le X < 40$), slightly sensitive ($40 \le X < 60$), tolerant ($60 \le X < 80$), and extremely tolerant ($80 \le X \le 100$).

To evaluate the genetic diversity of the rice population under heat stress conditions, we categorized the varieties into five survival rate groups: $0 \le X < 20$, $20 \le X < 40$, $40 \le X < 60$, $60 \le X < 80$, and $80 \le X \le 100$ (Fig. 2c). The distribution of *Indica*, *Japonica*, and Intermediate rice varieties among these groups showed significant differences in survival rates. Notably, the *Indica* subgroup had a higher proportion of varieties in the $60 \le X < 80$ and $80 \le X \le 100$ survival rate groups compared to *Japonica* ($p < 0.01$), indicating greater resilience to heat stress in *Indica* varieties. Conversely, *Japonica* varieties were more common in the $0 \le X < 20$ and $20 \le X < 40$ groups, with significantly lower survival rates ($p < 0.01$). The Intermediate subgroup had minimal representation across all groups, suggesting most varieties align closely with either *Indica* or *Japonica* genetic backgrounds. No significant differences (ns) were found between *Indica* and *Japonica* varieties in the $0 \le X < 20$ and $40 \le X < 60$ groups, indicating potential influences of additional genetic or environmental factors on survival rates in these ranges. The observed genotypic variation in survival rates underscores the genetic diversity within the population, supporting its suitability for genome-wide association studies (GWAS) targeting heat stress tolerance.

Principal component analysis (PCA) was performed on the GWAS data using the Mixed Linear Model (MLM) approach to examine the variance structure across SNPs. The PCA plot (Fig. 2d) illustrates the distribution of SNPs based on the first three principal components (PC1, PC2, and PC3), which collectively capture a significant portion of the genetic variance. SNPs are color-coded by their respective chromosomes, but no distinct clustering by chromosome is evident, indicating a widespread distribution of genetic variation across the genome. This pattern reflects the polygenic nature of the traits being studied, with no single chromosome exerting a dominant influence on the genetic architecture. The PCA results are noteworthy as they highlight the intricate genetic contributions, with numerous SNPs exhibiting modest yet significant effects, laying the groundwork for further exploration of potential candidate loci influencing the observed phenotypic variation. These findings offer valuable insights into the genetic underpinnings of traits and can inform future marker-assisted selection or functional validation studies.

Population Structure and Kinship

Population structure and genetic relatedness among rice accessions were assessed using genome-wide SNP markers to evaluate potential confounding effects in genome-wide association analysis. STRUCTURE analysis was performed using 2,858,438 SNP markers with assumed population numbers (K) ranging from 1 to 10. The highest ΔK value was observed at K = 4 (Fig. 3a), indicating the presence of four major genetic groups within the rice panel. Based on the estimated ancestry proportions, the accessions were assigned into four subpopulations (Fig. 3b), reflecting the genetic diversity present within the evaluated germplasm.

A kinship matrix was calculated to estimate pairwise genetic relatedness among accessions (Fig. 3c). The kinship analysis revealed variation in genetic relationships among individuals, with some accessions exhibiting higher relatedness and others showing greater genetic divergence. These results confirmed the presence of both population stratification and relatedness within the panel, which may influence marker–trait associations.

To account for these effects, genome-wide association analysis was performed using the mixed linear model (MLM) implemented in the rMVP package in R, incorporating the kinship matrix and the first five principal components (PCs) as covariates. This approach reduced the impact of population structure and relatedness, thereby improving the reliability of detected marker–trait associations.

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Discovery of QTLs Governing Survival Rate through GWAS

To elucidate the genetic basis of post-heat stress survival, a genome-wide association study (GWAS) was conducted using survival rate as the phenotypic trait across two independent experiments. Genome-wide association analysis was performed using a mixed linear model (MLM), incorporating population structure and kinship relationships to minimize spurious associations. Manhattan plots revealed multiple

genomic regions associated with heat stress resilience (Fig. 4). Using a significance threshold of −log10 (P) ≥ 3.0, with at least two significant SNPs defining a QTL, nine quantitative trait loci (QTLs) associated with heat stress resilience were identified. This suggestive threshold has been successfully applied in previous plant GWAS studies involving complex quantitative traits and provides a balance between detection power and the identification of biologically meaningful loci (Darmagaru et al., 2026; Javid et al., 2022; Sardouei-Nasab et al., 2026; Shewabez et al., 2025). The reliability of identified QTLs was further enhanced by considering loci detected across independent experiments and prioritizing regions supported by candidate gene and physiological evidence.

In the first trial, *qSR3, qSR6, qSR8*, and *qSR10* were identified (Fig. 4a), whereas the second trial revealed *qSR1.1, qSR1.2, qSR2, qSR3, qSR6, qSR8, qSR9*, and *qSR11* (Fig. 4c). Among these loci, *qSR3, qSR6*, and *qSR8* were consistently detected in both trials, indicating that these genomic regions represent robust loci contributing to heat stress resilience in rice seedlings. The repeated detection of these QTLs under independent experimental conditions increases confidence in their biological relevance

Several identified QTL regions overlapped with previously characterized stress-responsive genes. The *qSR1.2* region contained *OsSIRH2-23*, located 18.3 kb from the peak SNP, which has been associated with salinity stress tolerance (J. H. Kim et al., 2022), and *RGN1a*, located 46.9 kb from the peak SNP, which regulates grain number per panicle in japonica rice (Q. Zhang et al., 2022). The *qSR3* region harbored *OsPTR1*, located 54.7 kb from the peak SNP, a gene previously implicated in responses to cold, drought, and salt stress in rice(J.-Y. Kim et al., 2004). Similarly, the *qSR6* region contained *OsCGT*, located only 2.0 kb from the peak SNP, encoding CYP93G2 involved in C-glycosylflavone biosynthesis. The *qSR8* region contained *OMTN6*, located 10.8 kb from the peak SNP, which has been reported to negatively regulate drought resistance in rice (Fang et al., 2014). Additionally, *qSR10* contained *OsDSR-1*, located 2.5 kb from the peak SNP, a gene involved in reactive oxygen species scavenging and drought tolerance in rice, as well as lateral root development in Arabidopsis under high-potassium conditions(X. Yin et al., 2017; X. M. Yin et al., 2011).

In contrast, *qSR1.1, qSR9*, and *qSR11* did not overlap with previously characterized stress-related genes, suggesting that these loci may represent novel genomic regions associated with heat stress tolerance at the seedling stage. These novel loci provide potential targets for future fine-mapping and functional validation.

Collectively, the consistent identification *of qSR3, qSR6*, and *qSR8* across independent trials highlights their importance as stable genomic regions underlying heat stress resilience. These loci were therefore prioritized for subsequent candidate gene identification and characterization.

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Table 1
Genome-wide significant loci for survival

Trial	QTL	Chr.	No. of significant SNPs	position	p value
1st trial	*qSR3*	3	147	2129607	0.000314
	qSR6	6	48	180014	2.34E-05
	qSR8	8	105	5836014	0.000218
	qSR10	10	76	5323090	0.000208
2nd trial	*qSR1.1*	1	95	28555711	0.000305
	qSR1.2	1	153	28563475	5.30E-05
	qSR2	2	9	19382268	0.000259
	qSR3	3	16	2186626	0.000310
	qSR6	6	44	161174	3.91E-05
	qSR8	8	192	5839975	0.000128
	qSR9	9	23	10246270	0.000207
	qSR11	11	10	853188	9.65E-05

Identification of potential candidate genes of qSR3, qSR6, and qSR8

To identify candidate genes associated with heat stress tolerance in the *qSR3*, *qSR6*, and *qSR8* regions, we conducted an analysis within a ± 200 kb search interval around the peak SNP. Heat stress significantly impacts rice productivity, highlighting the importance of identifying genes linked to tolerance to improve crop resilience. In these regions, we identified 166 genes (Table S2). To refine our selection of candidate genes, we excluded retrotransposons, transposons, hypothetical proteins, and unknown proteins, ultimately identifying 82 genes across these QTLs (Table S3).

The selection of candidate genes for heat stress regulation in rice was conducted using a systematic approach combining bioinformatics analysis and a thorough literature review. The goal was to identify underexplored genes that may play crucial roles in heat stress adaptation. This process led to the identification of 9 novel candidate genes that had not been extensively studied or cloned in prior research (Table 2). The selection criteria included functional annotation and domain analysis, novelty, and functional diversity. Genes with known regulatory roles, such as transcription factors, protein kinases, and oxidoreductases, were prioritized. For example, *LOC_Os06g01370*, a DNA-binding protein, and *LOC_Os08g10150*, a SHR5-receptor-like kinase, were included due to their potential involvement in stress regulation. Excluding genes already well-characterized, such as WRKY transcription factors and GSTF proteins, ensured a focus on novel genes like *LOC_Os03g04370* (Protein-methionine-S-oxide reductase) and *LOC_Os08g10030* (KH domain-containing protein). The final list encompasses a variety of

gene types, including transcription factors, signaling proteins, and transporters, highlighting their potential roles in oxidative stress responses, protein stability, and cellular signaling during heat stress.

Table 2
Candidate genes from the three candidate QTLs

Number	Gene symbol	Gene annotation
1.	***LOC_Os03g04370***	**Protein-methionine-S-oxide reductase, oxidative stress repair potential**
2.	*LOC_Os08g10030*	KH domain-containing protein, putative regulator in stress adaptation
3.	*LOC_Os06g01360*	Homogentisate 1,2-dioxygenase, putative, involved in oxidative stress defense
4.	*LOC_Os03g04300*	Ankyrin repeat domain-containing protein, putative, potential role in stress signaling
5.	*LOC_Os08g09830*	BTB and MATH domain-containing protein, potential ubiquitin ligase activity under stress.
6.	*LOC_Os06g01370*	DNA-binding protein, regulatory potential
7.	*LOC_Os06g01170*	PHD-finger domain-containing protein, possible transcriptional regulator.
8.	*LOC_Os03g04750*	60S ribosomal protein L21-2, putative, involvement in stress-induced translation.
9.	*LOC_Os08g10150*	SHR5-receptor-like kinase, stress signaling

To further confirm the response of the nine candidate genes to heat stress, quantitative real-time PCR (qRT-PCR) was conducted in two rice varieties, BNP162 and BNP206, under heat stress conditions (Fig. 5). The qRT-PCR analysis revealed differential expression of selected genes under heat stress in the rice varieties BNP162 and BNP206. *LOC_Os03g04370* exhibited significant upregulation in both varieties, indicating its potential role in the oxidative stress response. Other genes, such as *LOC_Os08g10030* and *LOC_Os06g01360*, were also upregulated in BNP162, while several genes, including *LOC_Os08g09830* and *LOC_Os06g01370*, showed downregulation in both varieties, particularly in BNP206. These findings suggest that *LOC_Os03g04370* could be a key candidate for heat stress resilience, with distinct expression patterns observed between the varieties.

Further analysis of the genomic intervals flanking *LOC_Os03g04370*, with a region spanning from 1971226bp to 2266792bp as shown in the local Manhattan plot (Fig. 6a), revealed that the markers surrounding this gene were in strong linkage, suggesting low recombination rates in this region. This indicates that the genomic interval around *LOC_Os03g04370* is less likely to undergo recombination over generations, highlighting its conserved genetic nature. The LD heatmap (Fig. 6b) further supports this finding, showing significant LD between *LOC_Os03g04370* and surrounding markers, suggesting that these regions are genetically stable and could be important for heat tolerance mechanisms

Reactive Oxygen Species (ROS) Accumulation and Oxidative Stress Response in Heat-Stressed Rice Varieties

Following the qRT-PCR analysis, a local Manhattan plot, and an LD heatmap, which identified *LOC_Os03g04370* as a potential candidate gene associated with heat resilience, we delved deeper into its function in protecting rice cells from oxidative stress. *LOC_Os03g04370*, known as Protein-methionine-S-oxide reductase, plays a crucial role in scavenging reactive oxygen species (ROS), a key mechanism for heat tolerance. To validate its function, we compared ROS accumulation in two natural rice varieties with differing heat stress tolerance: BNP162 (heat-tolerant) and BNP206 (heat-sensitive). NBT and DAB staining revealed significant variations in ROS levels post heat stress. While both varieties exhibited minimal ROS accumulation under normal conditions, BNP206 displayed significantly higher levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) compared to BNP162. DAB staining (Figs. 7a and 7b) and NBT staining (Figs. 7c and 7d) confirmed these differences, with quantitative analysis indicating a significant ROS accumulation in BNP206 ($p < 0.01$). These results support the role of *LOC_Os03g04370* in regulating oxidative stress and suggest its contribution to heat resilience through ROS scavenging.

Discussion

Assessment of population structure and survival rate of the natural population

Heat stress impairs several growth stages and metabolic processes at the cellular level, ultimately leading to a loss of yield quantity/quality or death (Bita & Gerats, 2013). The natural population used in the study exhibited differential heat stress resistance due to notable genetic differences. These potential genetic variations can be used to develop rice plants with increased heat stress tolerance. The seedling stage is most important for plant establishment. However, the specific mechanisms underlying heat tolerance at the Seedling stage have not yet been elucidated.

For the purposes of the present study, it was ideal to test whether the natural population used in the study was suitable for GWASs of quantitative traits such as survival. This is of critical importance in reducing spurious associations (Sul et al., 2018). In this context, a population structure analysis was performed on 2,858,438 SNPs in the natural population. By definition, population structure causes genome-wide correlation between alleles (linkage disequilibrium), which can easily lead to genome-wide occurrence of false positives (Platt et al., 2010). In the analysis of population structure, the natural population was divided into four groups. This analysis appears to confirm the low relatedness as determined by kinship analysis, potentially suggesting that these data might be suitable for GWAS.

Heat tolerance is a quantitative trait influenced by the environment and genetics (TS et al., 2023). In the present study, the survival rate was used as an Indicator of the heat stress tolerance of the 210 rice

accessions. However, to improve the accuracy of heat tolerance determination, the study was conducted twice. Correlation analysis revealed a positive relationship between the two trials (Fig. 1a). This indicates that the results were more genetic than environmental. The survival rate did not significantly differ between the two trials, with longer survival in *Indica* rice than in *Japonica* rice (Fig. 1d). The survival rate under heat stress varied widely, ranging from 0 to 100%. Notably, *Japonica* rice variety showed a significantly lower survival rate during the recovery period, indicating its increased vulnerability to heat stress. This finding aligns with Zhang et al.'s (2022) research, supporting the notion that *Indica* rice varieties have better heat stress resilience than *Japonica* rice. The genetic differences between the rice subspecies likely play a role in this varying heat tolerance. As a result, these accessions exhibit significant genetic diversity and heat tolerance, making them promising candidates for future genetic enhancement projects focused on developing heat-tolerant rice varieties.

Our research highlights the crucial role of genetic background in heat stress resistance among rice accessions, making this population ideal for Genome-Wide Association Studies (GWAS). The phenotypic assessments enhance our understanding of the mechanisms underlying heat stress tolerance and offer valuable genetic resources for future breeding programs. Additionally, Principal Component Analysis (PCA) shows that the first three principal components (PC1, PC2, and PC3) collectively capture a substantial portion of the genetic variance. The distribution of SNPs does not exhibit distinct clustering by chromosome, indicating that genetic variation influencing heat stress resistance is widely spread across the genome. This polygenic nature underscores the complexity of heat stress tolerance mechanisms, with multiple SNPs contributing small yet significant effects. These findings provide a strong basis for identifying candidate loci and genes associated with heat stress resistance and guiding marker-assisted selection and targeted breeding strategies to enhance rice resilience to climate change.

Identification of QTLs for Heat Stress Resilience

This genome-wide association study (GWAS) identified nine quantitative trait loci (QTLs) linked to heat stress tolerance in rice seedlings in two separate trials. Among these, *qSR3*, *qSR6*, and *qSR8* were consistently detected in both trials, demonstrating their stability and robustness across experimental conditions and highlighting them as reliable genomic regions underlying heat stress resilience. The co-localization of these QTLs with previously cloned stress-responsive genes, including *OsPTR1* (*qSR3*), *OsCGT* (*qSR6*), and *OsDSR-1* (*qSR10*), which have established roles in regulating responses to cold, drought, and oxidative stress, suggests that conserved stress-response pathways may also contribute to heat tolerance(Fang et al., 2014; J.-Y. Kim et al., 2004; X. M. Yin et al., 2011). The repeated detection of *qSR3*, *qSR6*, and *qSR8*, together with the biological relevance of genes located within these intervals, makes them high-confidence QTLs for candidate gene identification and functional validation. These loci represent promising targets for marker-assisted selection, genomic selection, and gene-editing approaches aimed at improving heat resilience in rice. Furthermore, the identification of the putatively novel QTLs *qSR9*, *qSR11*, and *qSR1.1* expands the current understanding of the genetic architecture of heat tolerance and provides valuable genomic resources for future fine-mapping, gene cloning, and characterization of previously unexplored mechanisms governing heat stress adaptation.

Candidate genes identification and characterization

The identification of candidate genes underlying heat stress tolerance provides an important foundation for understanding the molecular mechanisms governing rice adaptation to elevated temperatures. We focused our analysis on the three stable QTLs (*qSR3, qSR6*, and *qSR8*), which were consistently detected across both GWAS experiments. Following the exclusion of retrotransposons, transposons, and hypothetical proteins, 82 annotated genes were retained for downstream analysis. This filtering strategy reduced annotation uncertainty and increased the likelihood of identifying biologically relevant genes, consistent with established practices in functional genomics(Li et al., 2024). By integrating GWAS signals, linkage disequilibrium analysis, and functional annotation with published literature, we prioritized candidate genes with potential roles in heat stress adaptation. Unlike previous studies that primarily emphasized already characterized stress-responsive genes (Biscarini et al., 2016; Islam et al., 2022; Nyasulu et al., 2024; H. Wang & Qin, 2017), our approach also identified less-explored loci that may represent novel components of heat tolerance pathways.

Among the prioritized candidates, *LOC_Os03g04370*, encoding a protein methionine sulfoxide reductase (MSR), emerged as the strongest candidate underlying *qSR3*. MSRs constitute an important antioxidant defense system by repairing oxidized methionine residues generated during oxidative stress, thereby maintaining protein integrity and cellular redox homeostasis (Tarrago et al., 2009). The conserved NAD(P)-binding domains identified within LOC_Os03g04370 further support its role in NADPH-dependent redox reactions, which are essential for regenerating antioxidant systems during stress (Chen et al., 2024). Heat stress is known to disrupt cellular redox balance through excessive accumulation of reactive oxygen species (ROS), resulting in protein oxidation, membrane damage, and impaired metabolism. The induction of LOC_Os03g04370 under heat stress, together with its localization within a strong LD block and its proximity to the peak GWAS association signal, provides multiple independent lines of evidence supporting its involvement in heat stress tolerance. Furthermore, the elevated accumulation of superoxide and hydrogen peroxide detected by NBT and DAB staining suggests activation of oxidative stress defense pathways, reinforcing the proposed role of LOC_Os03g04370 in mitigating ROS-induced cellular damage. Similar protective functions have been demonstrated for *AtMSRB5* in *Arabidopsis*, indicating that MSR-mediated oxidative stress protection may represent a conserved mechanism across plant species(Cai et al., 2023). Nevertheless, functional validation through gene knockout, overexpression, or genome-editing approaches will be necessary to establish its direct contribution to heat tolerance in rice.

Beyond oxidative stress regulation, several additional candidate genes suggest that heat tolerance involves coordinated regulation of multiple biological processes. Genes encoding transcriptional regulators and signaling proteins, including *LOC_Os06g01370* (DNA-binding protein) and *LOC_Os08g10150* (SHR5 receptor-like kinase), likely participate in stress signal perception and activation of downstream defense pathways(Gandhi & Oelmüller, 2023). Candidate genes associated with protein quality control, including *LOC_Os03g04300* (ankyrin repeat-containing protein) and *LOC_Os08g09830* (BTB-MATH domain-containing protein), indicate that proteostasis represents another critical

component of heat adaptation. BTB-MATH proteins commonly function as substrate-recognition components of ubiquitin ligase complexes, facilitating the removal of damaged proteins that accumulate during heat stress(L.-L. Zhang et al., 2024). Likewise, genes involved in ribosome function and protein synthesis, such as *LOC_Os03g04750* and *LOC_Os06g01170*, suggest that maintenance of translational capacity may be essential for sustaining cellular metabolism under elevated temperatures.

Interestingly, *LOC_Os08g10030*, encoding a KH-domain RNA-binding protein, was consistently downregulated following heat treatment. Although its precise biological function remains unclear, KH-domain proteins are widely implicated in RNA metabolism, including transcript stability, RNA processing, and translational regulation. Its suppression under heat stress may therefore reflect transcriptional reprogramming associated with stress adaptation rather than a direct contribution to heat tolerance. Further investigation will be required to determine whether this gene functions as a negative regulator or participates in post-transcriptional regulatory networks during thermal stress.

Collectively, the candidate genes identified in this study indicate that heat stress tolerance is governed by an integrated network involving ROS detoxification, stress signaling, transcriptional regulation, protein quality control, and translational maintenance rather than by a single defense pathway. Among these candidates, *LOC_Os03g04370* represents the most compelling target because it is supported by convergent evidence from GWAS, linkage disequilibrium, transcriptomic expression, ROS accumulation assays, and functional annotation. These findings provide valuable genetic resources for future functional characterization and offer promising targets for marker-assisted breeding, genomic selection, and genome editing aimed at developing heat-resilient rice cultivars under future climate scenarios

Conclusion

This study provides new insights into the genetic architecture of heat stress tolerance at the rice seedling stage through the integration of genome-wide association analysis, linkage disequilibrium mapping, transcriptomic evidence, and functional annotation. Nine quantitative trait loci (QTLs) associated with heat stress resilience were identified, with *qSR3, qSR6*, and *qSR8* consistently detected across independent experiments, demonstrating their stability and potential utility in rice improvement. Among the prioritized candidate genes, *LOC_Os03g04370*, encoding a protein methionine sulfoxide reductase, emerged as the strongest candidate due to converging evidence from GWAS, linkage disequilibrium analysis, differential gene expression, and its established role in maintaining cellular redox homeostasis. These findings suggest that oxidative stress regulation is a key mechanism underlying heat tolerance in rice seedlings. Collectively, this study expands the current understanding of the genetic basis of heat stress resilience and provides valuable genomic resources for future functional characterization. The identified QTLs and candidate genes represent promising targets for marker-assisted selection, genomic selection, and genome-editing strategies aimed at accelerating the development of heat-resilient rice cultivars to enhance food security under a warming climate

Declarations

Conflicts of interest:

The authors declare no conflicts of interest.

Ethics and Consent to Participate declarations

Not applicable

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Author Contribution

M.N. conducted the experiments, analysed the data and drafted the manuscript. Q.Z., Z.C., and Z.W., were involved in performing the experiments. H.H. and J.B. designed and supervised the experiments. All the authors have read and agreed to publish the manuscript.

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Data Availability

The variant data mentioned in this paper have been stored inthe Genome Variation Map (GVM) at the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, and China National Center for Bioinformation. The data can be accessed under the accession number GVM000815 and BioProject PRJCA028545. To view the data, please visit: https://bigd.big.ac.cn/gvm/getProjectDetail? Project=GVM000815

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Figures

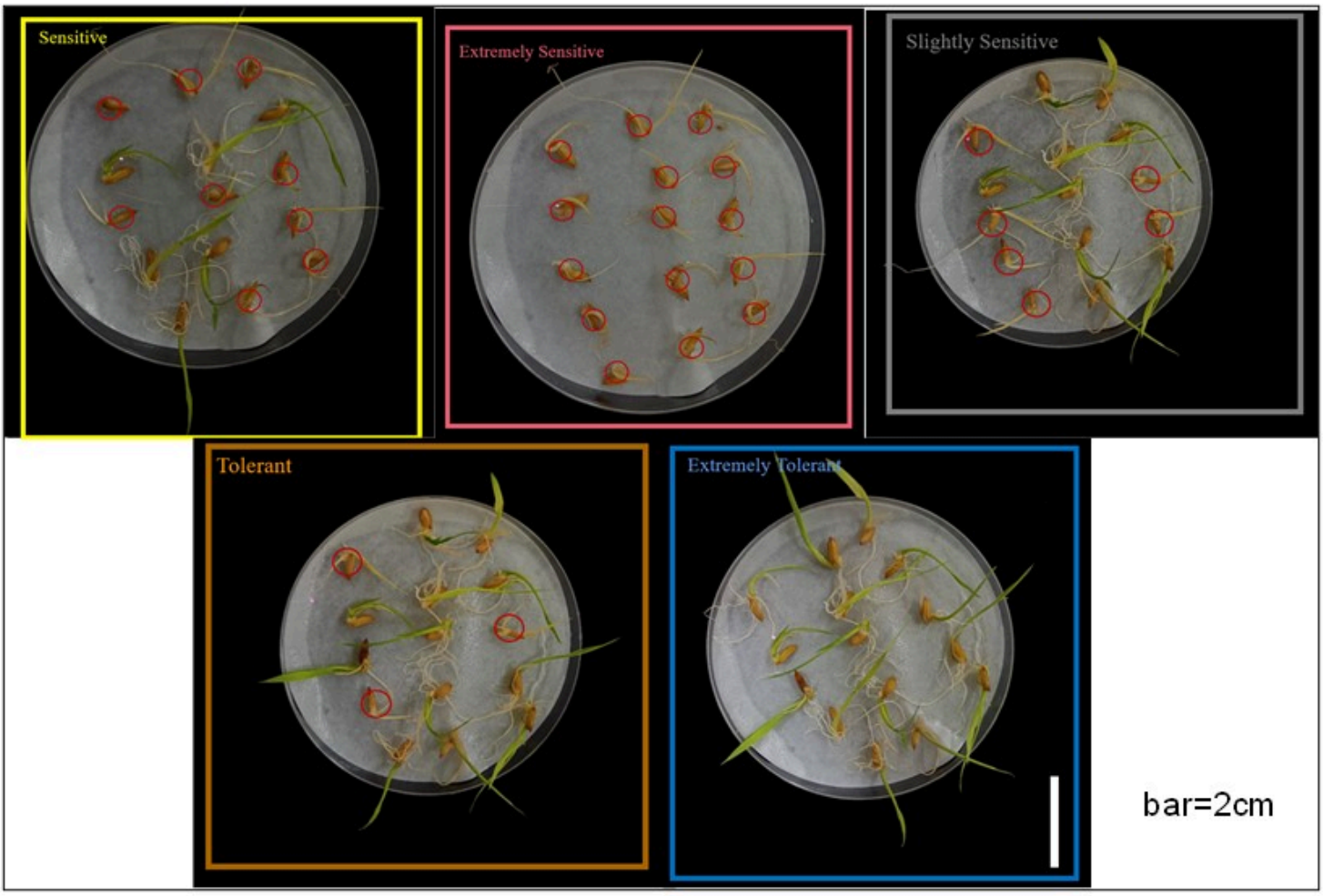


Figure 1

Variations in heat tolerance at the Seedling stage

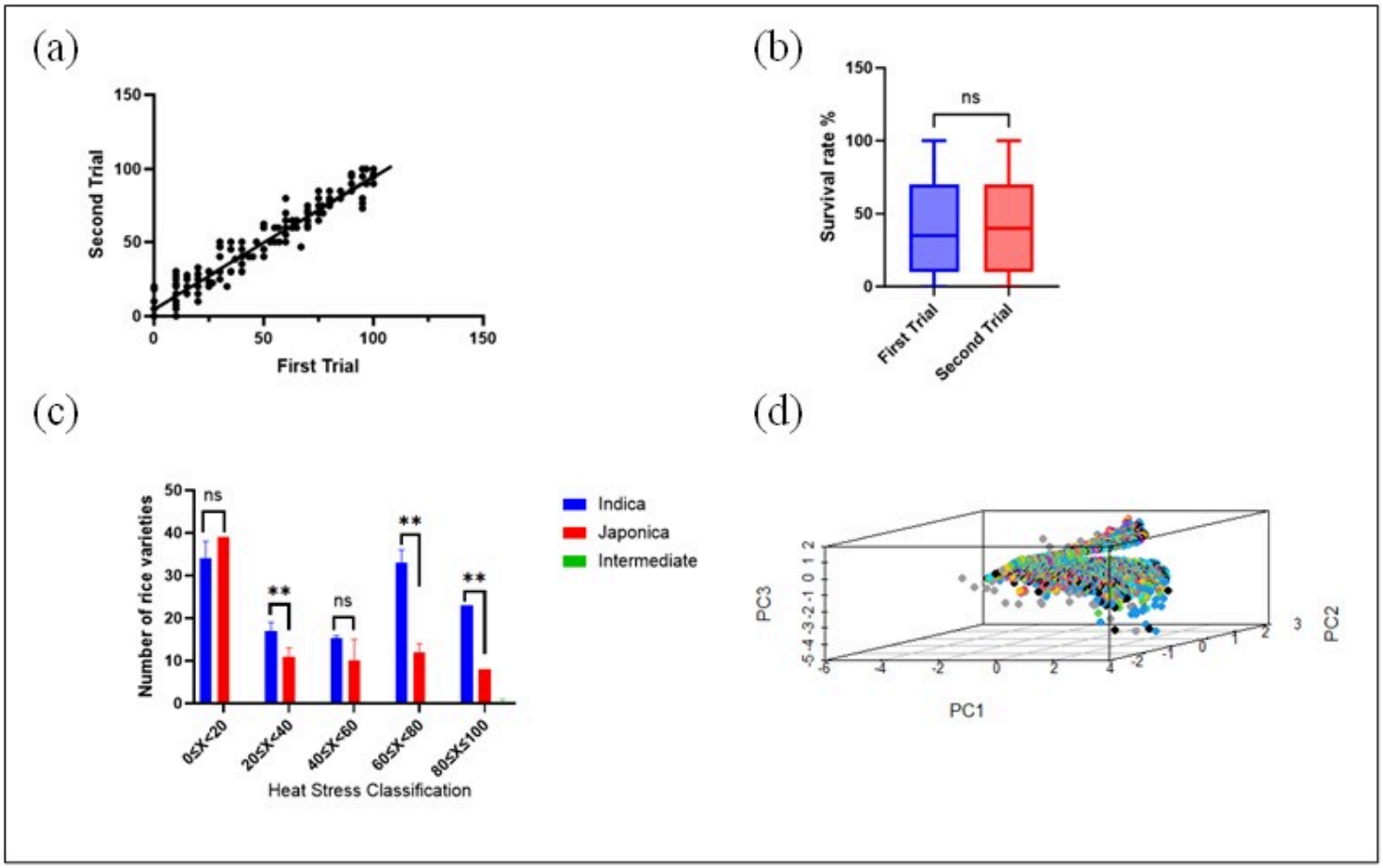


Figure 2

Phenotypic variations in rice accessions. (a) Correlation coefficients for the SRs of rice cultivars from the first and second trials, (b) Box plot for the SRs of the first and second trials, (c) Heat stress patterns for Japonica and Indica rice (d) Principal component analysis (PCA) of GWAS data using the Mixed Linear Model (MLM) approach

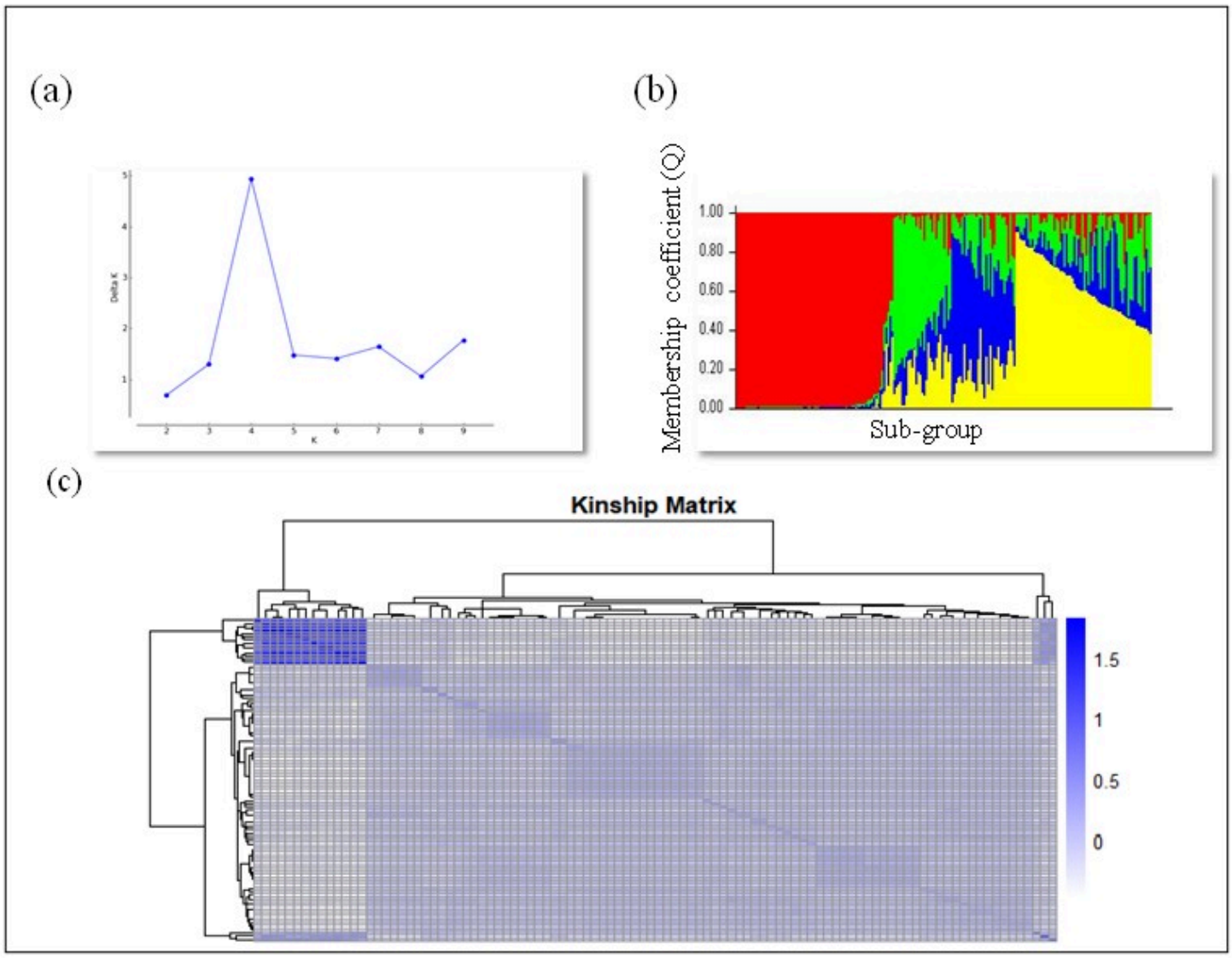


Figure 3

Analysis of population structure and relative kinship relationships (a) Relative kinships (delta-K plot), (b) population structure estimated by STRUCTURE (k =4), and (c) Kinship analysis

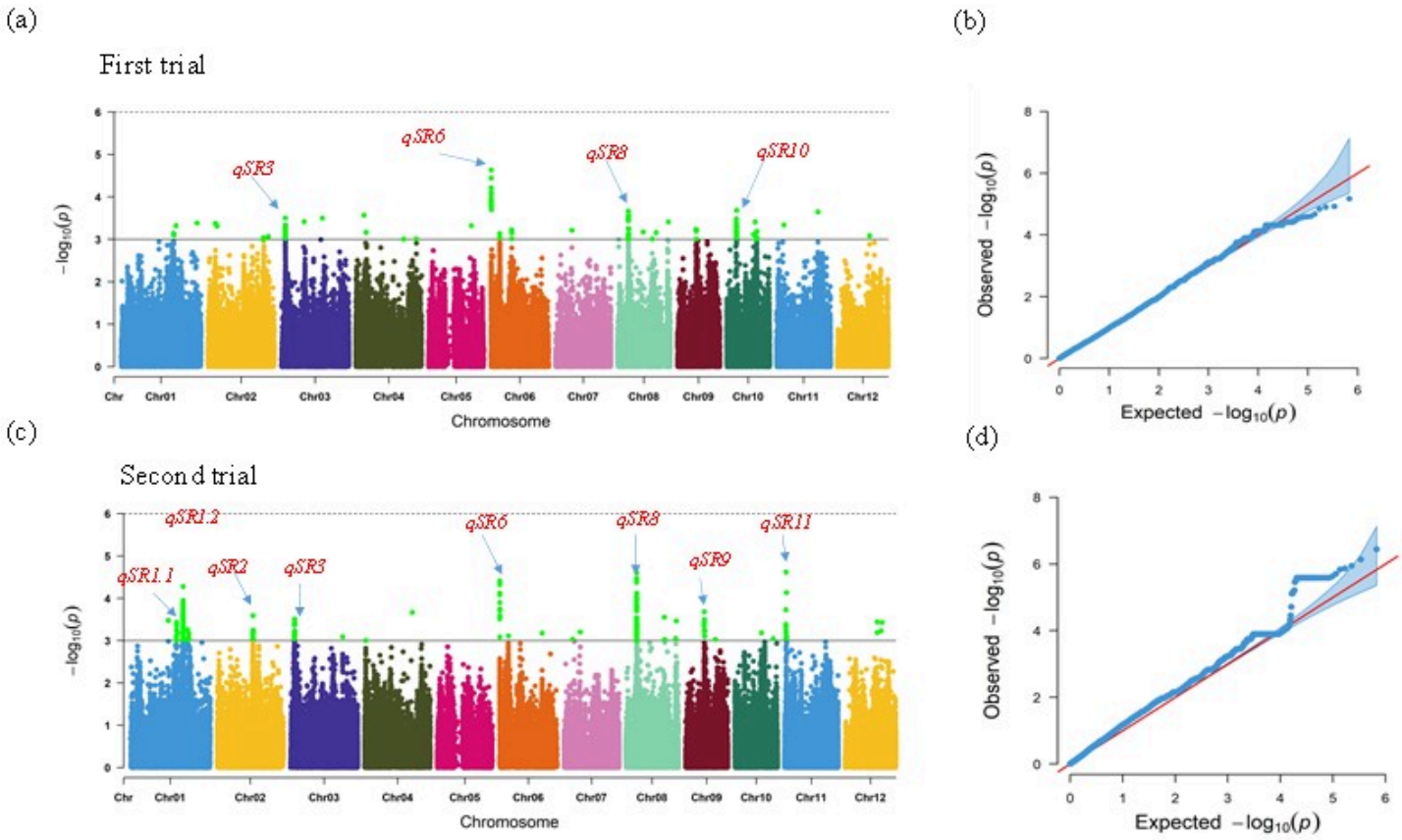


Figure 4

Genome-wide association mapping and LD block analysis for survival rate (SR) under heat stress. (a and c) Manhattan plots for the first and second trials, respectively. (b and d) QQ plots for the first and second trials of expected and observed P values, respectively.

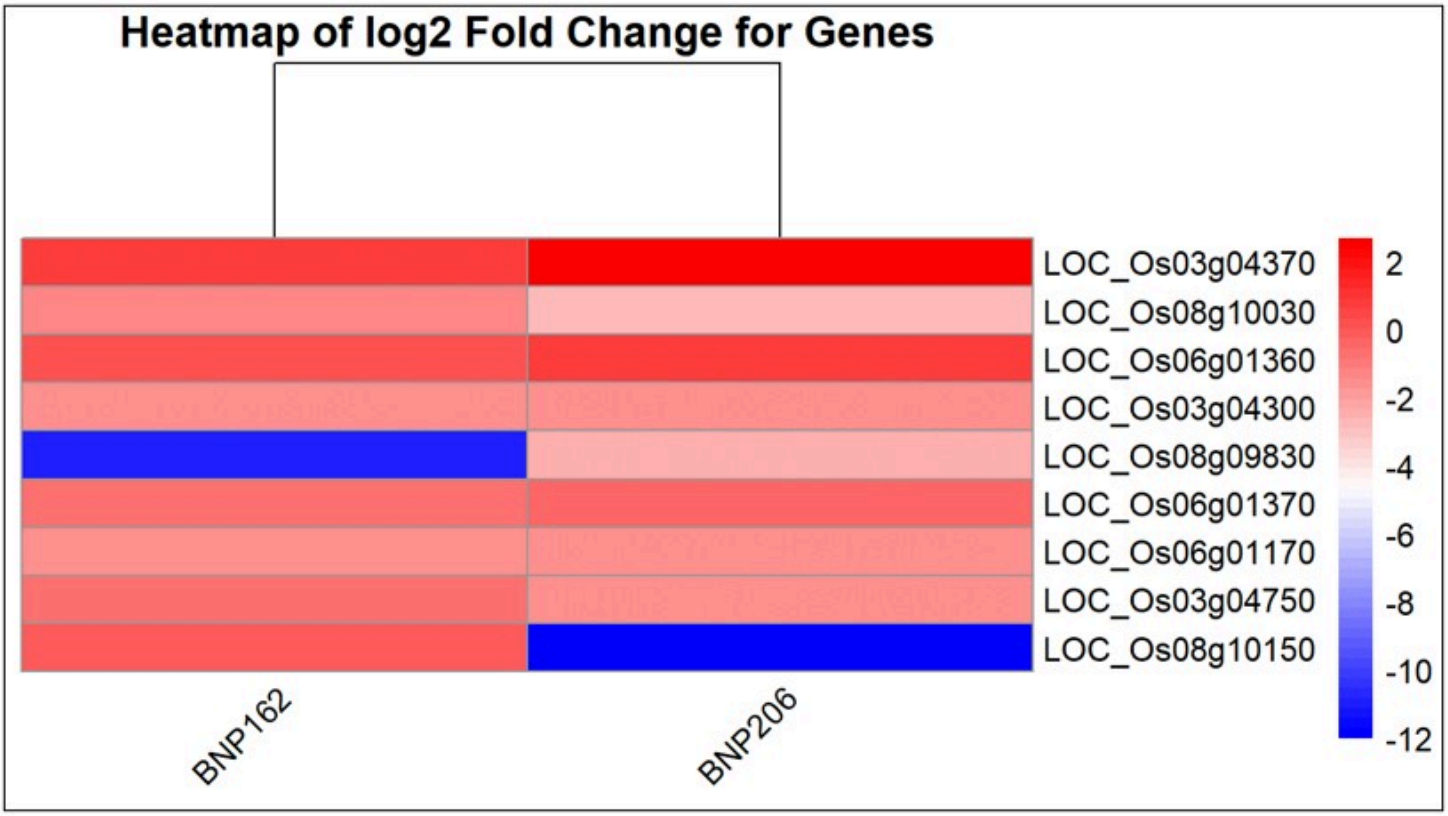


Figure 5

Differential expression of selected genes under heat stress in rice varieties BNP162 and BNP206

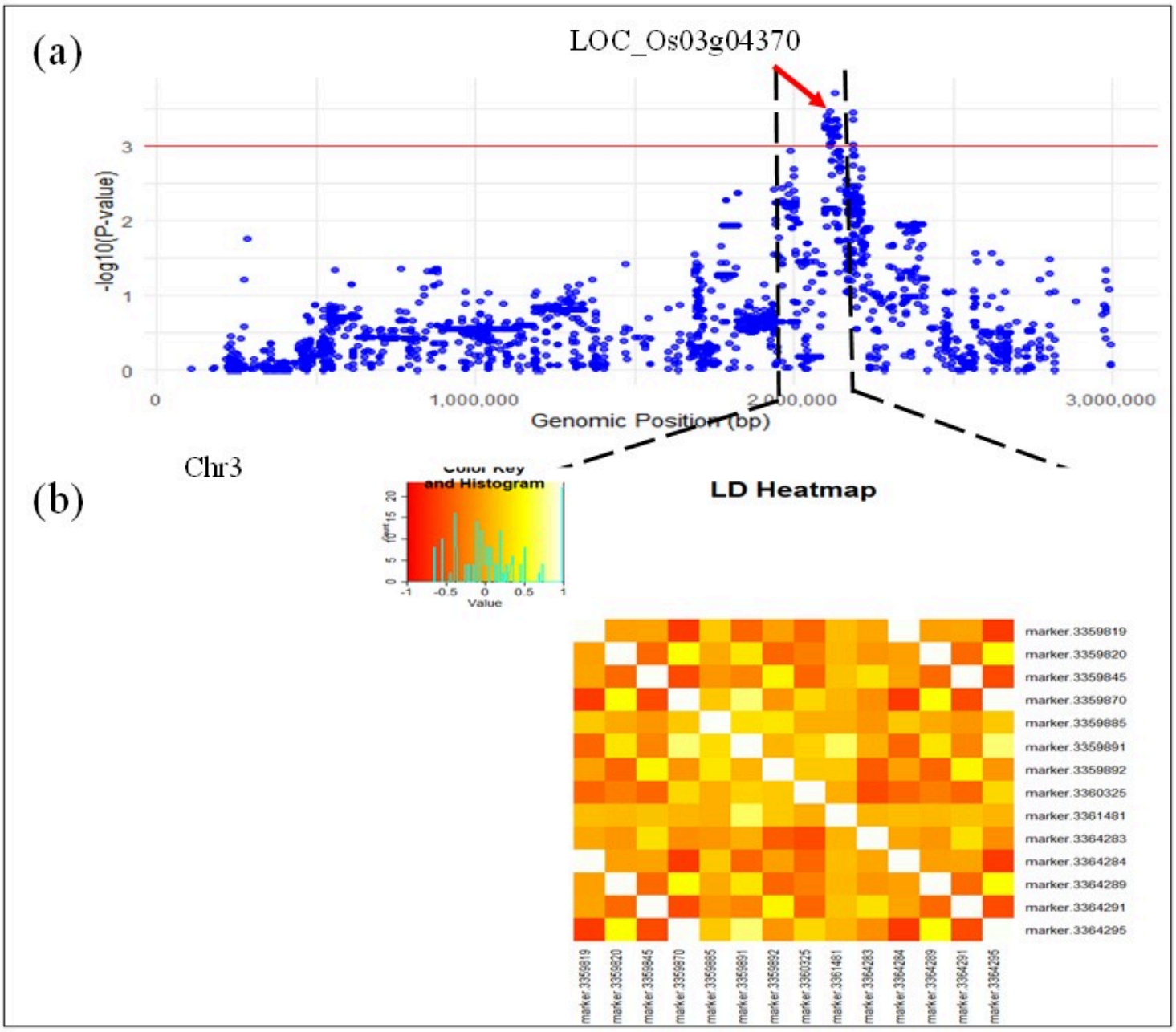


Figure 6

Local Manhattan plot and linkage disequilibrium (LD) heatmap. (a) Local Manhattan plot for chromosome 3 and (b) and linkage disequilibrium (LD) heatmap

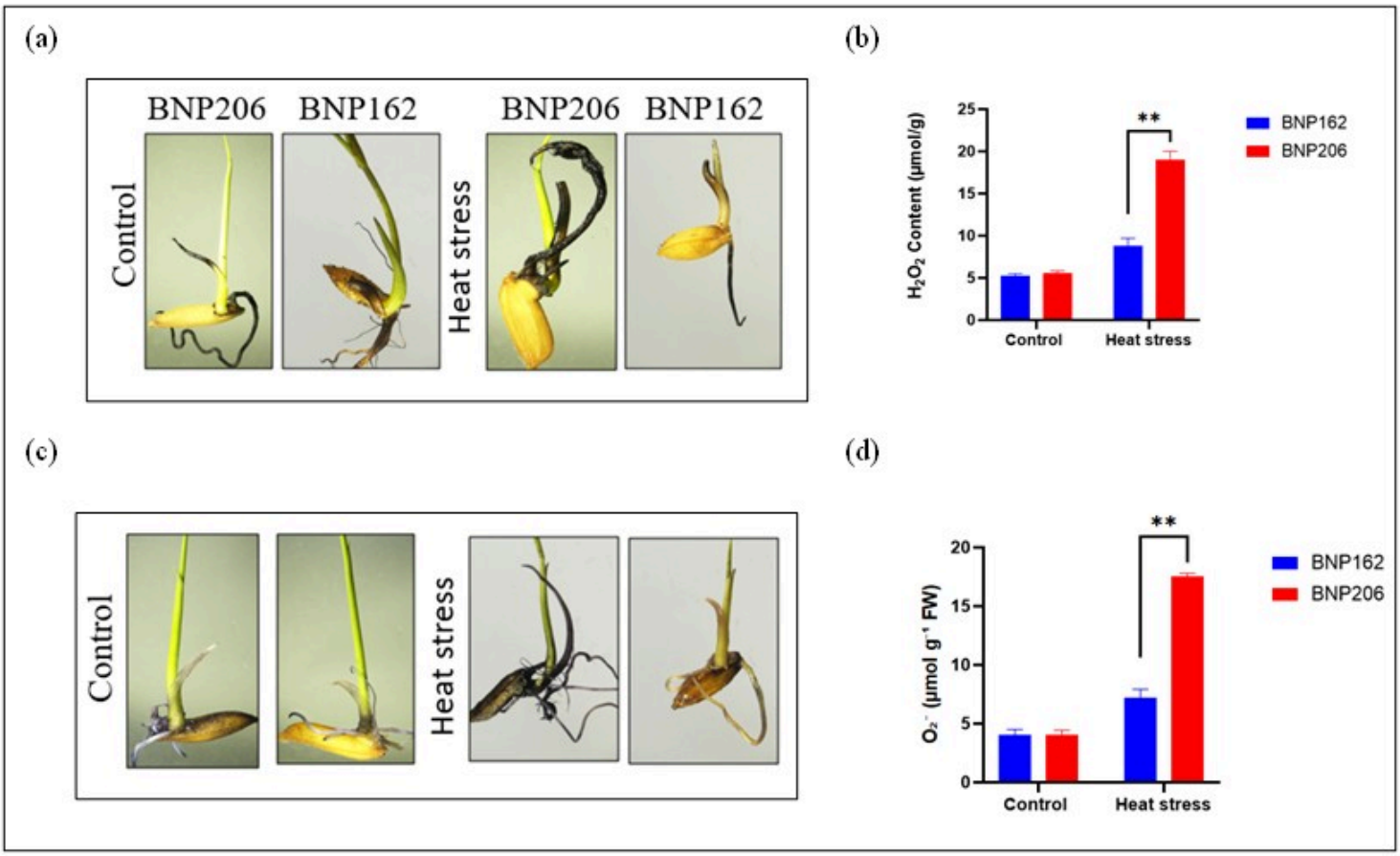


Figure 7

Analysis of superoxide and hydrogen peroxide. (a) DAB staining. (b) Hydrogen peroxide (H_2O_2) levels. (c) NBT staining. (d) Superoxide (O_2^-) levels

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2..docx
- TableS3..xlsx