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ORIGINAL ARTICLE

Enhancing grain shape, thermotolerance, and alkaline tolerance via Gγ protein manipulation in rice

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Abstract

Key message **Our findings highlight a valuable breeding resource, demonstrating the potential to concurrently enhance grain shape, thermotolerance, and alkaline tolerance by manipulating Gγ protein in rice.**

Abstract Temperate Geng/Japonica (GJ) rice yields have improved significantly, bolstering global food security. However, GJ rice breeding faces challenges, including enhancing grain quality, ensuring stable yields at warmer temperatures, and utilizing alkaline land. In this study, we employed CRISPR/Cas9 gene-editing technology to knock out the *GS3* locus in seven elite GJ varieties with superior yield performance. Yield component measurements revealed that *GS3* knockout mutants consistently enhanced grain length and reduced plant height in diverse genetic backgrounds. The impact of *GS3* on the grain number per panicle and setting rate depended on the genetic background. *GS3* knockout did not affect milling quality and minimally altered protein and amylose content but notably influenced chalkiness-related traits. *GS3* knockout indiscriminately improved heat and alkali stress tolerance in the GJ varieties studied. Transcriptome analysis indicated differential gene expression between the *GS3* mutants and their wild-type counterparts, enriched in biological processes related to photosynthesis, photosystem II stabilization, and pathways associated with photosynthesis and cutin, suberine, and wax biosynthesis. Our findings highlight *GS3* as a breeding resource for concurrently improving grain shape, thermotolerance, and alkaline tolerance through Gγ protein manipulation in rice.

Introduction

China is a pioneer in the domestication and cultivation of rice, currently holding the position of the world's largest rice producer and consumer (Muthayya et al. 2014). Remarkable advancements in rice productivity have characterized China's trajectory over the past century, witnessing a substantial increase in yields from 1.9 t/hm^2 in 1949 to 7.0 t/hm^2 in 2018 (http://faostat.fao.org/). This noteworthy growth is primarily attributed to the adoption of semi-dwarf and hybrid rice varieties coupled with the rapid proliferation of high-yielding temperate Geng/Japonica (GJ) cultivars. In national trials evaluating new rice varieties in northeast China, the average yield of emerging temperate GJ varieties demonstrated an upward trend, escalating from 8.3 in 2004 to 9.0 t/hm^2 in 2018 (Fei et al. 2020). Despite these impressive achievements in GJ rice production, challenges persist in GJ rice breeding.

GJ rice breeding has proven effective in enhancing grain yield, although the quality of grains often remains at a moderate level. Grain shape is a crucial breeding target because of its influence on both yield potential and appearance quality. Although many GJ rice varieties exhibit short and round grains, consumer preferences lean towards slender shapes given their superior appearance quality. Recent studies have revealed that the determination of rice grain shape involves multiple signaling pathways, including ubiquitination-mediated proteasomal degradation, phytohormones (cytokinin, brassinosteroid, and auxin), and G-protein signaling pathways (Ren et al. 2023). Among these pathways, *GS3* is pivotal, representing the first cloned QTL encoding a rice G-protein γ subunit with a negative regulatory role in

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grain length (Fan et al. 2006; Takano-Kai et al. 2009). The C/A SNP in the 2nd exon of *GS3* is a functional mutation that leads to elongated grain shapes, and its prevalence is widespread in rice germplasms (Fan et al. 2009; Wang et al. 2011). Consequently, manipulating *GS3* presents a viable avenue for elongating the grains of the GJ varieties.

Another challenge confronting GJ breeding is climate change, particularly global warming, which poses a severe risk to agriculture worldwide. Reports from the Intergovernmental Panel on Climate Change (IPCC) in 2021 and 2022 underscore that global temperatures have surged by 1.1 °C compared to the pre-industrialization era (1850–1900), with projections anticipating a further increase exceeding 1.5 °C in the next two decades (Deliang et al. 2021; Rawshan et al. 2022). Notably, the average temperature in China increased by 0.24 °C per decade from 1951 to 2020, surpassing the global average warming rate. Our recent research revealed a pronounced elevation of 0.48 ℃ in the daily average temperatures over the past two decades in the central rice region of Liaoning Province, a rate significantly surpassing the national average (unpublished data). This increasing temperature trend indicates that the Northeast region is one of the areas most profoundly affected by climate change in China and globally, imposing substantial constraints on rice growth, development, yield, and quality. Therefore, exploring gene resources related to heat tolerance in GJ rice holds both theoretical significance and practical application value, providing a means for GJ rice to adapt to the challenges imposed by climate change. Investigation of the molecular mechanisms underlying thermotolerance in African rice identified a QTL associated with thermotolerance, attributed to natural variations in the G-protein γ subunits *GS3/TT2*. Upon heat stress, *GS3* facilitates cytosolic Ca^{2+} elevation and fosters downstream interactions between calmodulin and transcription factor *SCT1*. This interaction suppressed *OsWR2* gene expression and diminished cuticular wax content, resulting in increased thermosensitivity. In the absence of *GS3*, the blockade of Ca^{2+} accumulation and CaM–*SCT1* interaction promotes the deposition of cuticular wax, thereby endowing plants with enhanced thermotolerance (Kan et al. 2022). Hence, the utilization of a knockout mutant of *GS3* holds promise for improving the thermotolerance of GJ rice varieties.

The third challenge involves the cultivation of GJ varieties in alkaline salt lands. According to 2015 research conducted by the Food and Agriculture Organization (FAO), more than 1 billion hectares of land worldwide are affected by salt, with approximately 60% of this area categorized as sodic due to elevated pH levels resulting from the presence of sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3). In China, saline-alkali land spans approximately 100 million hectares and is primarily situated in the northern provinces along the Yangtze River. Effective utilization of marginal lands is imperative for augmenting annual crop yields, given the limited availability of arable land. In rice plants, *GS3* interacts with aquaporin PIP2s and hinders its hydrogen peroxide (H_2O_2) export function, thereby causing an excessive accumulation of reactive oxygen species (ROS) and rendering plants susceptible to alkalinity. The non-functional gs3 protein fails to inhibit PIP2s, consequently enhancing alkaline tolerance (Zhang et al. 2023). This suggests that the non-functional *gs3* allele not only improves grain yield and thermotolerance but also enhances tolerance to alkaline soil. Such enhancements have the potential to expand the suitable areas for cereal cultivation.

Therefore, the concurrent improvement in grain shape, thermotolerance, and alkaline tolerance through the manipulation of Gγ protein in rice is theoretically feasible. To verify this hypothesis, we selected seven GJ varieties with high yield performance, which are widely distributed, as subjects for modification. The *GS3* locus was knocked out using CRISPR/Cas9 gene-editing technology. Grain shape, grain quality, thermotolerance, and tolerance to alkali were investigated. Downstream genes of *GS3* were identified using transcriptome sequencing.

Materials and methods

GWAS and haplotype assay

The grain length data for 529 diverse *O. sativa* accessions were obtained from a previous study (Xie et al. 2015). A mixed-model approach was employed, utilizing factored spectrally transformed linear mixed models (FaST-LMM) (Lippert et al. 2011). Comprehensive details regarding GWAS are available elsewhere (Yang et al. 2014). Haplotype analysis of *GS3* utilized sequencing data from 4726 accessions accessed through RiceVarMap2 (https://ricevarmap.ncpgr.cn/). Phenotype data for haplotype analysis were sourced from a previous study (Yang et al. 2014).

Plant materials

We chose six interrelated modern Chinese temperate GJ varieties and one related Japanese GJ varieties to be the genetic background of the present study. All seven varieties were in large-scale promotion. The Japanese GJ variety, Sasanishiki (Sasa), developed in 1963, was cultivated in the Tohoku region in Japan and gained status as high-quality rice. Toyonishiki was derived from the cross between Sasa and another Japanese GJ variety Aoyu329, and subsequently introduced to China and served as a backbone parent for a long time. A series of high-yield varieties were bred using Toyonishiki as a parental line, such as Shennong265 (SN265), the first released 'super rice variety' by the

Chinese Ministry of Agriculture, and Liaoxing1 (LX1), the variety with the largest promotion area in Liaoning Province in China. The other varieties used in the present study, such as Yanfeng47 (YF47), Beigeng1501 (BG1501), Beigeng1 (BG1), and Beigeng (BG1705) were bred using SN265 as a parental line. The pedigree relationship among the seven varieties is demonstrated in Fig. S1.

Vector construction and plant transformation

For CRISPR/Cas9 gene editing, we followed the vector construction protocol described by Li et al. (2017). A targeting sequence, including the PAM sequence (23 bp), was selected within the first exon of the *GS3* gene. To ensure the specificity of the targeting sequence, a BLAST search of the rice genome was conducted using the NCBI BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The rice transformation procedures followed the guidelines provided by Hsu et al. (2013). Genomic DNA extracted from the transformants was sequenced to identify the mutants. The resulting PCR products (200–500 bp) were sequenced and analyzed using the Degenerate Sequence Decoding method (Ma et al. 2015).

Yield components and quality investigation

Wild-type varieties and their *GS3* mutants were cultivated in the experimental paddy field of Shenyang Agricultural University, Shenyang, China (N41°, E123°). Planting commenced on April 24th in the seedling nursery, with transplantation occurring per hill on May 23rd. The plant materials were arranged in a randomized block design, with three replicates. Each plot, measuring 5.4 m^2, accommodated 120 plants at 30 cm × 15 cm intervals. The cultivation methods and field management followed previously established procedures (Li et al. 2018). Field examination plants were harvested 45 d after heading, with a total of 20 plants from the middle rows harvested for each line. The yield components and quality measurements followed the protocols outlined in our previous study (Li et al. 2018).

Thermotolerance and tolerance to alkali soil evaluation

Seeds designated for thermotolerance and alkali-soil tolerance evaluations were harvested simultaneously and stored under identical conditions. To mitigate dehydration stress associated with high temperatures, high relative humidity (RH > 90%) was maintained during the heat treatment. Twelve-day-old seedlings, at the two-leaf stage and grown in a hydroponic culture solution4, underwent a 42 °C treatment for the specified duration, followed by a week of recovery under normal conditions (28 °C). The survival rate was subsequently calculated. To assess alkali-soil tolerance, 75 mM mixed alkali ($NaHCO_3$:Na_2CO_3 at a molar ratio of 5:1) was applied to the soil. The relative survival rate after exposure to 75 mM alkali stress for three weeks was recorded. Additional details can be found in previous studies (Kan et al. 2022; Zhang et al. 2023).

RNA-seq and bioinformatic analysis

A transcriptome assay involving wild-type varieties and their *GS3* mutants was performed to elucidate the downstream genes of *GS3*, particularly in the mediation of grain size, grain quality, thermotolerance, and tolerance to alkali soil. Total RNA was extracted from the young panicles of wild-type varieties and their *GS3* knockout plants, with three biological replicates, each comprising 10 plants. The RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Paired-end libraries were constructed and subsequently sequenced on an Illumina HiSeq2500 platform at OneMore Tech Co., Wuhan, China. Transcripts exhibiting significant differential expression between wild-type varieties and their *GS3* mutants were identified using a False Discovery Rate (FDR) of less than 0.05, and an absolute fold change of at least 1.5. Differentially Expressed Genes (DEGs) were subjected to enrichment analysis based on Gene Ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results

GS3 is the major locus for grain length

To identify the major locus for grain length, we initially compiled grain length data for 529 diverse *Oryza. sativa* accessions from previous studies (Yang et al. 2014; Xie et al. 2015). The grain length, ranging from 6.13 mm to 10.97 mm, exhibited a normal distribution (Fig. 1A). Subsequently, we performed GWAS by integrating phenotypic and sequencing data based on the Illumina platform (Yang et al. 2014; Xie et al. 2015). A total of 16 loci exceeding a significant threshold ($-\log_{10}P \geq 5$) were detected (Fig. 1B), with a focus on the top peak located on chromosome 3, correlating with the known grain length regulation gene *GS3* (Fan et al. 2006). A dataset comprising 4,729 rice accessions, including 595 XI I, 465 XI II, 913 XI III, 786 XI intermediate, 767 GJ-Te, 504 GJ-Tr, 241 GJ intermediate, 269 Aus, 96 VI, and 90 intermediate, was subsequently employed to conduct haplotype network analysis of *GS3*. Allelic variations in *GS3* could be classified into three major haplotypes (Fig. 1C). Among these three haplotypes, the C/A SNP in the 2nd exon of Hap3 generated a stop codon, causing the formation of a truncated *GS3* protein. The grain length of

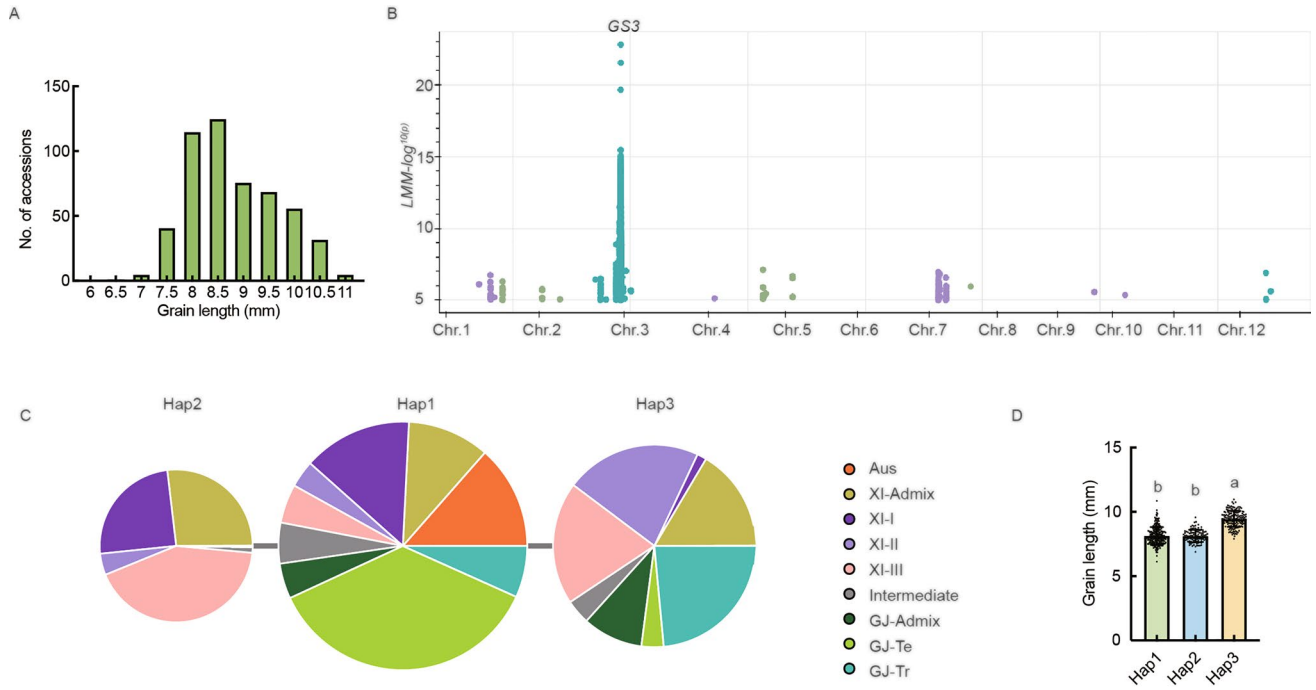


Fig. 1 GWAS for grain length and identification of the causal gene for the peak on chromosome 3. **A** Diversity in grain length among 529 *O. sativa* accessions. **B** Manhattan plot of grain length with $-\log_{10}P \geq 5$. **C** Haplotype network analysis of *GS3* using a dataset of 4729 rice accessions. **D** Comparison of grain length among the three haplotypes. The grain length data was derived from Fig. 1

Hap3 was significantly longer than those of Hap1 and Hap2 (Fig. 1D). Consequently, we deduced that *GS3* serves as the primary regulator of grain length.

Gene editing of *GS3* in high-yield rice varieties

To validate whether manipulating *GS3* could regulate grain length in widely cultivated commercial rice varieties, we selected six Chinese rice varieties and one Japanese GJ variety, all characterized by short and round grains. Single-guide RNAs designed for the target sites on the 1st exon of *GS3* were integrated into the CRISPR/Cas9 vector (Fig. 2). These constructs were introduced into seven varieties using *Agrobacterium*. Sanger sequencing of 20 individuals from each mutant in the T_0 generation was conducted to assess the mutation efficiency. Single peaks in Sanger sequencing results were interpreted as homozygous mutations, whereas double peaks signified heterozygous mutations. On an average, mutations were observed in 44.29% of the plants, with 18.57% exhibiting putative homozygous mutations (Table S1), except for Sasa, where at least two types of mutations were identified for each variety (Fig. 2).

Effect of *GS3* on yield related traits

We first observed the plant architecture of *GS3*-edited plants. The results demonstrated that all *GS3*-edited plants exhibited significantly reduced height compared to wild-type varieties, affirming the role of *GS3* knockout in lowering plant height (Fig. 3A–H). Although *GS3* had no discernible effect on panicles number in most varieties, exceptions were noted in SN265 and Sasa. In the SN265 genetic background, *GS3* knockout significantly decreased panicle numbers, whereas in the Sasa genetic background, panicle numbers were significantly increased (Fig. 3I–O). Regarding grain number per panicle, *GS3* exerted its influence primarily on YF47 and LX1, with *GS3* knockout significantly decreasing grain number per panicle (Fig. 4). The *GS3* mutant exhibited a disadvantageous effect on the setting rate in most studied varieties; however, the knockout of *GS3* under the YF47 genetic background surprisingly improved the setting rate (Fig. 4). Our gene-editing experiments underscored the robust and consistent impact of *GS3* on grain length across diverse genetic backgrounds (Fig. 5). The knockout of *GS3* led to a notable increase in grain length and 1,000 grain weight, with averages of 6.43% and 5.33%, respectively. Notably, the effect of *GS3* was relatively weaker in YF47 and LX1, with modest increases in grain length of 2.28% and 2.99%, respectively (Fig. 5D, F). Conversely, its impact was more pronounced

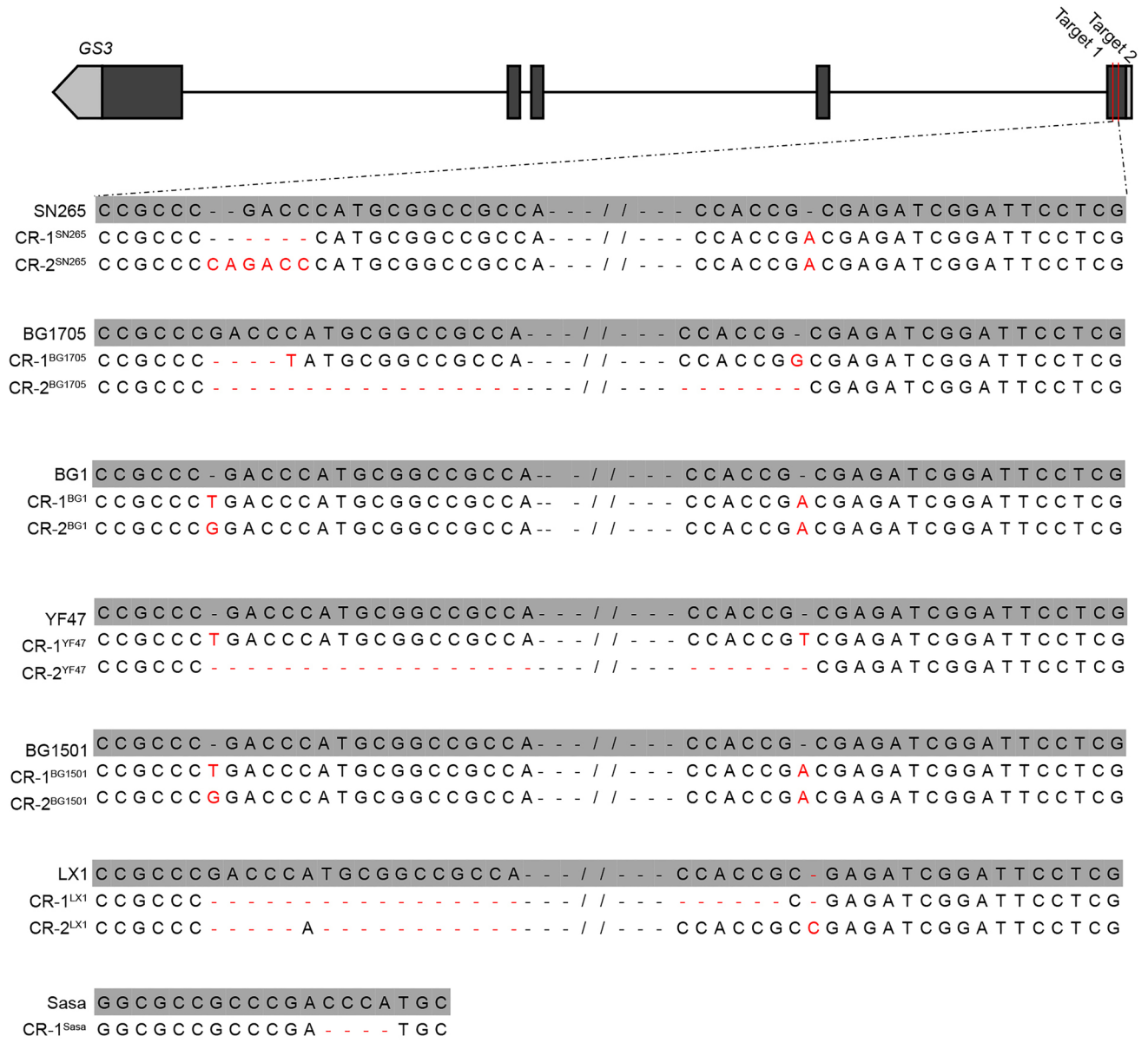


Fig. 2 Schematic diagram of *GS3* and sequence comparison between wild-type varieties and their *GS3* mutants. The coding region is depicted in gray, and the untranslated regions are shown in light gray. The lines represent introns. Sequences of the target sites in the wild-type and homozygous mutants were aligned below the gene structure

in Sasa, resulting in a substantial 14.29% increase in the grain length (Fig. 5G).

Effect of *GS3* on quality related traits

Grain length is closely related to milling and appearance quality, with slender grain shapes typically enhancing the appearance quality while influencing milling quality. Our findings revealed that despite the significant increase in grain length resulting from *GS3* knockout, there was no discernible effect on the brown rice and head rice ratios (Fig. 6). Interestingly, *GS3* exhibited varied effects on chalkiness-related traits across seven varieties. The chalkiness rice level and chalkiness rice ratio significantly improved under the genetic backgrounds of SN265 and YF47 but deteriorated under BG1705, BG1, and Sasa. No significant differences were observed between the gene-edited plants and their wild-type counterparts, such as BG1501 and LX1. To assess the impact of *GS3* on protein and amylose content, we observed significant increases in protein content and decreases in amylose content under the genetic backgrounds of SN265 and YF47 (Fig. 6A, D). Notably, *GS3* knockout displayed similar effects on chalkiness-related traits, protein content, and

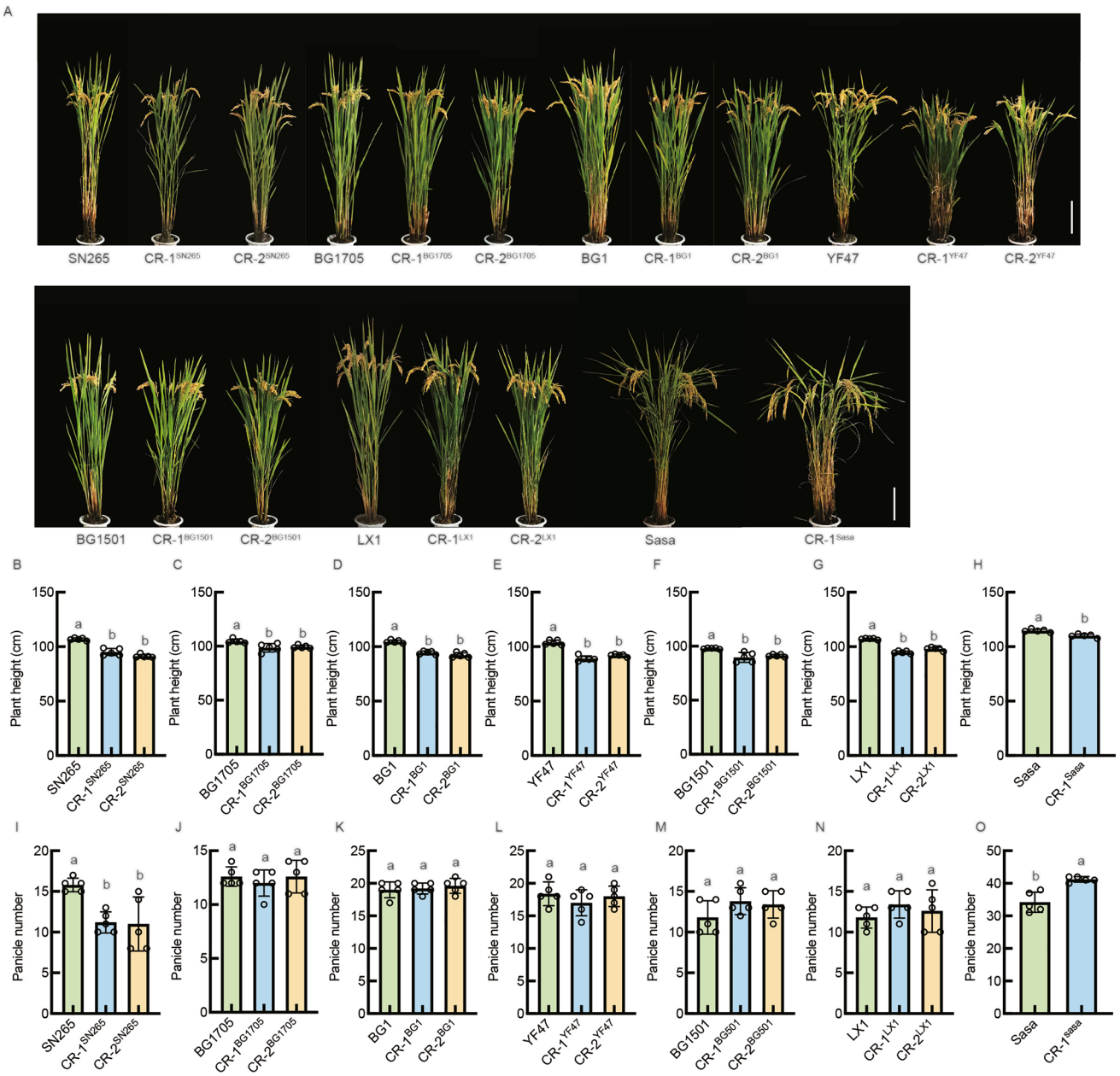


Fig. 3 Agronomic trait comparison between wild-type varieties and their *GS3* mutants. **A** Representative images of wild-type varieties and their *GS3* mutants. Scale bar = 20 cm. **B**–**O** Plant height and panicle number of wild-type varieties and their *GS3* mutants. The investigation was conducted using three biological replicates, with each replicate containing 10 plants. Different letters indicate significant differences ($P < 0.05$) from a Duncan's multiple range test

amylose content in SN265 and YF47 genetic backgrounds. Both protein and amylose contents increased in the BG1 genetic background (Fig. 6C). The amylose content of the *GS3* mutant decreased in the LX1 genetic background, whereas the protein content increased in the Sasa genetic background (Fig. 6F, G). No significant differences in protein and amylose contents were observed among the *GS3* mutants of BG1705, BG1501, and their wild-type varieties (Fig. 6B, E). In conclusion, *GS3* knockout did not affect milling quality and barely changed protein and amylose content but primarily influenced chalkiness-related traits.

Effect of *GS3* on thermotolerance and tolerance to alkali soil

Recent molecular studies have highlighted the involvement of *GS3* in regulating thermotolerance and tolerance to alkali soil in the genetic backgrounds of ZH11 (a GJ variety) and

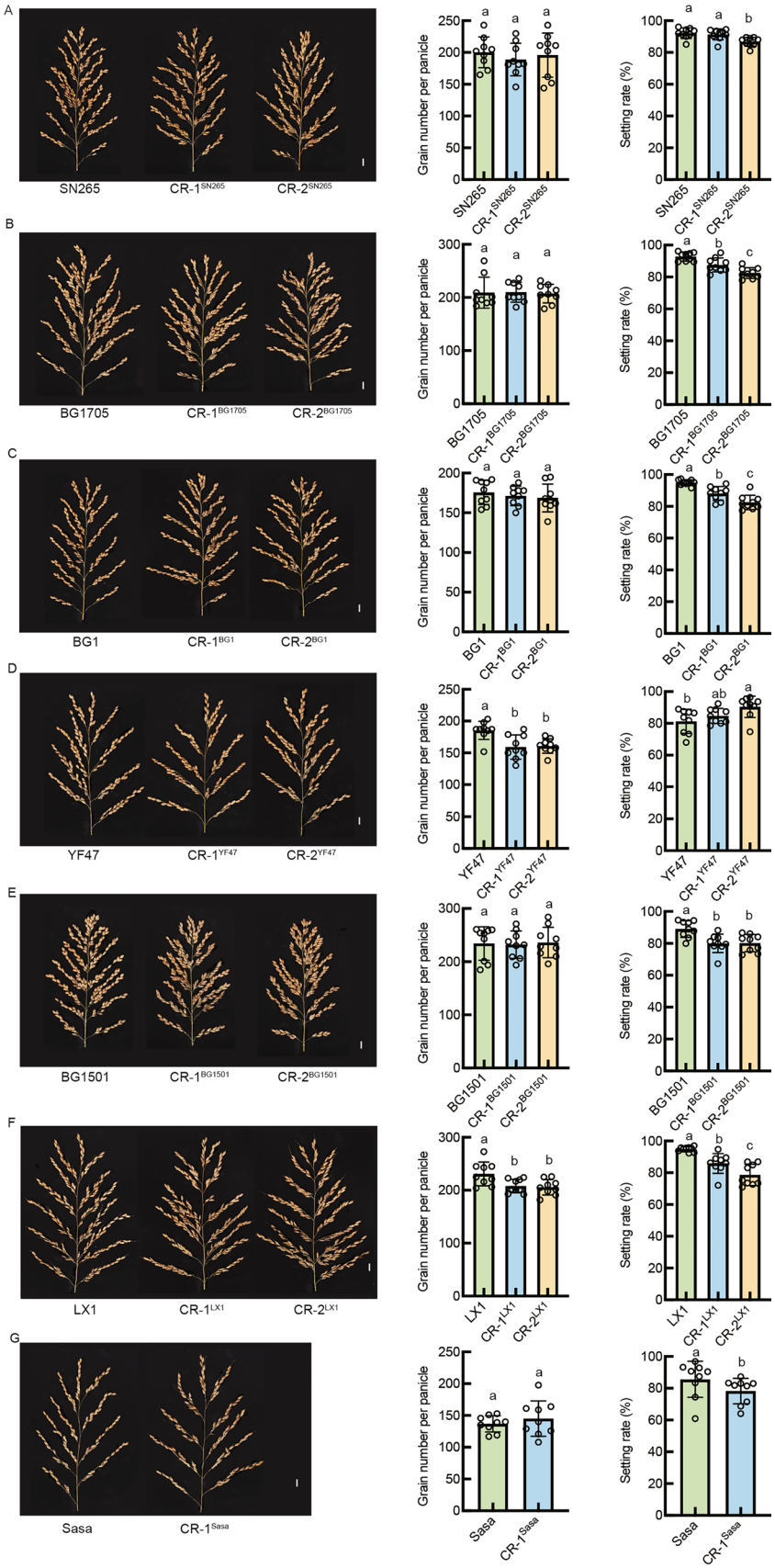
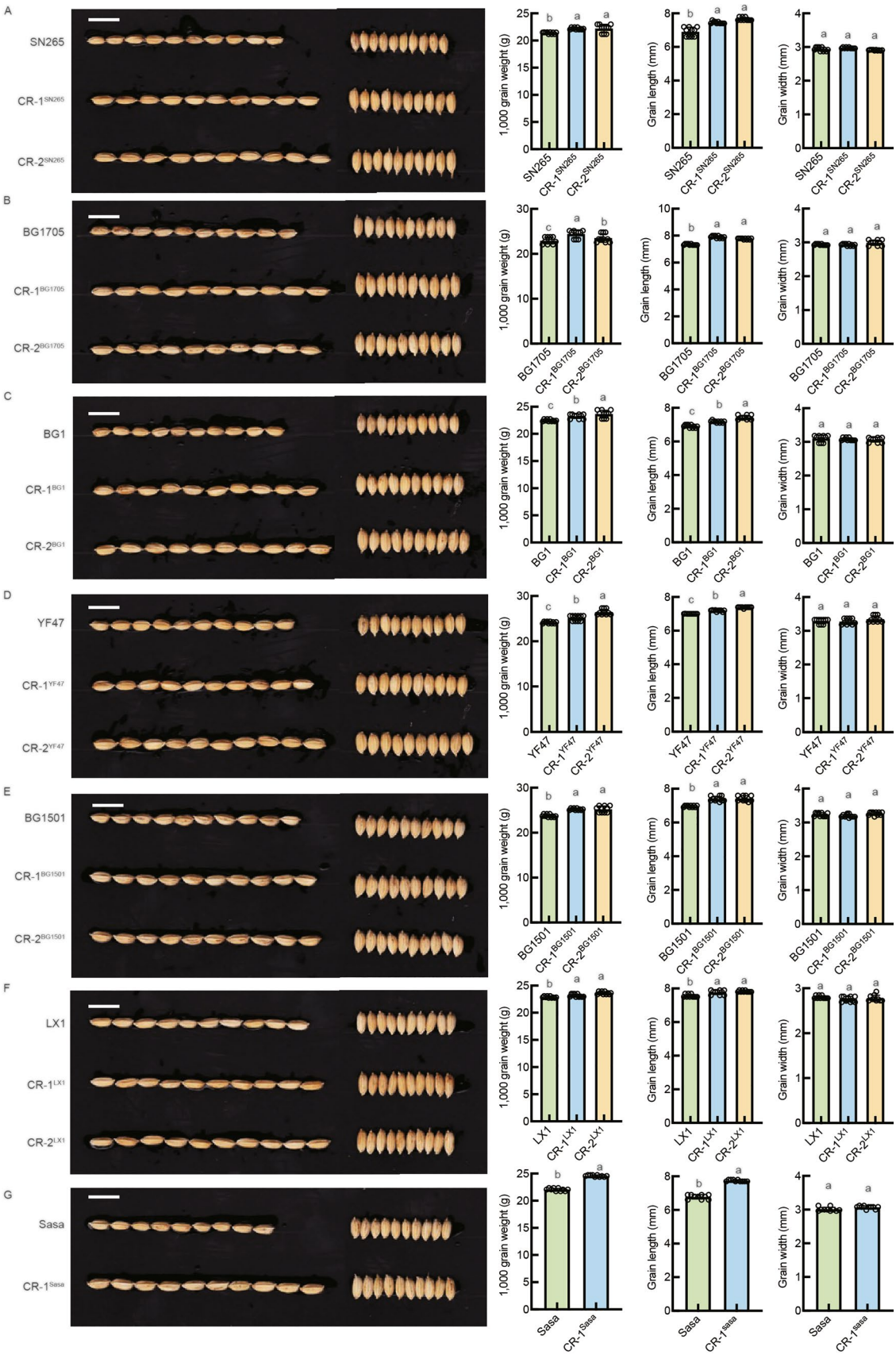


Fig. 4 Panicle architecture in wild-type varieties and their *GS3* mutants. **A**–**G** Representative images of panicles, grain number per panicle, and setting rate of SN265 (**A**), BG1705 (**B**), BG1 (**C**), YF47 (**D**), BG1501 (**E**), LX1 (**F**), and Sasa (**G**). Scale bar = 1 cm. Data are presented as mean ± s.e.m. (n = 10). Different letters indicate significant differences ($P < 0.05$) from a Duncan's multiple range test


A
SN265
CR-1SN265
CR-2SN265
B
BG1705
CR-1BG1705
CR-2BG1705
C
BG1
CR-1BG1
CR-2BG1
D
YF47
CR-1YF47
CR-2YF47
E
BG1501
CR-1BG1501
CR-2BG1501
F
LX1
CR-1LX1
CR-2LX1
G
Sasa
CR-1Sasa
1,000 grain weight (g)
Grain length (mm)
Grain width (mm)

◂**Fig. 5** Grain shape in wild-type varieties and their *GS3* mutants. **A**–**G** Representative images of grains, 1,000 grain weight, grain length, and grain width of SN265 (**A**), BG1705 (**B**), BG1 (**C**), YF47 (**D**), BG1501 (**E**), LX1 (**F**), and Sasa (**G**). Scale bar = 1 cm. Data are presented as mean ± s.e.m. (n = 10). Different letters indicate significant differences ($P < 0.05$) from a Duncan's multiple range test

Huajingxian (an XI variety) (Kan et al. 2022). To ascertain whether *GS3* knockout could uniformly enhance tolerance to heat and alkali stress, we assessed the thermotolerance and tolerance to alkali soil of *GS3* mutants across seven varieties. Although thermotolerance varied among the seven varieties, none exhibited a survival rate greater than 20% after heat treatment. BG1705 displayed the highest survival rate (16.67%), whereas LX1 exhibited the lowest survival rate (2.08%) (Fig. S2). Differential degrees of improvement were observed between the *GS3* mutants and their wild-type counterparts during heat treatment. Knockout of *GS3* increased the survival rate of BG1705 from 16.67 to 80.21%, which was the highest among the mutants. Conversely, the *GS3* mutant of LX1 exhibited the lowest improvement, increasing from 2.08 to 19.79% (Fig. 7). Subsequent assessments of alkali tolerance revealed that YF47 had the highest survival rate (18.60%), significantly surpassing that of the other six varieties (Fig. S3). Comparative analysis of alkali tolerance between mutants and wild-type varieties indicated significant improvement in the survival rates of all mutants. The *GS3* mutant of YF47 exhibited further enhancement, reaching a survival rate of 40.40%. Conversely, the lowest survival rate was observed for the Sasa mutant, which increased the survival rate from 1.35 to 6.42% (Fig. 7). Collectively, *GS3* knockout indiscriminately improved the tolerance to heat and alkali stress across the GJ varieties used in this study.

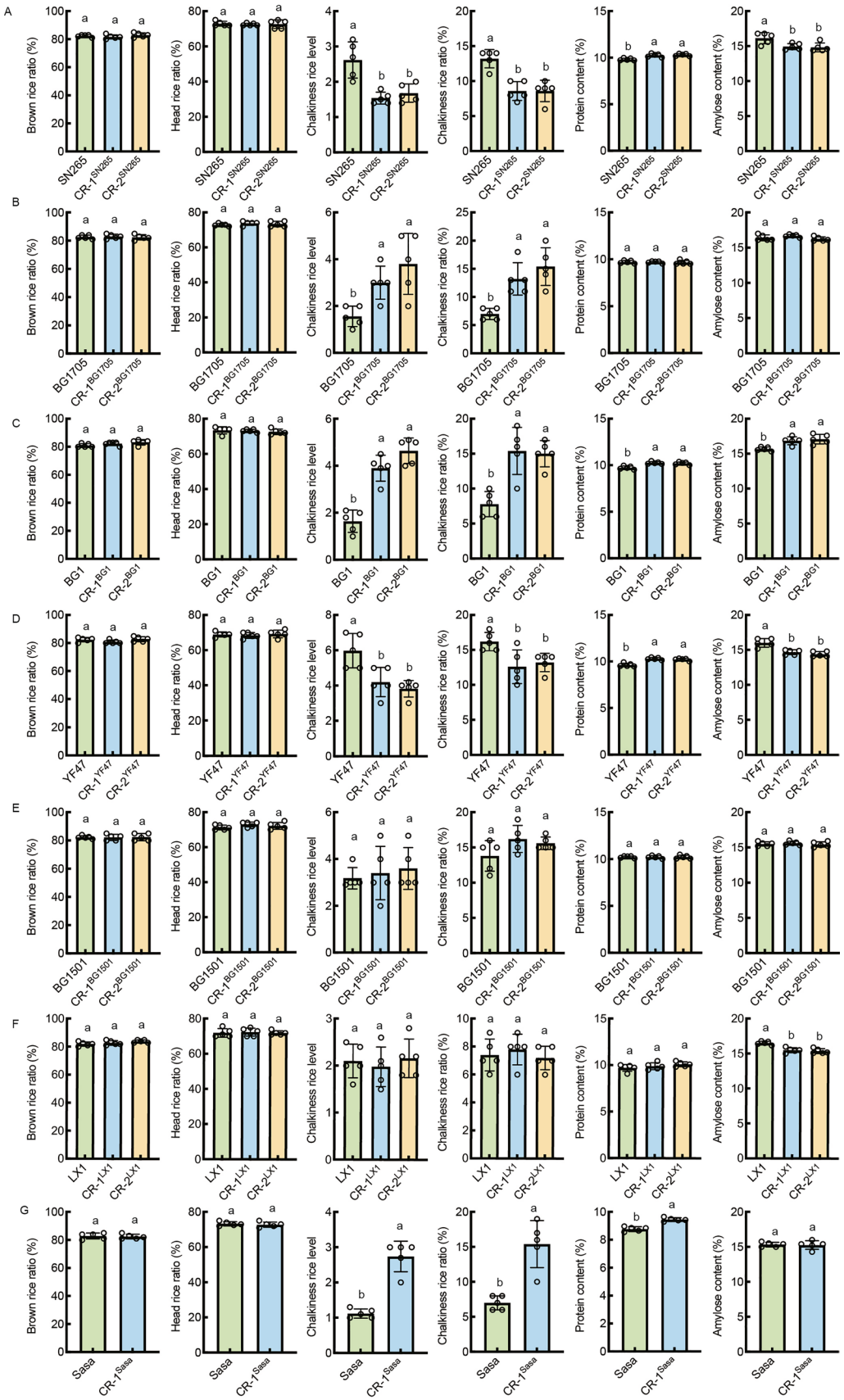
Identification of downstream genes regulated by *GS3*

Given *GS3*'s pleiotropic effects on plant height, grain length, and tolerance to heat and alkali stress, we sought to unveil its downstream genes. To achieve this, we conducted RNA-seq analysis of *GS3* mutants and their corresponding wild-type varieties. A total of 14,053 differentially expressed genes (DEGs) were identified, comprising 6599 upregulated and 7454 downregulated genes in *GS3* mutants compared with their wild-type counterparts. Gene Ontology (GO) analysis indicated that these DEGs were enriched in biological processes related to carbohydrate metabolic process, polysaccharide metabolic process, and cell wall organization or biogenesis (Fig. S4). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses further revealed enrichment in pathways associated with metabolic pathways, biosynthesis of secondary metabolites, and starch and sucrose metabolism (Fig. S5). Upon closer examination, only 46 genes exhibited differential expression in all seven comparisons between the mutants and their respective varieties (Fig. 8A). Subsequent GO analysis of these 46 DEGs highlighted the enrichment in biological processes related to photosynthesis and photosystem II stabilization (Fig. 8B and Fig. S6). Additionally, KEGG pathway analyses indicated that the 46 DEGs were enriched in pathways related to photosynthesis and cutin, suberine, and wax biosynthesis (Figs. 8 and S7).

Discussion

In plants, G proteins exhibit structural similarities to their animal counterparts but employ atypical mechanisms and effector proteins to regulate diverse processes such as growth, cell proliferation, defense, stomatal movements, channel regulation, sugar sensing, and hormonal responses (Urano et al. 2013). Within the rice genome, a single subunit of Gα (*RGA1*) and Gβ (*RGB1*) is encoded, along with two typical Gγ subunits (*RGG1* and *RGG2*) and three atypical Gγ subunits (*GS3*, *DEP1*, and *OsGGC2*) (Xu et al. 2016a, 2016b). The cloning of two important yield quantitative trait loci (QTLs), *GS3* and *DEP1*, has prompted researchers and breeders to speculate that manipulating G protein signaling could enhance yields not only in rice but also in other crop species (Botella 2012). Subsequent investigations have shown that the manipulation of three Gγ proteins, *DEP1*, *GGC2*, and *GS3*, can achieve the desired grain size (Sun et al. 2018). Additionally, modulation of the C-terminus of *DEP1* has been demonstrated to synergistically enhance grain quality and yield in rice (Huang et al. 2022). Recent studies have demonstrated that *GS3/TT2* regulates thermotolerance and alkali tolerance through SCT1-dependent alteration of wax biosynthesis and phosphorylation of aquaporins, thereby influencing the distribution of hydrogen peroxide (H_2O_2) (Kan et al. 2022; Zhang et al. 2023). These findings suggest that manipulating G proteins has the potential to achieve a high and stable yield performance in rice production.

In this study, we selected seven GJ varieties, six of which harbored a truncated *DEP1*, displaying a dense and erect panicle architecture. Given the opposing effects of truncated *DEP1* and truncated *GS3* on specific traits, such as reduced grain length with the truncated *DEP1* allele and significantly increased grain length with the truncated *GS3* allele (Sun et al. 2018; Wu et al. 2022), we introduced a variety, Sasa, with a full-length *DEP1*. This addition aimed to examine whether the truncated *DEP1* would also exhibit contrary effects on thermotolerance and alkali soil tolerance. Our findings indicated that *GS3* could perform its function independently of the *DEP1* genotype. Knockout of *GS3* consistently increased grain length and enhanced tolerance


A
B
C
D
E
F
G
Brown rice ratio (%)
Head rice ratio (%)
Chalkiness rice level
Chalkiness rice ratio (%)
Protein content (%)
Amylose content (%)
SN265
CR-1SN265
CR-2SN265
BG1705
CR-1BG1705
CR-2BG1705
BG1
CR-1BG1
CR-2BG1
YF47
CR-1YF47
CR-2YF47
BG1501
CR-1BG1501
CR-2BG1501
LX1
CR-1LX1
CR-2LX1
Sasa
CR-1Sasa

◂**Fig. 6** Quality traits of wild-type varieties and their *GS3* mutants. **A**–**G** Brown rice ratio, head rice ratio, chalkiness rice level, chalkiness ratio, protein contents, and amylose contents of SN265 (**A**), BG1705 (**B**), BG1 (**C**), YF47 (**D**), BG1501 (**E**), LX1 (**F**), and Sasa (**G**). Data are presented as mean ± s.e.m. (n = 5). Different letters indicate significant differences ($P < 0.05$) from a Duncan's multiple range test

to heat and alkali stress across all genotypes. However, the effects of *GS3* on chalkiness-related traits varied depending on the inherent performance of the varieties. For example, SN265 and YF47, the two varieties displaying the poorest chalkiness traits among the seven selected varieties, showed

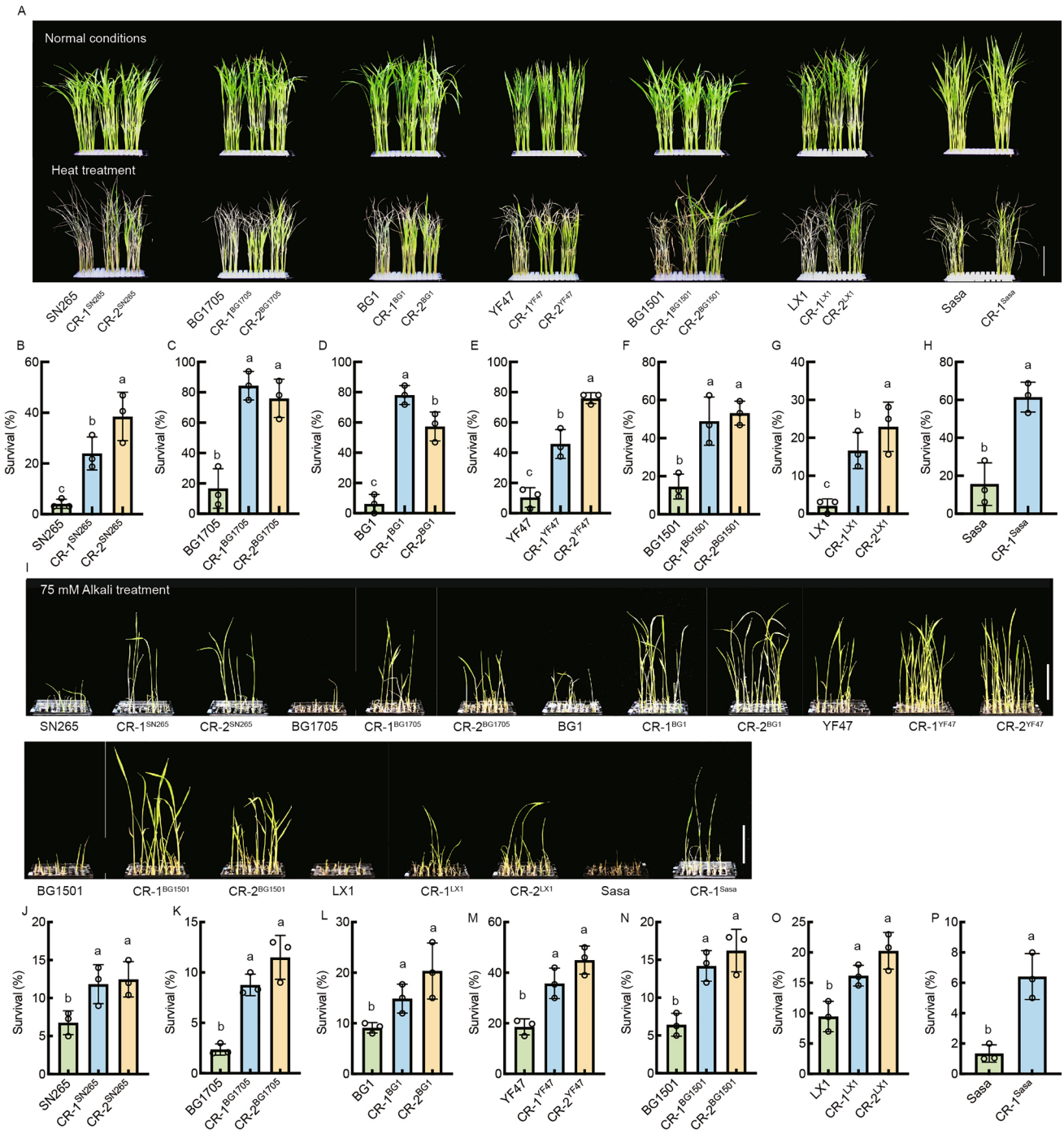


Fig. 7 Tolerance of wild-type varieties and their *GS3* mutants. **A** Representative images of wild-type varieties and their *GS3* mutants under normal and heat treatment conditions. Scale bar = 5 cm. **B**–**H** Survival ratio of wild-type varieties and their *GS3* mutants after heat treatment. **I** Representative images of wild-type varieties and their *GS3* mutants under 75 nm alkali treatment. Scale bar = 5 cm. **J**–**P** Survival ratio of wild-type varieties and their *GS3* mutants after alkali treatment. The investigations in B–H and J–P were conducted with three biological replicates, each replicate containing 10 plants. Different letters indicate significant differences ($P < 0.05$) from a Duncan's multiple range test

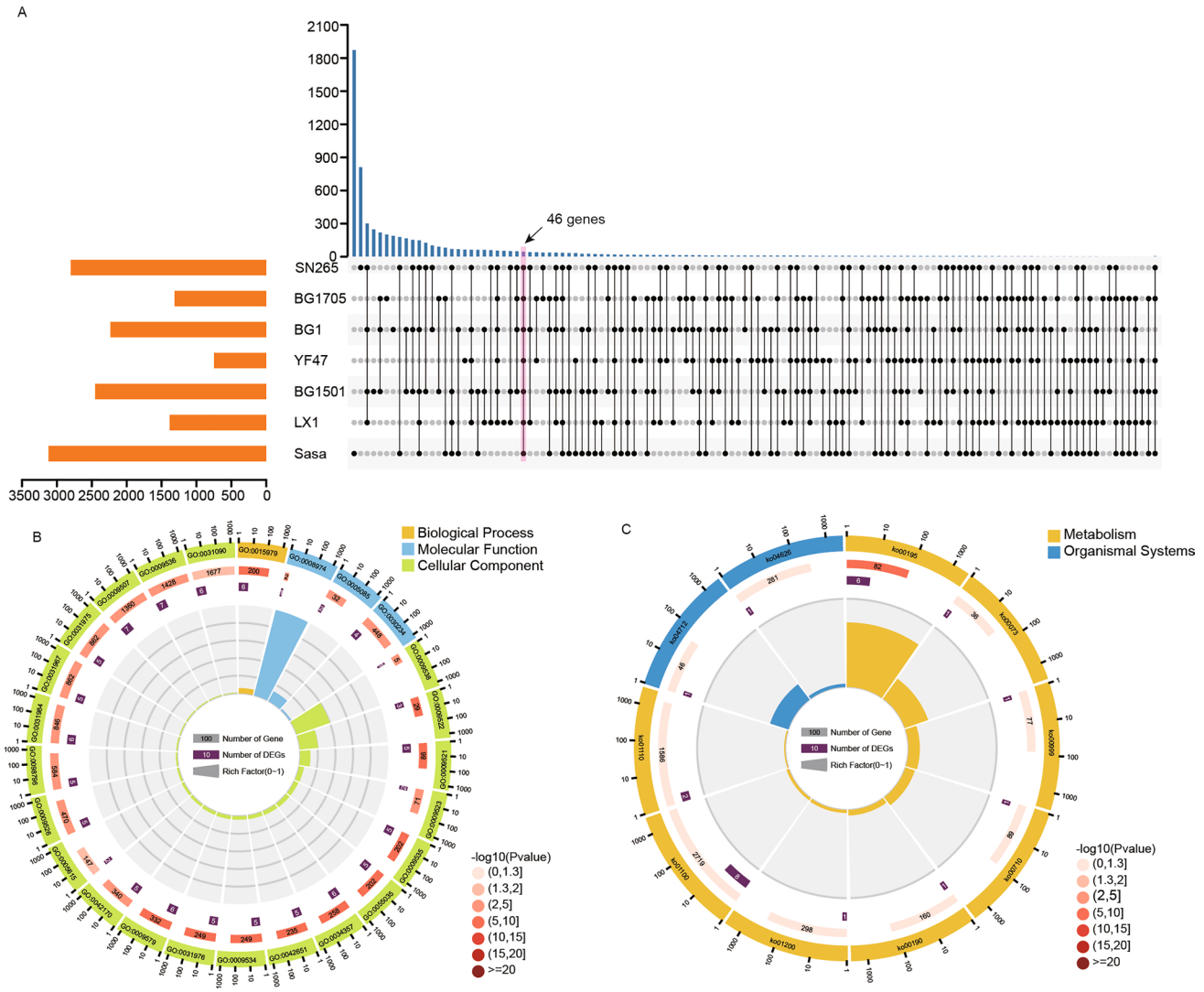


Fig. 8 Transcriptome analysis of wild-type varieties and their *GS3* mutants. **A** Upset plot represents the differentially expressed genes (DEGs) regulated by *GS3*. **B** GO analysis of the 46 DEGs. **C** KEGG pathway analysis of the 46 DEGs

significantly improved chalkiness rice levels and chalkiness rice ratios upon *GS3* knockout. In contrast, varieties such as Sasa, BG1705, and BG1, which initially exhibited an advantage in chalkiness-related traits, showed a decline in performance following the *GS3* knockout. Our previous study revealed that YF47 carried an XI-type allele at the *Chalk5* locus, responsible for encoding a vacuolar H^+-translocating pyrophosphatase influencing grain chalkiness in rice (Li et al. 2014; Wang et al. 2023). This indicates that the function of *GS3* in chalkiness-related traits in YF47 may be influenced by the distinct genotype of *Chalk5*. Thus, the strategic combination of a nonfunctional *GS3* allele with other chalkiness-regulating genes, such as *Chalk5*, *GS9*, *OsMADS1*, and *OsSPL16*, has the potential to achieve a balance between yield, quality, and tolerance at a higher level (Wang et al. 2012; Li et al. 2014; Liu et al. 2018; Zhao et al. 2018).

Natural genetic variation plays a pivotal role in advancing crop domestication and evolution. However, optimal variants that simultaneously enhance yield, quality, and tolerance may not exist in natural populations. For example, rice production often faces a trade-off between panicle number and grain number per panicle, as exemplified in IPA1 (Ideal Plant Architecture 1), a typical pleiotropic gene that increases grains per panicle while reducing tillers (Jiao et al. 2010). Addressing this tradeoff, the artificial deletion of a 54-base pair *cis*-regulatory region in *IPA1* resolves the conflict, substantially boosting the grain yield per plant (Song et al. 2022). A base-editing system at the *Wx* locus was utilized to generate a range of mutants with apparent amylose contents ranging from 1.4 to 11.9%. This approach has successfully achieved the goal of finely tuning rice AC within the range of 0–12%, thereby expanding

the diversity of breeding materials accessible to breeders (Xu et al. 2021). Editing the region near the TATA box of the Wx^b promoter has led to the creation of six novel *Wx* alleles, downregulation of *Wx* expression, and fine tuning of grain amylose content (Huang et al. 2020). Collectively, these studies highlight that targeted gene modification is a widely applicable and reliable approach for generating optimal alleles that may not naturally exist in populations. In this study, we identified three major haplotypes of *GS3*, with Hap3 carrying a C/A SNP mutation in the exon that generates a stop codon, causing a truncated *GS3* protein. This natural variation originated from an ancient GJ lineage and was later introgressed into Xian/Indica (XI) (Takano-Kai et al. 2009). Hap3 not only exhibits an enlarged grain size but also enhanced thermotolerance (Kan et al. 2022; Zhang et al. 2023). However, this truncated *GS3* allele displayed increased sensitivity to alkaline conditions (Zhang et al. 2023). In this study, we demonstrated that the knockout of *GS3* could simultaneously improve grain shape, thermotolerance, and alkaline tolerance.

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Author contribution statement QX conceived and designed the experiments. NX and YQ performed the gene editing, phenotypic analysis. XC and CF performed the quality investigation. QX analyzed the data and wrote the manuscript. QX agrees to serve as the author responsible for contact and ensures communication. All authors have read and approved the manuscript.

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Data availability All data generated during this study are included in this article and its supplementary files.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Botella JR (2012) Can heterotrimeric G proteins help to feed the world? Trends Plant Sci 17:563–568

Deliang C, Maisa R, Bjørn HS, Kim C, Aïda D-N, Paul E, EMORI S (2021) Climate Change 2021: The Physical Science Basis. *Intergovernmental Panel on Climate Change*

Fan C, Xing Y, Mao H, Lu T, Han B, Xu C, Li X, Zhang Q (2006) GS3, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor Appl Genet 112:1164–1171

Fan C, Yu S, Wang C, Xing Y (2009) A causal C-A mutation in the second exon of GS3 highly associated with rice grain length and validated as a functional marker. Theor Appl Genet 118:465–472

Fei C, Xu Q, Xu Z, Chen W (2020) Effect of rice breeding process on improvement of yield and quality in China. Rice Sci 27:363–367

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. Nat Biotechnol 31:827–832

Huang L, Li Q, Zhang C, Chu R, Gu Z, Tan H, Zhao D, Fan X, Liu Q (2020) Creating novel Wx alleles with fine-tuned amylose levels and improved grain quality in rice by promoter editing using CRISPR/Cas9 system. Plant Biotechnol J 18:2164–2166

Huang H, Ye Y, Song W, Li Q, Han R, Wu C, Wang S, Yu J, Liu X, Fu X, Liu Q, Wu K (2022) Modulating the C-terminus of DEP1 synergistically enhances grain quality and yield in rice. J Genet Genomics 49:506–509

Jiao Y, Wang Y, Xue D, Wang J, Yan M, Liu G, Dong G, Zeng D, Lu Z, Zhu X (2010) Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. Nat Genet 42:541–544

Kan Y, Mu XR, Zhang H, Gao J, Shan JX, Ye WW, Lin HX (2022) TT2 controls rice thermotolerance through SCT1-dependent alteration of wax biosynthesis. Nat Plants 8:53–67

Li Y, Fan C, Xing Y, Yun P, Luo L, Yan B, Peng B, Xie W, Wang G, Li X (2014) Chalk5 encodes a vacuolar H+-translocating pyrophosphatase influencing grain chalkiness in rice. Nat Genet 46(4):398–404

Li W, Zhu Z, Chern M, Yin J, Yang C, Ran L, Cheng M, He M, Wang K, Wang J (2017) A natural allele of a transcription factor in rice confers broad-spectrum blast resistance. Cell 170:114–126

Li X, Wu L, Wang J, Sun J, Xia X, Geng X, Wang X, Xu Z, Xu Q (2018) Genome sequencing of rice subspecies and genetic analysis of recombinant lines reveals regional yield- and quality-associated loci. BMC Biol 16:102

Lippert C, Listgarten J, Liu Y, Kadie CM, Davidson RI, Heckerman D (2011) FaST linear mixed models for genome-wide association studies. Nat Methods 8:833–835

Liu Q, Han R, Wu K, Zhang J, Ye Y, Wang S, Chen J, Pan Y, Li Q, Xu X, Zhou J, Tao D, Wu Y, Fu X (2018) G-protein βγ subunits determine grain size through interaction with MADS-domain transcription factors in rice. Nat Commun 9:852

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol Plant 8:1274–1284

Muthayya S, Sugimoto JD, Montgomery S, Maberly GF (2014) An overview of global rice production, supply, trade, and consumption. Ann N Y Acad Sci 1324:7–14

Rawshan AB, Robert L, Elham A, Tor Arve B, Thomas B, Avelino Gumersindo SR (2022) Climate Change 2022: impacts, adaptation and vulnerability. Intergovernmental Panel on Climate Change

Ren D, Ding C, Qian Q (2023) Molecular bases of rice grain size and quality for optimized productivity. Sci Bull 68:314–350

Song X, Meng X, Guo H, Cheng Q, Jing Y, Chen M, Liu G, Wang B, Wang Y, Li J, Yu H (2022) Targeting a gene regulatory element enhances rice grain yield by decoupling panicle number and size. Nat Biotechnol 40:1403–1411

Sun S, Wang L, Mao H, Shao L, Li X, Xiao J, Ouyang Y, Zhang Q (2018) A G-protein pathway determines grain size in rice. Nat Commun 9:851

Takano-Kai N, Jiang H, Kubo T, Sweeney M, Matsumoto T, Kanamori H, Padhukasahasram B, Bustamante C, Yoshimura A, Doi K, McCouch S (2009) Evolutionary history of GS3, a gene conferring grain length in rice. Genetics 182:1323–1334

Urano D, Chen J-G, Botella JR, Jones AM (2013) Heterotrimeric G protein signalling in the plant kingdom. Open Biol 3:120186

Wang C, Chen S, Yu S (2011) Functional markers developed from multiple loci in GS3 for fine marker-assisted selection of grain length in rice. Theor Appl Genet 122:905–913

Wang S, Wu K, Yuan Q, Liu X, Liu Z, Lin X, Zeng R, Zhu H, Dong G, Qian Q (2012) Control of grain size, shape and quality by OsSPL16 in rice. Nat Genet 44:950–954

Wang Y, Li F, Zhang F, Wu L, Xu N, Sun Q, Chen H, Yu Z, Lu J, Jiang K, Wang X, Wen S, Zhou Y, Zhao H, Jiang Q, Wang J, Jia R, Sun J, Tang L, Xu H, Hu W, Xu Z, Chen W, Guo A, Xu Q (2023) Time-ordering japonica/geng genomes analysis indicates the importance of large structural variants in rice breeding. Plant Biotechnol J 21:202–218

Wu L, Wang X, Yu Z, Cui X, Xu Q (2022) Simultaneous improvement of grain yield and quality through manipulating two type C G protein gamma subunits in rice. Int J Mol Sci 23:1463

Xie W, Wang G, Yuan M, Yao W, Lyu K, Zhao H, Yang M, Li P, Zhang X, Yuan J, Wang Q, Liu F, Dong H, Zhang L, Li X, Meng X, Zhang W, Xiong L, He Y, Wang S, Yu S, Xu C, Luo J, Xiao J, Lian X, Zhang Q (2015) Breeding signatures of rice improvement revealed by a genomic variation map from a large germplasm collection. Proc Natl Acad Sci USA 112:E5411-5419

Xu H, Zhao M, Zhang Q, Xu Z, Xu Q (2016a) The DENSE AND ERECT PANICLE 1 (DEP1) gene offering the potential in the breeding of high-yielding rice. Breed Sci 66:659

Xu Q, Zhao M, Wu K, Fu X, Liu Q (2016b) Emerging insights into heterotrimeric G protein signaling in plants. J Genet Genomics 43:495–502

Xu Y, Lin Q, Li X, Wang F, Chen Z, Wang J, Li W, Fan F, Tao Y, Jiang Y, Wei X, Zhang R, Zhu QH, Bu Q, Yang J, Gao C (2021) Fine-tuning the amylose content of rice by precise base editing of the Wx gene. Plant Biotechnol J 19:11–13

Yang W, Guo Z, Huang C, Duan L, Chen G, Jiang N, Fang W, Feng H, Xie W, Lian X, Wang G, Luo Q, Zhang Q, Liu Q, Xiong L (2014) Combining high-throughput phenotyping and genome-wide association studies to reveal natural genetic variation in rice. Nat Commun 5:5087

Zhang H, Yu F, Xie P, Sun S, Qiao X, Tang S, Chen C, Yang S, Mei C, Yang D, Wu Y, Xia R, Li X, Lu J, Liu Y, Xie X, Ma D, Xu X, Liang Z, Feng Z, Huang X, Yu H, Liu G, Wang Y, Li J, Zhang Q, Chen C, Ouyang Y, Xie Q (2023) A Gγ protein regulates alkaline sensitivity in crops. Science 379:eade8416

Zhao DS, Li QF, Zhang CQ, Zhang C, Yang QQ, Pan LX, Ren XY, Lu J, Gu MH, Liu QQ (2018) GS9 acts as a transcriptional activator to regulate rice grain shape and appearance quality. Nat Commun 9:1240