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Rice

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Identifying Heat Adaptability QTLs and Candidate Genes for Grain Appearance Quality at the Flowering Stage in Rice

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Abstract

High temperature significantly impacts grain appearance quality, yet few studies have focused on identifying new quantitative trait loci (QTLs)/genes related to these traits under heat stress during the flowering stage in rice. In this study, a natural population of 525 rice accessions was used to identify QTLs and candidate genes associated with grain appearance quality using a Genome-Wide Association Study under heat stress. We identified 25 QTLs associated with grain length (GL), grain width (GW), and grain chalkiness (GC) under heat stress across 10 chromosomes in the three rice populations (full, *indica*, and *japonica*). Notably, three sets of overlapping QTLs were identified (set 1: *qHTT-L3* and *qHTT-XL3*; set 2: *qHTT-C5* and *qHTT-XC5*; set 3: *qHTT-L11.1* and *qHTT-GL11*), located on chromosomes 3, 5, and 11, respectively. Haplotype analysis indicated that Hap1 is the superior haplotype, and pyramiding more than two superior alleles improved rice grain appearance quality (longer GL, wider GW, and lower GC) in high-temperature environments. Based on RNA-seq, qRT-PCR and functional annotations analysis, *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* were highly expressed, identifying them as the high-priority candidate genes for QTLs linked to grain appearance quality (GL, GW, and GC) under heat stress. Expression analysis revealed that *LOC_Os05g06920*, which encodes a relA-SpoT-like protein RSH4, and *LOC_Os11g28104*, which encodes a protein kinase with a DUF26 domain, were highly expressed in seeds, leaves, and shoots. And *LOC_Os05g06970*, encoding a peroxidase precursor, exhibited high expression levels in roots. Compared to the wild-type (WT) plants, the mutants of *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* exhibited increased GL and grain length-to-width ratio, but reduced GW under both natural and heat stress conditions, while the *LOC_Os05g06970* and *LOC_Os11g28104* mutants significantly increased the chalky grain rate and grain chalkiness degree under natural conditions. Furthermore, the *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* mutants showed a lower decline in grain appearance quality traits than the WT after high-temperature treatment. These findings suggest that *LOC_Os05g06920*, *LOC_Os05g06970*,

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and *LOC_Os11g28104* play crucial roles in regulating both grain development and heat tolerance under heat stress at anthesis, thus affecting grain appearance quality in rice. Our results provide a promising genetic resource for improving rice grain appearance quality under heat stress.

Keywords GWAS, Heat stress, Grain appearance quality, Gene pyramiding, Rice

Background

Rice is the staple food for over 50% of the global population (Muthayya et al. 2014). Climate change, particularly rising temperatures, poses a significant challenge to rice production. The optimal conditions for rice flowering and spikelet development occur at 25–30 °C (Sánchez et al. 2014). Each 1 °C increase in global mean temperature reduces rice yield by 3.2% (Zhao et al. 2017). Key factors influencing rice yield include effective panicle number, grains per panicle, and grain weight (Xing and Zhang 2010). Grain weight depends on grain size and plumpness, with grain length and width being critical determinants. Chalkiness significantly affects rice appearance quality (Li et al. 2014), and physical appearance traits such as GL, GW, and GC are important to growers and consumers. However, heat stress during flowering or before impedes grain development, reducing seed size or weight (Folsom et al. 2014; Wu et al. 2022). Elevated temperatures also hinder grain filling, increasing chalkiness and the chalky area of rice grains (Morita et al. 2016). Rice glumes reach their final size at anthesis, so heat treatment during the grain-filling stage does not significantly affect grain length and width (Counce et al. 2005; Morita et al. 2005).

In recent decades, significant progress has been made in understanding the genetic basis of rice appearance quality under heat stress. Many QTLs and genes related to GL, GW, and GC have been identified in various high temperature environments. A previous GWAS study identified several loci specific to high night temperature (HNT) that regulate grain size (length and width) and identified loci common to both optimal and HNT stress conditions (Dhatt et al. 2021). Additionally, several QTLs and genes influencing grain quality (GL, GW, and % chalk) under HNT stress at the booting stage were located on chromosomes 1, 6, and 7, respectively (Kumar et al. 2023). QTL mapping for chalkiness under high-temperature conditions identified two QTLs on chromosomes 9 and 11, with more pronounced additive effects in the second cropping season compared to the first (Yang et al. 2021). Furthermore, the heat-induced gene *OsIAA29* was found to regulate seed development under high temperature, and the *osiaa29* mutant exhibited a higher percentage of shrunken seeds (with decreased GL and GW) and increased chalkiness compared to the WT under high-temperature conditions (Chen et al 2024). The heat-tolerant gene *TT3.1* was found to control rice heat tolerance, with mutant lines showing significant variations in grain length, width, and weight compared to the WT under high-temperatures (Zhang et al. 2022). A meta-analysis of QTLs for low grain chalkiness under high temperatures during the grain-filling stage underscored the important roles of two starch synthesis genes, *GBSSI* and *SSIIa* (Kumari et al. 2023). Another novel gene, *OsCG5*, was identified as regulating natural variation in GC under five days of heat stress (36/32 ± 1 °C), beginning one day after flowering (Chandran et al. 2022). Although previous research has largely focused on pre- and post-flowering stages (panicle initiation and grain-filling periods), little is known about potential causal genes influencing grain appearance quality under heat stress specifically during the flowering stage in rice.

In this study, we performed a GWAS on a diverse panel of 525 rice core germplasms to explore the natural variation in heat stress tolerance. We assessed grain appearance quality-related traits under high temperature and natural conditions, which led to the identification of 25 QTLs associated with grain appearance quality under heat stress. Haplotype analysis illustrated the allele and pyramiding effects of QTLs with heat tolerance. Additionally, three candidate genes were preliminarily functionally characterized and confirmed to play roles in heat tolerance and grain development during the flowering stage in rice. These findings highlight the importance of heat-tolerant germplasms and genes in plant adaptability to environmental stress, and provide abundant germplasm and gene resources for breeding rice varieties with strong heat tolerance and superior appearance quality.

Materials and Methods

Plant Materials

A total of 525 rice materials from the 3 K Rice Genome Project (3 K-RGP) were used to assess heat stress response in this study (Additional file 1: Table S1). These *Oryza sativa* accessions, with an average sequencing depth exceeding 12×had well-defined geographic origins. Ecotype information for these rice accessions was obtained from the Catalog of Rice Germplasm Resources in China and IRRI. The genomic data of these 525 rice accessions from the 3K-RGP can be downloaded from https://aws.amazon.com/public-data-sets/

3000-rice-genome/. The study adhered to institutional, national, and international guidelines and legislation.

The WT and mutant rice lines of the candidate genes, derived from the *japonica* cultivar Zhong-Hua 11 (ZH11), were used in the experiments. Furthermore, seven other varieties GH998, HHZ, 9311, X21, MR167, and Cisadane were selected for candidate gene screening. All materials were cultivated in the experimental field of the Rice Research Institute of Guangxi Academy of Agricultural Sciences in Nanning, China.

Phenotyping

In the summer of 2021, 525 materials were cultivated in the rice paddy field under natural field conditions. Upon reaching the flowering stage, three uniform-looking plants per accession were relocated to a phytotron (Fujian Jiupo Biotechnology Co., Ltd., Fuzhou, Fujian, China; Additional file 2: Fig. S1). The phytotron was set to 28.0 ± 0.5 °C, a light/dark 12/12-h cycle, and 75% relative humidity to facilitate rice plant growth. Subsequently, panicles were tagged and subjected to high-temperature treatment, consisting of 6 h (from 09:30 am to 3:30 pm) of 38.0 ± 0.5 °C daily. Following three consecutive days of high-temperature exposure, the treated plants were returned to the experimental field, and grain phenotypes were assessed evaluated post-harvest. A minimum of three panicles per plant and three plants per accession were marked and subjected to heat stress (Pan et al. 2023). Upon reaching maturity, panicles were harvested and air-dried for 3 months. Subsequently, the appearance quality characteristics of the filled grain sample were analyzed using the automatic seed analyzer (SC-G and SC-E, Hangzhou Wanshen Test Technology Corporation, China). These characteristics included GL, GW, GLWR, GCR, GCD, and grain transparency (GTP). The ratios of GL, GW, GLWR, GCR, GCD, and GTP under high-temperature treatment to those under natural conditions in the same rice cultivar were calculated and denoted as the relative GL (RGL), relative GW (RGW), relative GLWR (RGLWR), relative GCR (RGCR), relative GCD (RGCD), and relative GTP, respectively. Cultivars with RGL, RGW, and RGLWR values < 0.90, between 0.90 and 1.10, and > 1.10 were defined as heat-sensitive, moderate, and heat-tolerant cultivars, respectively. The RGCR and RGCD values < 1.00, between 1.00 and 2.00, and > 2.00 were defined as heat-tolerant, moderate, and heat-sensitive cultivars, respectively (Additional file 1: Table S2). All trait measurements were repeated at least thrice to ensure accuracy.

GWAS

To select heat-tolerant germplasms for breeding, 525 cultivated rice accessions were evaluated for heat tolerance at the flowering stage using a GWAS. Two panels, Panel 1 and Panel 2, were utilized, comprising 3,851,692 and 3,002,287 high-quality single nucleotide polymorphisms (SNPs) (minor allele frequency $\geq 5\%$, missing rate $< 25\%$), respectively. The GWAS was performed using both the general linear and compressed mixed linear models (CMLM) in the GAPIT package within an R environment. The significance threshold for genome-wide association was determined through conditional permutation tests with 1000 replications (Additional file 3: Fig. S2) (Zhao et al. 2018a, b). The conditional permutation test was executed using CMLM with the same parameters. SNPs with $-\log(P) \geq 2$ in the GWAS using original phenotypes were extracted to improve the computational efficiency. Finally, a threshold of $-\log(P) = 4$ was determined, which was higher than the 95th percentile of 1000 conditional permutation tests (Zhao et al. 2018a, b).

Due to different genome-wide linkage disequilibrium (LD) decay rates in *indica* and *japonica* at 123 kb and 167 kb, adjacent significant SNP with distances less than 170 kb were merged into single association signals (Huang et al. 2010). An associated signal was defined as a region with three or more consecutive significant SNPs, and the lead SNP was identified as the SNP with the minimum *P* value within this signal. The signal interval is the region between two significant SNPs located at the boundary.

Gene Genotyping by PARMS

This study employed the Penta-primer amplification refractory mutation system (PARMS) for genotyping 525 *Oryza sativa* accessions, as previously described (Pan et al. 2023).

RNA-Seq Analysis

Total RNA extracted from the panicles of the *indica* rice variety GH998 under normal conditions was utilized for RNA-seq analysis. The young panicle samples of approximately 5, 10, 15, and 20 cm in length were collected. The analysis was conducted in triplicates. The RNA library construction and RNA-seq were performed by Gene Denovo Biotechnology Co., Ltd (Guangzhou, China). To assess gene expression levels, fragments per kilobase of exon per million mapped fragments (FPKM) values were computed. Heatmap analysis was performed using OmicShare tools (https://www.omicshare.com/tools). The RNA-Seq expression data of selected candidate genes in different rice tissues were obtained from the Rice Expression Database (https://ngdc.cncb.ac.cn/red/).

RNA Extraction and qRT-PCR

Two rice varieties with different heat tolerance, HHZ (heat-tolerant) and 9311 (heat-sensitive), were selected to identify candidate genes responsive to heat stress. These varieties were subjected to heat stress for three days during the flowering stage in the phytotron. RNA samples were collected at 12 time points to document dynamic gene expression changes under heat stress conditions (Chen et al. 2021). Three other varieties, X21, MR167, and Cisadane, were subjected to heat stress for 6 h at anthesis in the phytotron. At 0 and 6 h after heat treatment, young panicles were collected and sampled for RNA extraction. Furthermore, different tissues from ZH11 cultivated in natural conditions were sampled for qRT-PCR analysis, including root, shoot, internode, leaf, and seed. Subsequently, RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) were conducted to examine gene expression profiles, following the protocols described by Pan et al. (2023). Ubiquitin (*UBQ*) was used as the internal control and at least three replicates were performed for each experiment. Primers for qRT-PCR are listed in Table S3.

CRISPR/Cas9 Editing Vector Construction and Rice Genetic Transformation

Mutants of the candidate genes were generated using the CRISPR/Cas9 technology in ZH11 background. For generating mutants, a specific nucleic acid sequence in the coding region of the candidate genes was chosen as the target sequence for the CRISPR/Cas9 system, and then designed single-guide RNA (sgRNA) in the first exon for each candidate gene. Based on the target and sgRNA sequences, the Oligo sequence was synthesized according to the Biogle tool (http://biogle.cn/index/excrispr). Next, the Oligo dipolymer was created through an annealing reaction and then introduced into the CRISPR/Cas9 Vector BGK03 to create plasmid. Finally, the recombinant plasmid was transformed into ZH11 calli via *Agrobacterium tumefaciens*, and transgenic rice plants were regenerated through hygromycin screening. Genome fragments were amplified with candidate gene specific primers, sequenced, and screened for mutant plants, with seeds from transgenic positive plants selected for further functional study (Lu et al. 2017). The CRISPR/Cas9 gene editing work was performed by Biogle Gene Technology Co., Ltd., Changzhou, Jiangsu, China.

Statistical Analysis

Significant differences in measured traits were assessed using analysis of variance (ANOVA), followed by Student's *t*-test ($P < 0.05$ and $P < 0.01$).

Results

Grain Development of Both *indica* and *japonica* Rice was Negatively Affected Under High-Temperature Stress

To evaluate the effect of high temperatures on grain appearance quality, 525 rice accessions obtained from the 3 K Rice Genome Project (3 K-RGP) were subjected to high temperatures during the flowering stage (Additional file 1: Table S1). Population structure analysis was performed to comprehend the genetic relationship among 525 rice accessions for further phenotypic analysis and GWAS. Results showed that these germplasm resources were classified into two subpopulations ($K = 2$), *indica* and *japonica*, (Fig. 1A), which was further confirmed by the principal components analysis (PCA) and heatmap of kinship matrix (Fig. 1B, C). These results demonstrated the population's extensive genetic diversity.

Six traits related to appearance quality were measured, including GL, GW, GLWR, GCR, GCD, and GTP (Table 1; Fig. 2A–D). Under high-temperature treatment, the full population exhibited average decreases of 8.55%, 3.30%, and 5.36% in GL, GW, and GLWR respectively, and average increases of 26.26% and 78.25% in GCR and GCD, respectively, compared to normal temperatures. For the *indica* subspecies, high temperature treatment reduced GL, GW, and GLWR by an average of 9.18%, 2.54%, and 6.42%, respectively, while GCR and GCD increased by an average of 21.20% and 65.04%, respectively. In contrast, for *japonica* subspecies, GL, GW, and GLWR decreased by an average of 6.86%, 4.47%, and 2.39%, respectively, while GCR and GCD increased by an average of 36.71% and 105.88%, respectively. Moreover, high temperature also degraded GTP and disrupted the endosperm structure (Table 1; Fig. 2E). Therefore, phenotypic comparisons between field and phytotron conditions indicate that elevated temperatures during the flowering stage significantly deteriorate rice grain appearance quality (Fig. 2A–E; Table 1).

To assess cultivar-specific responses to high-temperature treatment, the relative values of grain appearance quality for germplasm were used to evaluate the heat tolerance. Relative values RGL, RGW, RGLWR, RGCR, and RGCD were calculated for each trait under heat stress and natural conditions (Fig. 2F, G). *Indica* rice exhibited heat-sensitivity ranging from 14.50% (RGW) to 45.62% (RGL), moderate responses from 26.45% (RGCD) to 75.53% (RGW), and heat-tolerant from 5.44% (RGL) to 25.62% (RGCR). In *japonica* rice, heat-sensitivity ranged from 14.69% (RGLWR) to 63.64% (RGCD), moderate responses from 22.73% (RGCD) to 78.32% (RGW), and heat-tolerant from 4.90% (RGW) to 19.09% (RGCR) (Fig. 2F, G; Additional file 1: Table S2). Furthermore, for each grain appearance quality trait, we identified five heat-tolerant germplasms that exhibited long, slender or low-chalky grains under both natural and heat stress conditions (Additional file 1: Table S4).

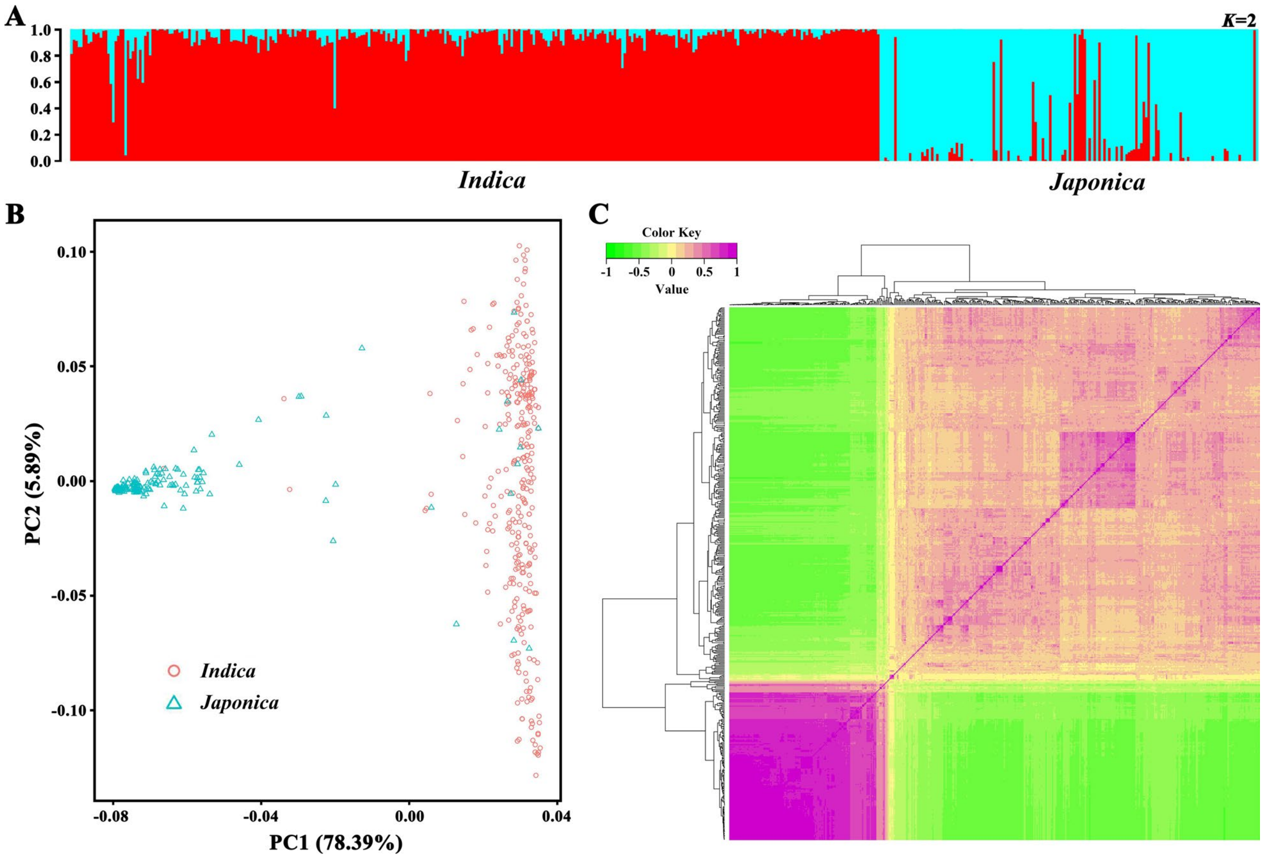

Fig. 1 Population structure analysis of 525 rice accessions. **A** The structure of germplasm population. $K=2$. **B** Principal Component Analysis (PCA). **C** Kinship analysis of the association panel

QTLs in Different Rice Populations and Overlapped QTLs Responsible for RGL, RGW, and RGCD

To explore potential QTLs associated with rice grain appearance quality under heat stress, a GWAS approach was used to examine RGL, RGW, and RGCD in three populations. A significance threshold of $P<0.0001$ was set based on permutation tests, with efficient control of population structure and relationships (Additional file 3: Fig. S2).

A total of 25 rice grain appearance quality-associated QTLs under heat stress were identified across ten chromosomes in the three GWAS panels (Table 2; Fig. 3A–I; Additional file 4: Fig. S3). Within the full population, QTLs for RGCD were located on chromosomes 1, 3, 5, 7, and 12; for RGL on chromosomes 3, 8, and 11; for RGW on chromosomes 4 and 9 (Fig. 3A–C). In *indica* subpopulation, RGCD QTLs were found on chromosomes 3, 4, 5, 7, and 9; RGL QTLs on chromosomes 3 and 11; and RGW QTLs on chromosomes 1, 5, and 10 (Fig. 3D–F). *Japonica* subpopulation did not exhibit RGCD QTL; RGL QTLs were located on chromosome 11; and RGW QTLs on chromosomes 4, 8, and 10 (Fig. 3G–I). Among them, overlapping QTLs included *qHTT-L3* and *qHTT-XL3*, *qHTT-C5* and *qHTT-XC5*, and *qHTT-L11.1* and *qHTT-GL11* co-localized across different genetic backgrounds (Table 2). Therefore, these findings collectively support the presence of numerous new valuable heat-tolerance QTLs/genes related to grain appearance quality that warrant identification, cloning, and utilization.

Accumulation of SA is Beneficial to Improve Grain Appearance Under Heat Stress

To identify the allele effects and pyramiding effects of QTLs with heat tolerance, haplotype analysis was conducted using GWAS and validation populations. In the GWAS panel, two haplotypes (Hap1 and Hap2) were identified. Hap2 was found to be a minority, with Hap1 predominating (Fig. 4A–H). In the full population, Hap1 emerged as the dominant haplotype (Fig. 4A, D, G). In QTLs for RGL and RGW, Hap1 of *qHTT-L3*/*L8*/*L11*/*W9* was significantly more prevalent than Hap2 (Fig. 4A). However, for RGCD, Hap1 of *qHTT-C1*/*C3*/*C5*/*C7*/*C12* was lower and significantly different from Hap2. In *indica* rice, for RGCD, the prevalence Hap1 in *qHTT-XC7* was

Table 1 Phenotype of rice grain apperance quality traits under natural condition and heat sress during the flowering stage

Type	GL (mm)		GW (mm)		GLWR		GCR (%)		GCD (%)		GTP	
	Natural condition	**Heat stress**	**Natural condition**	**Heat stress**	**Natural condition**	**Heat stress**	**Natural condition**	**Heat stress**	**Natural condition**	**Heat stress**	**Natural condition**	**Heat stress**
Full population	5.15	4.71	2.19	2.12	2.40	2.27	60.29	76.13	22.35	39.83	2.87	3.25
Indica population	5.31	4.82	2.09	2.03	2.57	2.40	59.79	72.47	22.18	36.60	2.79	3.17
Japonica population	4.75	4.42	2.43	2.32	1.99	1.94	61.05	83.46	22.74	46.81	3.06	3.53

GL, grain length; GW, grain weight; GLWR, grain length-to-width ratio; GCR, chalky grain rate; GCD, grain chalkiness degree; GTP, grain transparency

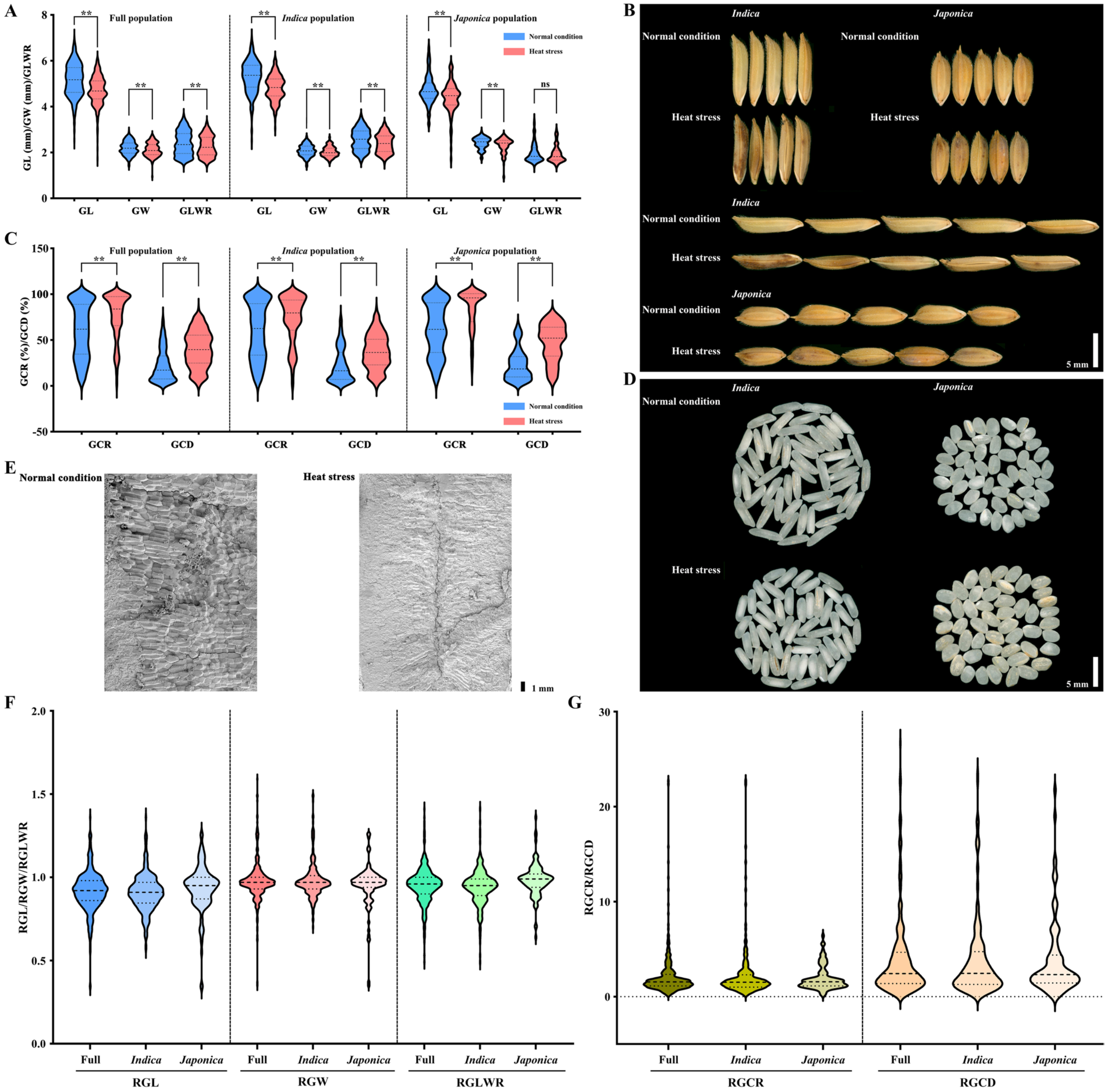

Fig. 2 Phenotypic variation in grain appearance quality between the normal and heat stress in different rice populations. **A**–**D** GL, GW, GLWR, GCR, and GCD under normal condition and heat stress in full, *indica*, and *japonica* populations, respectively. $^{**}P < 0.01$. Scale bar = 5 mm. GL, grain length; GW, grain weight; GLWR, grain length-to-width ratio; GCR, chalky grain rate; GCD, grain chalkiness degree. **E** Scanning electron microscopy analysis of the endosperm under normal condition and heat stress. Scale bar = 1 mm. **F**–**G** Distribution of RGL, RGW, RGLWR (**F**), RGCR, and RGCD (**G**) among different rice populations (full, *indica* and *japonica*). RGL, relative grain length; RGW, relative grain weight; RGLWR, relative grain length-to-width ratio; RGCR, relative chalky grain rate; RGCD, relative grain chalkiness degree

significantly higher than that of Hap2. These results demonstrate the existence of superior haplotypes in natural variation, which can be leveraged in breeding new rice varieties with preferred grain appearance quality.

To study haplotype effects of different QTLs, we analyzed pyramiding effects of dominant SA and inferior alleles (IA) (Table 2; Fig. 5A–G). In the full population, SA0 has the largest, but SA accumulation showed a superposition effect. More than two IA reduces RGL ($r = 0.8109$) (Fig. 5A). RGW increased with the increase of SA ($r = 0.9879$) and decreased with IA (Fig. 5C). For RGCD, more than two SA initially sharply decreased

Table 2 Summary of QTLs associated with grain appearance quality under heat stress

QTL	Population	Trait	Chr	Position (bp)		Signal under CMLM	Alleles	Elite allele
				Start	End			
qHTT-C1	Full	GCD	1	41,612,306	41,736,821	Chr1_41691238	T/A	T
qHTT-C3	Full	GCD	3	21,115,589	21,266,552	Chr3_21207192	A/G	A
qHTT-C5	Full	GCD	5	3,555,144	3,759,653	Chr5_3759644	G/T	G
qHTT-C7	Full	GCD	7	25,969,500	26,097,217	Chr7_26025435	C/G	C
qHTT-C12	Full	GCD	12	15,107,002	15,207,401	Chr12_15107401	A/G	A
qHTT-L3	Full	GL	3	23,147,658	23,210,248	Chr3_23210232	A/T	A
qHTT-L8	Full	GL	8	16,538,072	16,586,398	Chr8_16554234	C/T	C
qHTT-L11.1	Full	GL	11	16,104,814	16,246,186	Chr11_16147593	G/A	G
qHTT-L11.2	Full	GL	11	27,477,614	27,673,911	Chr11_27478002	/	/
qHTT-W4	Full	GW	4	31,599,696	31,664,952	Chr4_31643378	T/A	T
qHTT-W9	Full	GW	9	15,608,860	15,748,832	Chr9_15704704	C/T	C
qHTT-XC3	*Indica/Xian*	GCD	3	21,152,028	21,226,252	Chr3_21207192	A/G	A
qHTT-XC4	*Indica/Xian*	GCD	4	30,886,003	30,942,173	Chr4_30897372	C/T	C
qHTT-XC5	*Indica/Xian*	GCD	5	3,756,474	3,759,653	Chr5_3759644	G/T	G
qHTT-XC7	*Indica/Xian*	GCD	7	25,498,215	25,594,018	Chr7_25594018	G/A	G
qHTT-XC9	*Indica/Xian*	GCD	9	21,061,099	21,078,498	Chr9_21076238	T/C	T
qHTT-XL3	*Indica/Xian*	GL	3	23,204,318	23,210,248	Chr3_23210232	A/T	A
qHTT-XL11	*Indica/Xian*	GL	11	27,673,807	27,674,270	Chr11_27673911	G/T	G
qHTT-XW1	*Indica/Xian*	GW	1	28,473,846	28,587,568	Chr1_28568301	C/T	C
qHTT-XW5	*Indica/Xian*	GW	5	825,750	828,405	Chr5_827597	G/T	G
qHTT-XW10	*Indica/Xian*	GW	10	13,107,874	13,124,978	Chr10_13124483	T/C	T
qHTT-GL11	*Japonica/Geng*	GL	11	16,141,593	16,274,577	Chr11_16227657	C/A	C
qHTT-GW4	*Japonica/Geng*	GW	4	5,495,502	5,584,077	Chr4_5525995	G/A	G
qHTT-GW8	*Japonica/Geng*	GW	8	16,539,560	16,581,280	Chr8_16553137	A/G	A
qHTT-GW10	*Japonica/Geng*	GW	10	1,266,141	1,266,179	Chr10_1266167	A/G	A

GL, grain length; GW, grain weight; GCD, grain chalkiness degree; Chr., chromosome

RGCD to a stable level ($r = 0.9099$), while IA increased rapidly ($r = 0.9266$) (Fig. 5F). In the *indica* rice population, with the increase of SA, RGL increased slightly ($r = 0.8308$), whereas the increase of IA reduced the RGL (Fig. 5B). RGCD decreased with the increase of SA ($r = 0.9984$) and slightly increased with IA (Fig. 4G). In *japonica* rice, more than two SA increased RGW ($r = 0.9618$) and decreased IA more significantly ($r = 0.9545$) (Fig. 5E). These results suggest grain appearance quality was controlled by additive effects of causal genes. Heat-tolerance QTLs with dominant alleles for grain quality traits showed excellent phenotype and pyramiding effects, potentially enhancing heat tolerance in superior rice breeding through SA introduction.

Three Candidate Genes were Identified as Genes Affecting Grain Development Under Heat Stress

To screen valuable genes related to grain appearance quality under heat stress, we conducted a comprehensive functional analysis of the candidate genes in the overlapped QTLs. Among them, *qHTT-C5* and *qHTT-XC5*, *qHTT-L11.1* and *qHTT-GL11* were selected to further screen the candidate genes which can affect spikelet development under high temperatures. *qHTT-C5* and *qHTT-XC5* were located at 3.55–3.76 Mb on chromosome 5, with 25 putative genes (Table 2; Additional file 1: Table S5). *qHTT-L11.1* and *qHTT-GL11* were located at 16.10–16.27 Mb on chromosome 11, with 20 putative genes (Table 2; Additional file 1: Table S6).

To investigate the expression profile of predicted genes involved in rice spikelet development, we performed RNA sequencing (RNA-seq) using young panicles at four developmental stages (5 cm, 10 cm, 15 cm, and 20 cm in length) from the *indica* cultivar Guang-Hui 998 (GH998) grown under natural conditions. As a result, a total of 22 genes exhibited differential expression and were categorized into three classes. Notably, 14 genes were highly expressed in the young panicles 10–20 cm in length, which was exactly the period of heading and flowering stages in rice, suggesting their involvement in rice grain development (Fig. 6A). Subsequently, qRT-PCR analysis was conducted on rice spikelets subjected to heat

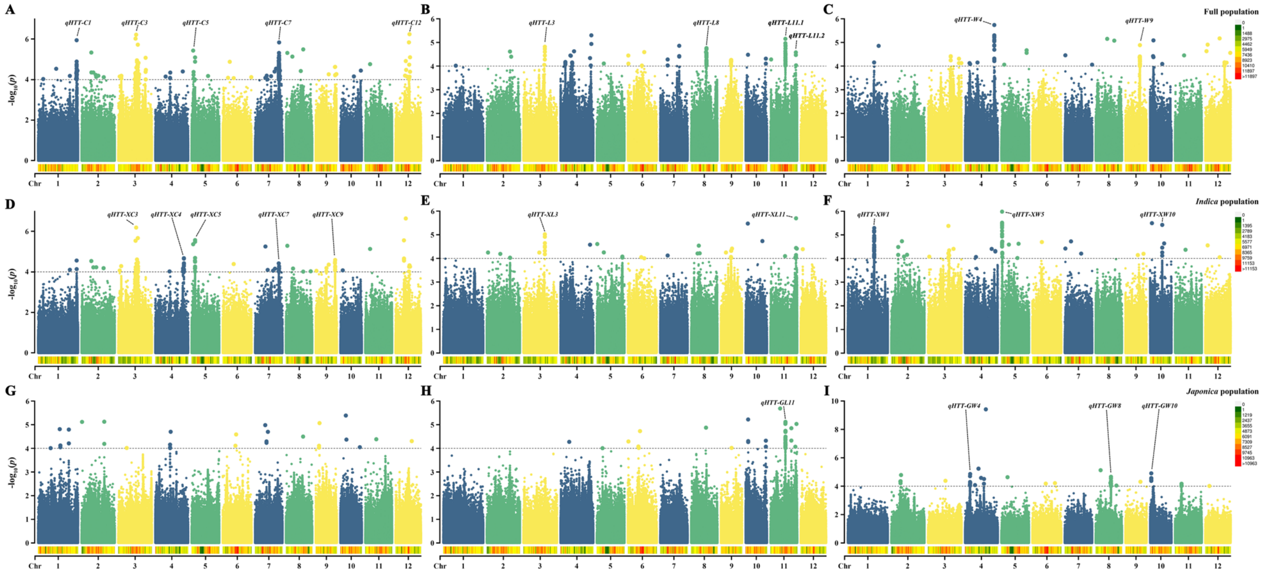

Fig. 3 GWAS of grain appearance quality under the control and high-temperature treatment conditions in different rice populations (full, *indica* and *japonica*). **A**–**C** Manhattan plots for the RGCD (**A**), RGL (**B**), and RGW (**C**) in the full population. **D**–**F** Manhattan plots for the RGCD (**D**), RGL (**E**), and RGW (**F**) in the *indica* population. **G**–**I** Manhattan plots for the RGCD (**G**), RGL (**H**), and RGW (**I**) in the *japonica* population. The horizontal dashed lines represent the significance threshold value ($P = 1.0 \times 10^{-4}$). Different colors (gray to red) indicate the different density distribution of SNPs within 1 Mb window size across 12 chromosomes (Chr) in rice. RGCD, relative grain chalkiness degree; RGL, relative grain length; RGW, relative grain weight

treatment at various time points to identify promising candidate genes influenced by heat stress. This analysis revealed ten genes with differential expression levels between the heat-tolerant variety HHZ and the heat-sensitive variety 9311, while the remaining genes showed minimal or no expression in spikelets. Under heat stress, expression levels of three genes (*LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104*) were significantly higher in HHZ compared to 9311 (Fig. 6B; Additional file 5: Fig. S4A–G). We further compared the expression levels of these three genes across three more materials used in the GWAS population with different heat-tolerance (X21 > MR167 > Cisadane) under heat stress (38 °C, 6 h). Results indicated that *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* were induced by heat stress and displayed different expression levels in these germplasms. Specifically, the expression levels of *LOC-Os05g06920* and *LOC-Os05g 06970* in tolerant variety were higher than those in susceptible varieties while *LOC-Os10g28104* showed higher expression levels in moderate and susceptible varieties, further illustrating the complexity of gene expression patterns under different stress times (Additional file 6: Fig. S5A–C). Additionally, to determine tissue-specific expression patterns, we analyzed the gene expression using the Rice Expression Database (https://ngdc.cncb.ac.cn/red/). *LOC_Os05g06920* showed prominent expression in seed, shoot, leaf, panicle, and aleurone. *LOC_Os05g06970* was also detectable in all examined tissues, with the highest expression observed in root. *LOC_Os11g28104* showed highly expressed in seed, anther, root, leaf, shoot, and panicle (Fig. 6C). These findings were further verified by qRT-PCR on different tissues (root, shoot, node, leaf, and seed) from ZH11 (Additional file 6: Fig. S5D–F). Together, these results confirm that *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* are considered to be the underlying candidate genes likely responsible for grain development and heat tolerance.

To confirm the function of underlying candidate genes in conferring grain appearance quality and heat tolerance, mutant lines of *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* were generated in ZH11 using the CRISPR/Cas9 system. These mutants harbored one or more base substitutions/deletions resulting in loss of gene function (Additional file 7: Fig. S6A). Under natural conditions, phenotypic analysis showed that the *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* mutants exhibited an increase in GL, GLWR, GCR, and GCD, but decreased GW compared to the WT. (Fig. 6D, F–K). Under heat stress, phenotypic analysis showed that, compared to the WT, the *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* mutants exhibited increased GL and GLWR, but decreased GW, GCR, and GCD (Fig. 6E, F–K). To validate whether *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104*

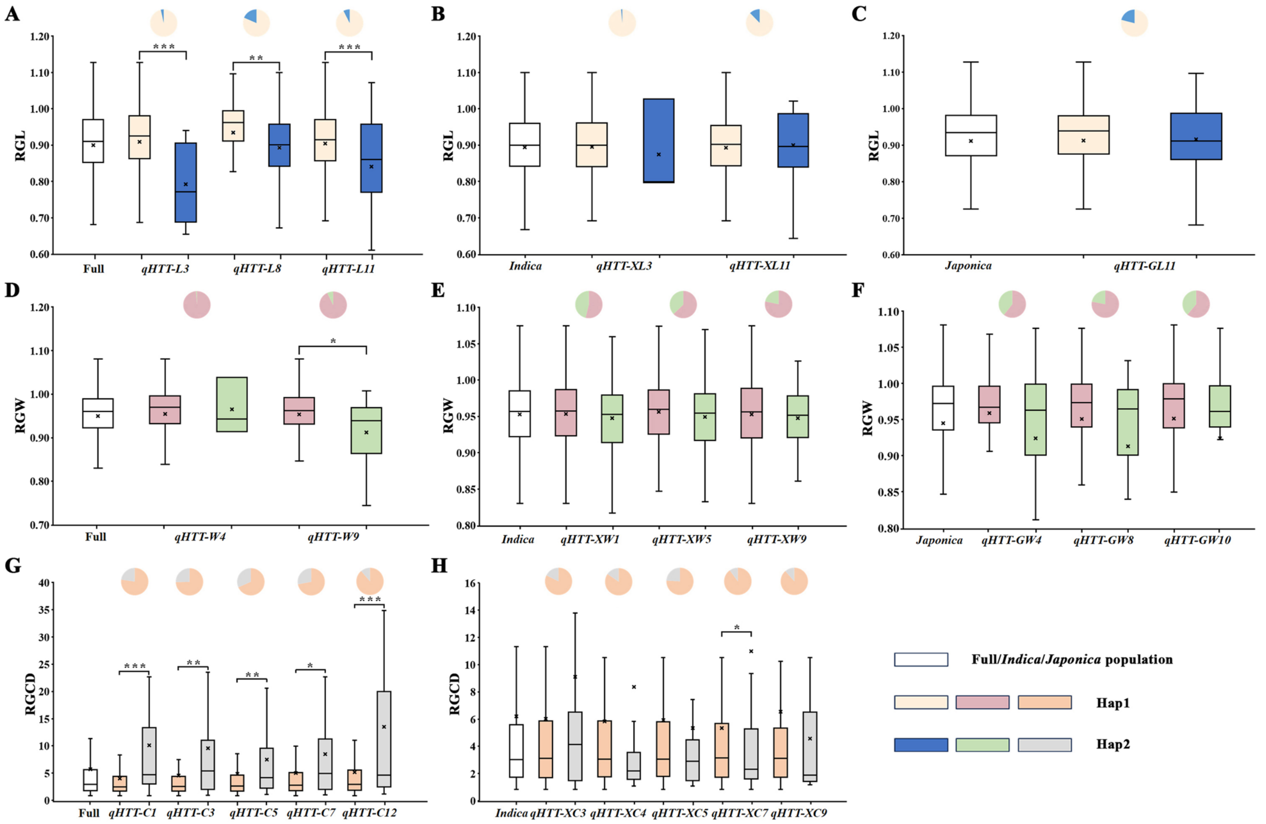

Fig. 4 Effect of Hap1 and Hap2 of heat tolerance QTL for RGL (**A**–**C**), RGW (**D**–**F**), and RGCD (**G**–**H**) in full (**A**, **D**, **G**), *indica* (**B**, **E**, **H**), and *japonica* (**C**, **F**) populations, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. RGL, relative grain length; RGW, relative grain weight; RGCD, relative grain chalkiness degree

function in regulating heat tolerance, we further compared the differences in appearance quality traits of WT and mutant lines under natural and heat stress conditions. Results showed that, after heat stress treatment, the WT showed a significant decrease in GL, GW, and GLWR, and a significant increase in GCR, GCD, and GTP. Both the *LOC_Os05g06920* and *LOC_Os05g06970* mutant exhibited a significant decrease in GW, but a significant increase in GCR. And the *LOC_Os11g28104* mutant displayed a significant increase in GCR, GCD, and GTP. No significant differences were observed in other appearance quality traits of the *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* mutants. (Fig. 6E–K). However, compared to the WT, the three mutant lines revealed significantly higher in RGL, RGW, and RGLWR while RGCR and RGCD were significantly lower, suggesting that loss-of-function of these genes improved the heat tolerance and maintained the stability of grain appearance quality traits under heat stress (Additional file 7: Fig. S6B–G). These results together suggest that *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* are considered high-priority candidate genes for regulating grain development and heat tolerance during the flowering stage.

Discussion

Heat stress during the reproductive stage adversely affects rice grain quality as extensively studied (Arshad et al. 2017; Liu et al. 2021; Wu et al. 2021, 2023; Zhang et al. 2023). Previous studies found that grain quality under heat stress varies substantially among different rice varieties, with improvement of heat tolerance identified as a highly effective counter measure against extreme high temperature in the future (Wu et al. 2023). Therefore, the discovery of heat-tolerant QTLs/genes and the development of genetically modified heat-tolerant varieties are paramount for rice breeders. Fortunately, numerous heat-tolerant cultivars and QTLs/genes have been identified in various rice germplasms or varieties. For example, the *aus* variety N22 exhibits exceptional heat tolerance during the flowering–grain filling stage (Jagadish et al. 2010; Prasad et al. 2006; Wu et al. 2023). Several heat-tolerant genes, such as *TT1*, *TT2*, *TT3*, and *HTH5*, have

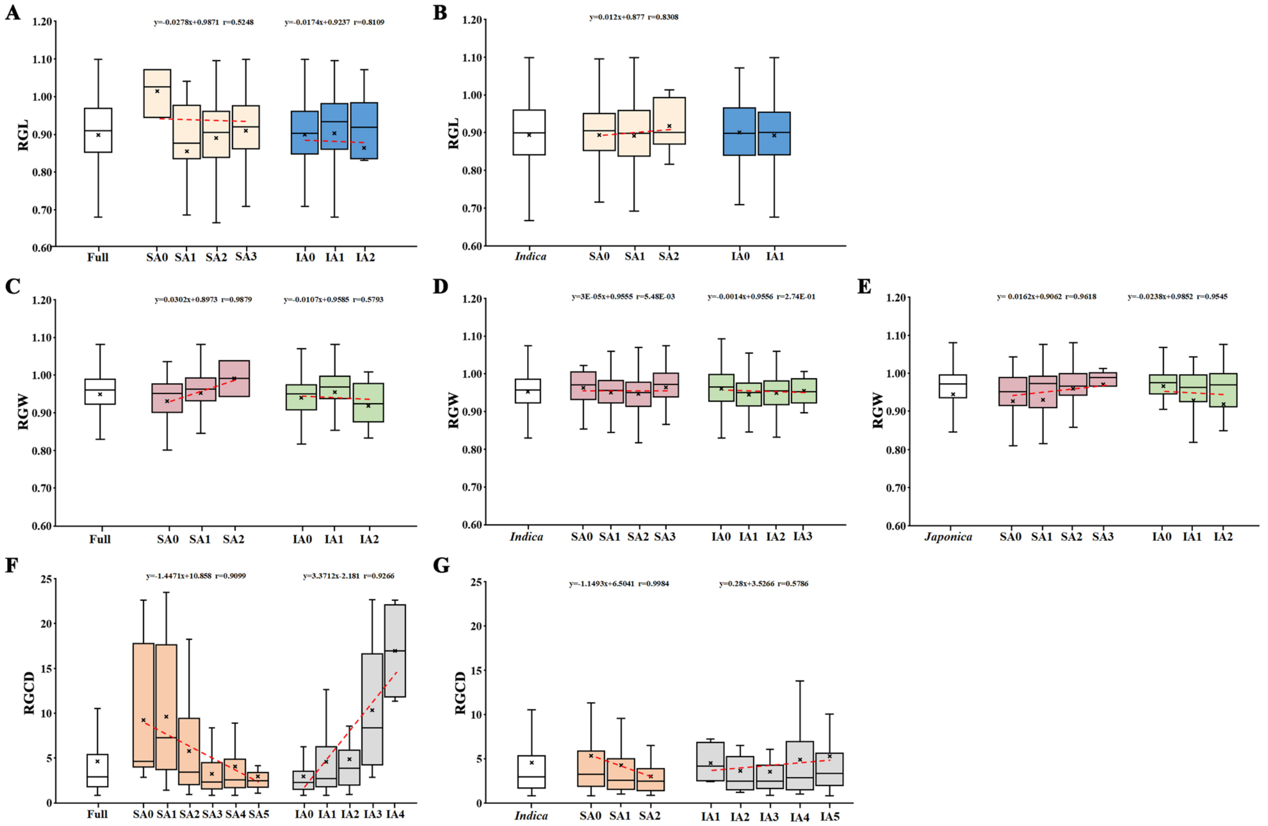

Fig. 5 Pyramiding effect of superior alleles (SA) and inferior alleles (IA) of heat tolerance QTL for RGL (**A**–**B**), RGW (**C**–**E**), and RGCD (**F**, **G**) in full (**A**, **C**, **F**), *indica* (**B**, **D**, **G**), and *japonica* (**E**) populations, respectively. RGL, relative grain length; RGW, relative grain weight; RGCD, relative grain chalkiness degree

been already fine-mapped and cloned (Cao et al. 2022; Kan et al. 2022; Li et al. 2015; Zhang et al. 2022). In this study, we investigated a large collection of rice accessions to explore the natural variation in grain shape (length and width) and chalkiness under heat stress. Panicle initiation and grain-filling stages are critical for rice grain development and grain quality (Wu et al. 2016, 2021, 2023). However, previous studies primarily examined the effects of high temperatures on rice grain quality during the grain-filling stage, with limited focus on quality changes following high-temperature treatment during the flowering stage. Our results demonstrate that heat stress during the flowering stage significantly diminishes rice grain appearance quality, producing shrunken (decreased GL and GW) and chalky seeds. Thus, it is crucial to recognize the pivotal role of the flowering stage, along with panicle initiation and grain-filling stage, in determining rice grain appearance quality. In general, varieties with longer, narrower, and lower chalkiness grains typically exhibit superior appearance quality (Gong et al. 2017; Zhao et al. 2022). Notably, as shown in Table S4, we have screened several heat-tolerant germplasms, such as Min Hui 3301, Feng Hua Zhan, and Inga, which exhibited good grain appearance quality even under high-temperature stress at anthesis, providing valuable resources for the future breeding of new heat-tolerant and superior appearance quality rice varieties.

Grain appearance quality and heat tolerance are complex, polygenic traits sensitive to environmental factors. Introducing one or few minor-effect QTLs into a rice variety may not significantly improve appearance quality under heat stress (Chen et al. 2021; Yang et al. 2021). Recent studies, however, have shown that pyramiding low chalkiness QTLs can develop high-quality rice varieties with reduced chalkiness even in high-temperature environments. The percentage of grain chalkiness decreased with the number of QTLs in the pyramiding lines increases (Yang et al. 2024). Similarly, rice plants with five heat-tolerant QTLs (*qHTSF1.2*, *qHTSF2.2*, *qHTSF4.1*, *qHTSF6.1*, and *qHTSF11.3*) exhibited greater heat tolerance and comparable or slightly higher mean spikelet fertility than the tolerant parent Giza178 (Ye et al. 2015). Introgression of several prominent QTLs or superior haplotypes into high-yielding,

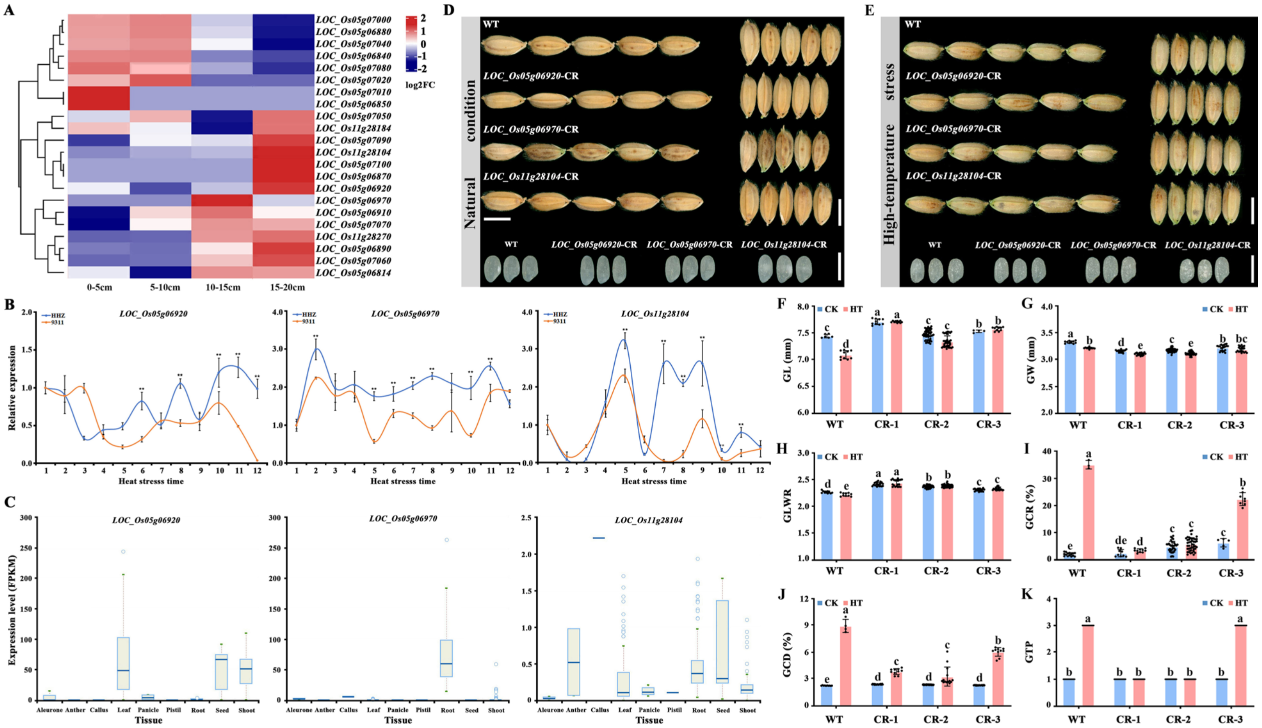

Fig. 6 Identification and functional validation of candidate genes in regulating grain appearance quality-related traits under normal and high-temperature conditions. **A** Results of the hierarchical cluster analysis and the expression levels of predicted genes in spike at 0–20 cm of GH998 plants grown under normal conditions. The heatmap was generated by taking $\log_2$ fold change of the FPKM ratio. Higher expression values are represented in red, and lower values are represented in blue. **B** Expression analysis of *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* in HHZ and 9311 under heat stress for different time points. $^{*}P < 0.05$, $^{**}P < 0.01$. **C** Expression profiles of the candidate genes (*LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104*) in different tissues. **D**–**E** Comparison of GL, GW, and GC in ZH11 (WT) and *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* mutants under natural conditions (**D**) and high-temperature stress (**E**). Scale bars = 5 mm. GL, grain length; GW, grain weight; GC, grain chalkiness. **F**–**K** GL (**F**), GW (**G**), GLWR (**H**), GCR (**I**), GCD (**J**), and GTP (**K**) in *LOC_Os05g06920*-CR (CR-1), *LOC_Os05g06970*-CR (CR-2), *LOC_Os11g28104*-CR (CR-3), and WT under natural and heat stress conditions. Different letters indicate significant differences at $P < 0.05$. GLWR, grain length-to-width ratio; GCR, chalky grain rate; GCD, grain chalkiness degree; GTP, grain transparency

drought-susceptible varieties also improved yield under drought stress (Singh et al. 2024). Furthermore, another study also revealed that pyramiding eleven heat-tolerant genes achieved the highest tolerance to heat stress (Yang et al. 2022). However, it is important to note that a recent study demonstrated that gene pyramiding performance depended on the comparison between the algebraic sum of the additive and epistatic effects of QTLs in the pyramidal line and the additive effect value of the single QTL. Knocking out one gene can also alter the function of other genes with similar functions (Liang et al. 2021). Our study detected 25 novel QTLs for RGL, RGW, and RGCD via GWAS, offering fresh insights into the intricate molecular regulation of grain appearance quality and heat tolerance. Further haplotype analysis revealed that varieties carrying Hap1 displayed longer GL, wider GW, and lower chalkiness under heat stress, particularly in the full population. The pyramiding of the SA for RGL, RGW, and RGCD demonstrated that combinations of multiple QTLs significantly enhanced the phenotypic effect in high-temperature environments, thereby improving grain appearance quality. Thus, it is essential to continue identifying favorable genes to enhance rice's appearance quality in high-temperature environments. The priority is to selectively polymerize multiple QTLs/genes to effectively acquire heat tolerance and boost adaptability to various high-temperature environments.

Previous studies demonstrate that numerous genes related to grain quality influence both grain size and chalkiness in rice (Gong et al. 2017; Zhao et al. 2022). For instance, *GW2* significantly increases GW while increasing GCR, with its allele, *gw2.1*, capable of increasing GL and GW but decreasing GCD (Huang 2022a, b; Song et al. 2007; Verma et al. 2021). Additionally, several genes controlling slender grain shape, including *gw8*, *GW7*, *lgy3*, and *gs9*, can reduce GC and significantly enhance grain appearance quality in rice (Liu et al. 2018; Wang et al. 2012, 2015; Zhao et al. 2018a, b). In this study, we identified three genes, *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104*, that exhibited high expression in reproductive tissues during the flowering stage under heat stress. Utilizing the CRISPR-Cas9 method, we generated transgenic plants with knockouts of these genes. Subsequent phenotypic analyses indicated that, compared with WT ZH11, the mutant grains displayed significant differences in both grain shape and chalkiness, implying that *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* participate in regulating seed development. Furthermore, under heat stress, the WT and mutant lines exhibited a deteriorated grain appearance quality, resulting in shorter, narrower, and more chalky grains. Notably, the mutants demonstrated a significantly smaller decline in appearance quality traits compared to the WT plants, indicating their enhanced heat tolerance relative to WT. The previous studies revealed that *LOC_Os05g06920* (*OsCRSH2*), encoding a relA-SpoT-like protein RSH4, is implicated in rice's hormonal response and seed development/maturation (Prusińska et al. 2019; Tozawa et al. 2007). *LOC_Os05g06970* encodes a peroxidase precursor, crucial in plant immunity for scavenging stress-induced reactive oxygen species (Kidwai et al. 2020). *LOC_Os11g28104* encodes a protein kinase with a DUF26 domain, implicated in signal transduction and stress response (Huang et al. 2022a, b; Lv et al. 2023). However, the genetic and molecular mechanisms by which *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* regulate grain appearance quality traits under heat stress during flowering stage remain unclear. To mitigate the negative effects of climate change on rice production, it is essential to breed high-yield superior-quality and climate-resilient rice cultivars in the future. However, simultaneous improvement of grain quality and abiotic stress resistance remains a huge challenge for rice breeders. Therefore, our results provide promising genes controlling grain appearance quality and heat tolerance for rice breeders, which can be used in marker-assisted selection for improving grain yield and quality stability under heat stress in rice.

Conclusions

This study observed significant variations in grain appearance quality traits (GL, GW, and GC) between high-temperature treatment and natural conditions in 525 rice accessions. A GWAS analysis identified 25 QTLs for RGL, RGW, and RGCD, including three overlapping QTLs across different populations. Haplotype analysis revealed Hap1 as the superior haplotype, and pyramiding more than two SA benefited improving rice grain appearance quality in high-temperature environments. Three novel genes, *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104*, were obtained and confirmed as the most likely candidates regulating grain development and heat tolerance by RNA-seq analysis, qRT-PCR verification, and functional analysis. Compared to the WT, the mutants of *LOC_Os05g06920*, *LOC_Os05g06970*, and *LOC_Os11g28104* exhibited enhanced heat tolerance and effectively reduced the negative impacts of high temperatures on rice grain appearance quality. Therefore, these findings provide a theoretical foundation and offer promising genetic targets for breeding strategies aimed at maintaining grain appearance quality under heat stress in rice.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00770-y.

Additional file 1.
Additional file 2.
Additional file 3.
Additional file 4.
Additional file 5.
Additional file 6.
Additional file 7.

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Not applicable.

Author Contributions

YP, TL, CC, and G(x)D designed the experiments. LC, WC and YW conducted the experiments. JL, DQ, JH, XY, MT, ZZ, JY, and G(f)D participated in the material preparation. YP and LC performed the data analyses. LC and YP wrote and revised the paper. All authors read and approved the manuscript.

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Data Availability

The sequencing data of 525 rice accessions used for our analyses can be obtained via project accession PRJEB6180 from NCBI (https://www.ncbi.nlm.nih.gov/sra/?term = PRJEB6180), accession ERP005654 from DDBJ (https://www.ddbj.nig.ac.jp/index-e.html) and from the GigaScience Database (https://doi.org/https://doi.org/10.5524/200001). All data generated or analyzed during this study are included in this manuscript, its supplementary information files and publicly available repositories.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors declare no competing interests.

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