Genetic editing of GS3 via CRISPR/Cas9 Accelerates the Breeding of Three-line Hybrid Rice With Superior Yield and Grain Quality

Juan Huang
Jiangxi Agricultural University https://orcid.org/0000-0002-5329-8467
Lijun Gao
Guangxi Crop Genetic Improvement and Biotechnology Laboratory/Guangxi Key Laboratory of Geneti Improvement of Crops, Nanning 530007, China
Shuming Luo
Guangxi Academy of Agricultural Science
Kaiqiang Liu
Guangxi Academy of Agricultural Science
Dongjin Qin
Guangxi Academy of Agricultural Science
Yinghua Pan
Guangxi Academy of Agricultural Science
Gaoxing Dai
Guangxi Academy of Agricultural Science
Guofu Deng
Guangxi Academy of Agricultural Science
Changlan Zhu (zhuchanglan@163.com)
Jiangxi Agricultural University

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Abstract

Grain size is one of the major traits that control rice grain yield and quality. The GS3 gene, a major QTL regulating grain length and weight, is the first one been identified. A mutation occurred in its N-terminal organ size regulating domain (OSR)results in a loss of function of the mutant allele *gs3* and rice varieties carry this *gs3* allele often produce longer grains. In this study, we exploited the CRISPR/Cas9 gene editing technology to introduce an edited *gs3* allele, two guide RNAs(gRNA) targeted at the OSR of the *GS3* gene were transformed into one of our *indica* maintainer lines, GM1B, for its grain yield and quality improvement. Through molecular analysis and sequencing, in T_1 generation, a homologous edited-*gs3* mutant line without tansgene was obtained and name as GM2B, then converted to CMS line GM1A by backcrossing to obtain another superior male sterile line GM2A for further tests. GM2B showed improved grain quality and yield compared to the WT GM1B, with grain length increased by 7.9%, length/width ratio increased from 3.89 to 4.19, TGW increased by 6.7%, and grain yield per plant increased by 14.9%. Meanwhile, genetic improvement of other quality traits including rice length (6.83mm), rice grain length/width ratio (3.61), matched the appearance standards set for traditional Simiao (silk seedling) type cultivars. Two restorer lines were outcrossed to both GM1A and GM2A to produce hybrid rice. Compared to GM1A's two hybrids, the hybrids of GM2A had longer grains, higher length/width ratio, higher TGW and yield per plant. In addition, hybrids of GM2A showed better performance on grain appearance including better translucency, lower chalky rice rate and chalkiness degree than hybrids of GM1A.These data strongly demonstrate that the introduction of an elite *gs3* allele into GM1A via CRISPR/Cas9 gene editing technology leads to significant genetic improvement. The resultant CMS line GM2A(*gs3*) performs much better than the original GM1A on grain quality and yield. Thus, our study proves that targeted genetic improvement using gene editing technology can expedite rice breeding, especially the breeding of three-line hybrid rice.

Introduction

Rice is an important crop grown in more than 100 countries across the world, and supplies staple food for 2/3 of the world population. The characteristics of grain size (GS) including grain length (GL), grain width (GW), grain thickness(GT) and grain length/width ratio are important factors related to yield and also closely linked to the quality of rice product such as appearance, processing, cooking and taste, etc.. (Xu et al.1993;Tan et al, 2000; Xu et al., 2004; Li et al., 2004; Song et al., 2007; Shomura et al., 2008; Wang et al., 2008; Huang et al. 2013; Huang et al. 2017). In the filling process of big and round grain, it is easy to generate chalkiness because of the long transportation route from back to abdomen. Therefore, the transparent and high-quality rice grains are mostly produced in slender grain varieties (Gu et al., 2001; Xu et al., 2004). In recently years, the demand for high-quality slender grain rice has increased significantly in consumer market, which has led to a research hotspot for breeders to cultivate high grain length/width ratio or Simiao-type variety(rice length≥ 6.5mm and rice length/width ratio≥ 3.5, T/GDSMM 001—2019) (Fitzgerald et al., 2009; Wang et al., 2012).

Traditional rice breeding methods heavily rely on breeder's experience , and are often inefficient in complex trait selection, which makes the traditional breeding labor and time consuming. The advance of MAS (marker-assisted selection) breeding technology greatly increases breeding efficiency. However, it is somehow limited when low recombination rate exists and genetic drags are hard to break at the target gene/QTL locus. Recently, a genome editing tool derived from an adaptive immune mechanism of microorganisms, the CRISPR-Cas9(clustered regularly interspaced short palindromic repeats/CRISPR-associated protein9)system, has been successfully applied to plants(Shan et al., 2013; Li et al., 2013;Nekrasov et al., 2013). It can precisely modify a plant's own genes without the introduction of foreign genes(Jiang et al.2013; Liang et al., 2014;Cai et al., 2015;Lawrenson et al., 2015;Iqbal et al., 2016), with which desired traits can be quickly introduced into a target variety , thus, greatly improving breeding efficiency(Tang et al., 2017; soyk et al.,2018; Kuang et al., 2020).

More than 500 QTL genes related to grain size have been identified(http://www.ricedata.cn/ Index. HTM), and 19 QTL genes have been cloned in rice(Chen et al., 2020). Among them, *GS3*、*qGL3/GL3.1*、*TGW3/GL3.3*、*LGY3* and *GS9* are major QTLs as negative factors for grain length(Fan et al., 2006; Zhang et al., 2012; Qi et al., 2012; Ying et al., 2018; Xia et al., 2018; Liu et al. 2018; Zhao et al., 2018). *GS3* plays an important role in controlling grain length and grain weight. Loss-of-function at its N-terminal organ size regulating domain (OSR) leads rice plants to produce long type grains. By analyzing the *GS3* locus of 78 rice varieties, about half of the *indica* and one tenth of the *japonica* contained the long grain allele *gs3*, indicating the gene's great potential in production(Fan et al., 2006; Mao et al., 2010). Previous studies have showed that the knockout of *GS3* gene can obtain longer grain lines which mainly focused on the oval-shaped *japonica* varieties for germplasm creation, but few on long-shaped *indica* rice(Han et al., 2018), especially *indica* hybrid rice(Shen et al., 2016 and 2017; Li et al., 2016; Chen et al., 2020).

The application of three-line hybrid rice(cytoplasmic male sterile line, maintainer line and restorer line) based on nucleo-cytoplasmic interaction has made great contributions to food security in China(Yuan and Tang, 1999). Up to now, the breeding of three-line hybrid rice still faces great challenges including low efficiency to create excellent maintainer lines and high cost of seed production despite breeders' great endeavor. In particular, when the traditional cross-selection methods are used to improve maintainer lines and sterile lines, introduction of exogenous elite genes into improved lines often accompanies the introgressive risk of major or minor restoring genes, which greatly decreases efficiency of hybrid rice breeding (Ren et al., 2016). In this study, to test whether introducing a *gs3* allele into an *indica* CMS (cytoplasmic male sterile) line GM1A could improve the grain quality and yield of its hybrids, we decided to edit the GS3 gene of its maintainer line GM1B with CRISPR/Cas9 technology. GM1A is a derivative of an elite CMS line, MeiA, whose hybrid varieties used to dominate the rice industry with the concept of slender grain size for better quality at the beginning of this century in Guangxi, China (Liang et al., 2001). GM1A inherits many favorable traits including high yield potency, high GCA (general combination ability) from MeiA. as its disadvantage in grain length, it is difficult to use this accession as parent to breed hybrid rice competitively in the current rice market favoring longer, slender(Simao-type) grains and better taste. Thus, we present data to show that introduction of a *gs3* allele into GM1A via CRISPR/Cas9 technology did improve grain quality and yield for both GM1A itself and its hybrids. This genetic improvement system could rapidly establish superior parental lines from small grain to long grain, which laid a foundation for sustainable utilization of existing resources.

Materials And Methods

Plant materials

Plant materials included an *Indica* maintainer line GM1B and its corresponding sterile line GM1A. Restorer lines Guanghui 998(GH998) and Gui 715(G715) were used as the testing parents. These plant materials were grown in the transgenic isolation greenhouse of Guangxi Academy of Agricultural Sciences during the whole experiment.

GS3 genotype detection and its target design in test

According to the *GS3* (Os03g0407400) gene sequence, primer pairs GS3-F1, GS3-R1 and GS3-F2, GS3-R2 were designed near the exons 1 and 2. Sequences of the primers were provided in Table 1. These primers were applied to amplify the DNA templates of the maintainer line GM1B, and the amplified products were sequenced. The sequencing results were then compared with the *GS3* (Os03g0407400) gene sequence on the NCBI data base. The exon2 of the GS3 gene in maintainer line GM1Bdid not have the long grain mutation that caused by the C-A bases shift. Using the sequences of the exons 1 and 2 of the GS3 gene in GM1B, gRNA target sites could be designed on a website (http://www.rgenome.net/cas-designer/). To improve the effectiveness of detecting loss-of-function of the target gene, we have selected two targets, target 1(cctcgaggaatccgatctcgcgg) in the exon 1 and target 2 (tgcagcatctggaggcagcgtgg)in exon 2. The GC content of targets 1 and 2 was 55.0% and 70.0%, respectively. The sequences of these two targets were also screened for off-target using the BLAST program on the NCBI website, and there were no off-target sequences matched, indicating an extremely low probability of the off-target.

Vector construction and genetic transformation

As for the target site sequence, we synthesized ECO31I restriction site with two primer pairs GS3-Y1+, GS3-Y1-, GS3-B1+, and GS3-B1-, respectively (Table 1, the underlined section means the sequence of the restriction site); then mixed equal amount of primer pairs GS3-Y1+ /GS3-Y1 and GS3-B1+/GS3-B1-, respectively; constructed the double-stranded DNA fragments at the Eco31I restriction site after denaturation and annealing treatments; ligated the double-stranded DNA fragments to the vectors pBWA and pBWD by restriction endonuclease enzyme and ligase; partially recombined the two constructed vectors containing both the editing elements and the binary vectors to obtain the recombinant plasmid CRISPR-Cas9 -GS3(Fig. 1).

Vector primers Yl-R+ and Pbw2- (Table 1) were used for PCR amplification of the 1100 bp editing element in the recombinant plasmid. Amplified products were verified by sequencing and then transferred to the *Agrobacterium* EHA105. Through the *Agrobacterium*-mediated transformation method, the whole system (recombinant plasmid CRISPR-Cas9-GS3) was transformed into the callus of the rice variety GM1B. Finally, hygromycin was used to screen the T_0 transgenic lines. The whole transformation experiment was entrusted to BioRun company in Wuhan.

Target-site mutations and grain shape variations of the T_0 transgenic lines

Positive lines were screened and selected using a hygromycin resistant gene primer pair (Hyg-F/Hyg-R) (Table 1). Initially, primers GS3-F1, GS3-R1, GS3-F2, and GS3-R2 were used to amplify the DNA templates of the positive lines selected, and the PCR products were sequenced. Seedlings of the positive lines and the wild-type materials were thereby planted at the same time, and harvested at maturity stage. Ten full and consistent grains from each single line were selected and measured for grain length using an automatic grain test instrument (Wanshen SC-G, China). Meanwhile, results could be compared with that of the wild type.

Establishment of the long-grain maintainer lines and the sterile lines

Harvested seeds of the T_0 generation were sown into plots, and each plot contained 20 specific lines labeled for testing. Primers Hyg-F/Hyg-R were used to amplify all the DNA template of each individual line in the T_1 generation, and WT Nipponbare was used as control. Lines tested with no amplification of the target band were concluded as the transgene-free mutants. Also in the T_1 generation, individual GM1B lines characterized with homozygous mutation, significant increase of grain length and no marker gene were selected to outcross to sterile line GM1A. Hybrid seeds or seeds from the mutants of the corresponding maintainer lines were harvested. After multiple backcrossing, the stable sterile line with increased grain length together with its corresponding maintainer line could be selected. Before backcrossing in each generation, the sterile line and the corresponding mutant maintainer were tested for the marker genes to ensure there is no transgene components.

Testing and trait characterization of potential hybrid rice combinations

After repeated backcrossing and selection, sterile lines with stable long-grain traits were obtained, and each line was tested by sequencing to confirm the edited *gs3* loci and free of transgene component. Two restorer lines GH998 (widely used by the hybrid rice industry) and G715(long grain bred by our team) were selected to test the improved long-grain sterile lines together with the original sterile line for hybrid rice combinations. Ten individual plants from each of the 4 hybrid combinations and their female parents, and 5 ones from each of the two restored lines were selected to examine the panicle length and the number of grains for each panicle. Ten full seeds were randomly selected from individual line for trait characterizations including measurements of the grain length, grain width, and the kernel weight of 100 full seeds was measured randomly and then converted into a weight for 1000 grains. Analysis and statistics of the agronomic traits were performed in the GraphPad Prism 8 software, and rice quality analysis for individual lines was in accordance with the standards set in the Quality for Cooking Rice Variety NY/T 593-2013. Fluorescent labeled primers were designed from the penta-primer amplification refractory mutation system (PARMS)(Ye et al., 2001; Zhang et al., 2019; Lu et al., 2020), and these specific lists of primers were used to test the parental genotypes relevant to some rice quality genes such as *GS3*, *Wx*, *ALK* and*Chalk5*(Fan et al., 2006; Hirano et al., 1998; Isshiki et al., 1998; Gao et al., 2003; Li et al., 2014). Detailed sequences of these primers were provided in Table 1.

Results

Targeted mutation of GS3 gene generated edited *gs3* loci

Twelve hygromycin resistant primary transformants were obtained and PCR amplification of the hygromycin-resistant gene confirmed that they all carried the selection marker gene. DNA sequencing results of these 12 mutant lines revealed that biallelic mutation occurred in 3 seedlings, two(P437-2, P437-13) were homozygous *gs3* mutants at target 1 and the other(P437-6) was homologus *gs3* mutant at target 2 (Fig. 2). T_1 seeds from each line were harvested individually from T_0 plants. Grain length and grain width for each line were measured and compared with that of the control(GM1B). Results showed that the average grain length of each three homozygous mutant lines(P437-2, P437-6, P437-13) were longer than 10.00mm whilst the average grain length of WT GM1B was 9.40mm, indicating a >5% increase in grain length (Fig. 2). Sequence alignments of these three homozygous mutants against that of the WT further showed that insertions occurred at both of the two target sites, and disrupted the *GS3* open reading frame and resulted premature translation termination. Thus, we generated 3 different edited *gs3* loci relevant to the long grain trait.

Selection of transgene-free, homozygous *gs3*mutants

Because three primary transformants, P437-2, P437-6 and P437-13, were already homozygotes for *gs3* loci, we just needed to identify transgene-free lines from their T_1 seedling using PCR amplification of the selection marker, hygromycin-resistant gene (Fig.3). The PCR amplification results allowed us to identify a new line without selection marker from T_1 offspring of P437-6. After comprehensive survey of target trait (grain length) and other agronomic traits, and sequencing confirmation of the mutation site, line P437-6-2 were selected for further constructing new long-grain sterile line. We named it as Guimei2B (GM2B) for convenience.

Conversion of superior long-grain sterile line GM2A from maintainer GM2B

The maintainer GM2B carrying the edited *gs3* loci was backcrossed to sterile line GM1A. In the BC_1F_1 segregated population, sterile lines with homozygous mutation and significantly increased grain length were selected to cross with GM2B for propagation and the later generation was named as GM2A (Fig.5). The selection process of male sterile line was presented in Fig.4. To test how the newly-generated mutant CMS GM2A and the WT CMS GM1A perform in agronomic traits, we selected two restorer lines GH998 and G715 to form four F_1 hybrid rice combinations(Fig.6-7).

Agronomic trait comparison of mutant maintainer GM2B and WT maintainer GM1B

To investigate whether the knockout of the *GS3* gene affects agronomic traits, the major relevant agronomic traits of maintainers GM1B and GM2B were characterized and compared. Traits including the grain length, grain width, ratio of grain length to width, panicle length, grain number per panicle, filled grain number per panicle, seed setting rate, 1000 grain weight, effective tiller number, tiller number at active stage, plant height and weight per plant were scored and data were presented in Fig.5 and Table 3. The statistical analyses of results showed that there was no significant difference between GM1B and GM2B in tillering number, grain width and filled grain number per panicle, but grain length,1000 grain weight and grain number per panicle increased by 7.9%, 7.7% and 25.5%, respectively. Compared to GM1B, although the seed setting rate of GM2B was decreased by 13.6%, its weight per plant was significantly increased by 14.9%. In term of grain yield per plant, the negative effect of decrease in Comparing of relevant traits between their corresponding CMS lines, GM1A and GM2A, resulted in similar conclusions.

Hybrids of GM2A carrying edited *gs3* loci performed better in grain length, length/width ratio and yield than hybrids of WT GM1A

Four hybrid combinations (GM2A/GH998, GM1A/GH998, GM2A/G715and GM1A/G715) were developed by long-grain sterile line GM2A and original sterile line GM1A with two restorer lines GH998 and G715, respectively. Statistical analysis of relevant traits was shown in Table 3, Fig.6, and Fig.7. Compared with GM1A/GH998, the combination of GM2A/GH998 showed 5.6%, 8.2%, 7.1% and 15.4% increase in grain length, ratio of grain length to width, 1000-grain weight and grain weight per plant, respectively, but no significant differences in grain width, panicle length, plant height, grain number, filled grain number, seed-setting rate and effective tillers. In the two combinations of GM2A/G715 and GM1A/G715, there was no significant difference in grain width, grain number, filled grain number, seed setting rate, plant height and effective tiller. However, GM2A/G715 presented a significant increase in grain length, ratio of grain length to width, panicle length, 1000-grain weight and weight per plant than GM1A/G715, by 11.2%,12.6%, 3.8% and 8.1%, 15.0% respectively(Fig.7).

Rice quality analysis

Due to the grain length and ratio of grain length to width are important factors affecting the appearance quality of rice, the rice length, ratio of rice length to width, translucency grade, chalky rate and chalkiness degree of polished rice grain were performed and the results indicated that the appearance qualities of GM2B(GM2A) and its combinations (GM2A/GH998 and GM2A/GH715) were superior or close to GM1B(GM1A) and its combinations (GM1A/GH998 and GM1A/GH715) (Fig 8). Especially, the improved parental line GM2B(GM2A, *gs3*) got the rice length to 6.83mm, and the ratio of rice length to width to 3.61 comparable to Simiao-type standard. Moreover, the alkali spreading value, gel consistency and amylose content of rice quality were also analyzed (Table 5). Compared with original GM1B(GM1A), the alkali spreading value and gel consistency of improved line GM2B(GM2A) were significantly increased, but there is no difference in amylose content, which is similar to the results of hybrid rice GM1A/GH998 and GM2A/GH998. In the combinations of GH715, GM2A/GH715 exhibited lower amylose content and higher gel consistency, which showed these quality traits of GM2A/GH715 were superior to that of GM1A/GH715. These results indicated that although the head rice rate of processing quality decreased slightly in GM2B(GM2A)and GM2A/GH715(Fig 8), the overall appearance quality and rice qualities of GM2B(GM2A) and its combination (GM2A/GH998 and GM2A/GH715) had been improved, especially in GM2A/GH715. In addition, the genotypes of *GS3* and three quality related genes (*Wx*^{b}, *ALK*, *Chalk5*) were also consistent with rice quality testing in Tables 4 and 5.

Discussion

The characteristics related to rice yield are still important indicators in the new varieties' breeding and previous reports demonstrate that introducing a*gs3* gene into a variety substantially increases rice grain yield (Shen et al., 2016; Li et al., 2016; Chen et al., 2020). Comparing the yield related traits of the maintainer

line GM1B(*GS3*) and the improved line GM2B(*gs3*) demonstrated a significant increase in thousand grain weight (TGW) and grain yield per plant. Even though the seed setting rate of GM2B(*gs3*) decreased, increases in grain number per panicle, filled grain per panicle and TGW offset the adverse effect of lower seed setting rate on grain yield. The seed setting rate decrease occurred in our improved maintainer line resembles results of previous studies. Two reports have pointed out that according to the source-sink-flow theory for rice plant growth or cultivation, the increase in grain length, grain weight, and the total number of grains per panicle could have enhanced the 'sink' capacity of the whole grain structure resulting in insufficient photosynthesis in an individual plant, causing more empty grains or a lower seed setting rate (Shen et al., 2016; Chen et al., 2020). However, contrast to the hybrids of original parental line GM1A(*GS3*), the ones of our improved line GM2A(*gs3*) had similar seed setting rate and relevant increase of grain length, 1000 grain weight and total yield per plant, thereby ensuring the overall stability of the yield(Table 3).The slender type Simiao or 'silk seedling rice' is very popular in the South China rice market and has high market value. After the introduction of a *gs3* allele, the grain length of the resultant male sterile line GM2A meted the grain length standard set of Simiao, which will greatly increase its market competitiveness. Indeed, when GM2A was combined with a slender restorer G715, not only was the yield of their F_1 product enhanced, but also did the appearance of their F_1 product matched the high value Simiao standard. In addition, our data also indicated that the edited *gs3* allele improved the eating and cooking quality of the male sterile line and its hybrid combinations. Together, our results demonstrated that improvement of GM1A through introducing a *gs3* allele via CRISPR/Cas9 technology indeed lead to better grain appearance and yield in both male sterile line itself and its hybrid combinations.

The values of the rice quality traits for the tested combinations are commonly determined or influenced by the male sterile line used(Mao et al., 2007) and the key to the success of male sterile line breeding is to completely maintain the characteristics of male sterility. Through the CRISPR-Cas9-mediated genome editing technology, some limitations in traditional crop breeding programs can be avoided so as to achieve desired traits quicker and more accurately in the current era of crop genetic improvement(Haque et al. 2018; Mishra et al. 2018). In our rice breeding project, this CRISPR/Cas9-mediated genome editing method was used to knock out the *GS3*gene in the maintainer line GM1B, and successfully obtained a *gs3* mutant with a specific loss of function of the grain size gene. In the T_1 mutant generation of GM1B, specific line with increased grain length and homozygous mutated loci, but without transgene was selected to be the new maintain line GM2B. In the BC_1F_1segregated populations, the modified sterile lines were selected base on long grain morphological trait and homozygous mutants and backcrossed with GM2B to obtain the corresponding new line GM2A in BC_2F_1 generation. We know that conventional three-line rice breeding system needs a long cycle to evaluate and achieve stable sterile lines, but this report established a breeding system to obtain superior sterile line with target trait and further to develop new cross combinations in only two years, which significantly shortened the breeding cycle for releasing new hybrid rice varieties.

The influence of restoration and conservation relationship on the heterosis of three-line hybrid rice led to more restrictions on trait improvement than conventional rice(Zhou, 1994; Gong et al.,2020). In this experiment, We chose strong restorer line GH998 to further consider the effect of the restored line and improved male sterile line. Otherwise, G715 was selected under the concept of long grain breeding in order to obtain a long grain hybrid combination. The analysis of combinations showed that the maintainer line with single gene knockout kept a good restoration relationship with the restorer line. The improved male sterile lines by gene knockout could be outcrossed to obtain some near isogenic line combinations with single gene mutation for further analyzed (Han et al., 2018), and then some important QTL genes related to rice yield and quality in the tested parents could be detected by PCR-based markers or SNP(single nucleotide polymorphism) primers from high-density arrays in genome-wide association studies (GWAS), to associate the subtle phenotypic effects for different alleles of the gene analyzed. Our results further showed that the test combination with *gs3*/*gs3* allele had more advantages in yield and quality, indicating that the main QTL gene with negative regulation should pay more attention to the selection of genotype at the corresponding locus of the restorer lines. This experiment is an application attempt of gene editing technology to guide three-line hybrid rice breeding practices from selection of the parents to cross combinations improvement. In the future, these results could not only quicken the effective use of the existing germplasm, shorten the breeding process to release hybrid rice combinations, but also offered new ideas for rice breeders to shift their focus from marker assisted selection (MAS) to genomic selection (Meuwissen et al., 2001), so as to reduce the challenges of phenotyping (Poland and Rutkoski, 2016), and higher the accuracy of genomic prediction using such genetic markers in pre-emptive breeding program (Emebiri et al., 2021).

Abbreviations

GM1B	Guimei 1B	GM2B	Guimei 2B
GM1A	Guimei 1A	GM2A	Guimei 2A
CMS	Cytoplasmic male sterility	WT	Wild type
GH998	Guanghui998	G715	Gui715
TGW	Thousand grain weight	FAM V	FAM value
LC	Low chalkiness	HEX V	HEX value
HC	High chalkiness	LG	long grain
LASV	Low alkali spreading value	SG	short grain
HASV	High alkali spreading value	AC	amylose content

Declarations

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Authors' contributions J H, D Q and Y P mainly carried out the sequencing, agronomic traits statistics and rice quality analysis. LG was responsible for crossing and back-crossing experiments in field. J H, S L, K L and G D performed data analysis and manuscript writing. G D and C Z contributed to designing the research.

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Availability of data and material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate Not Applicable

Consent for publication Not Applicable

Competing interests **The authors declare that they have no competing interests.**

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Tables

Table 1. Primers used in this study

Primer name	Sequence(5'-3')	Fluorescence value and corresponding genotype
GS3-F1	Tccgccattcaaagcaaagc	-
GS3-R1	Gagtttaggtggagggacgc	-
GS3-F2	Acagtacttgctgtctagcttt	-
GS3-R2	actcccaacgttcagaaattaaatg	-
GS3-Y1+	cagtggtctcaggcaatgggcatgaaccaactcc	-
GS3-Y1-	cagtggtctcaaaacggagttggttcatgcccat	-
GS3-B1+	cagtggtctcaggcatcaagactgtccagaaggc	-
GS3-B1-	cagtggtctcaaaacgccttctggacagtcttga	-
YI-R+	accggtaaggcgcgccgtagt	-
Pbw2-	gcgattaagttgggtaacgccaggg	-
Hyg-F	acgtctgtcgagaagtttctgatc	-
Hyg-R	agtcaatgaccgctgttatgc	-
Chalk5b-FT	gaaggtcggagtcaacggattagagagaagtgccaaggatctgt	HEX V:LC
Chalk5b-FC	gaaggtgaccaagttcatgctagagagaagtgccaaggatctgc	FAMV:HC
Chalk5b-R1	tgcatctagctaccttcatttcg	-
RGs3-RT	gaaggtcggagtcaacggattcagcaggctggcttactctctt	FAM V: LG
RGs3-RG	gaaggtgaccaagttcatgctcagcaggctggcttactctctg	HEX V: SG
RGs3-F	acacatgcccatctccctcg	-
alk-Ftt	gaaggtgaccaagttcatgcttacaaggagagctggaggggtt	FAM V: LASV
alk-Fgc	gaaggtcggagtcaacggatttacaaggagagctggagggggc	HEX V:H ASV
alk-R	ctgaggtcctgcgacatgc	-
RWx-Fg	gaaggtgaccaagttcatgcttcatcaggaagaacatctgcaagg	FAM V: HAC
RWx-Ft	gaaggtcggagtcaacggatttcatcaggaagaacatctgcaagt	HEX V: L AC
RWx-R	ggaaaaacgagcaatgaaagatgc	-

Table 2 Single plant of homozygous mutations and their grain length in the T_0 generation

Accession Numbers	Target sequences	Chromosome Numbers	Sequence of mutation(5'-3')	Grain length(mm)
P437-2	cctcgaggaatccgatctcgcgg	2	CGCGAGATCGGATTCCTTCGAGGGTGAAATAAAT (insertion)	10.33±0.36
P437-6	tgcagcatctggaggcagcgtgg	2	ATCCACGCTTGCCTCCAGATGCTGCAGAGAGGTTGACGAAT (insertion) (insertion)	10.22±0.45
P437-13	cctcgaggaatccgatctcgcgg	2	CGCGAGATCGGATTCCCTCGAGGGTGAAATAAAT (insertion)	10.24±0.41
GM1B	-	-	-	9.40±0.37

The inserted bases are highlighted in gray.

Table 3 Statistic of variety traits

variety	Grain length (mm)	Gain width (mm)	Ratio of grain length to width	Panicle length (cm)	Grain number per panicle	Filled grain number per panicle	Seed-setting rate(%)	1000 grain weight(g)	Effective tiller number	Tiller number at active stage	Plan heig (cm)
GM1B	9.40±0.37	2.42±0.11	3.89±0.20	25.6±2.0	163.0±44.6	141.8±49.8	84.5±12.8	18.0±0.6	10.5±1.7	-	111.
GM2B	10.14±0.40	2.42±0.09	4.19±0.25	27.7±2.0	204.5±34.3	148.4±26.0	73.0±8.8	19.2±0.4	11.0±1.0	-	112.
GH998	9.59±0.36	2.65±0.13	3.63±0.25	22.9±1.5	161.6±11.7	129.8±12.2	80.5±8.6	21.7±0.5	8.6±0.9	-	104.
G715	11.30±0.37	2.62±0.14	4.32±0.33	27.0±1.3	227.8±52.7	181.8±37.7	80.2±4.3	22.5±0.7	7.4±1.1	-	123.
GM1A/GH998	9.49±0.30	2.70±0.08	3.52±0.14	25.4±1.0	160.2±30.2	126.9±25.9	79.0±2.9	22.4±0.7	9.0±1.5	10.2±1.1	126.
GM2A/GH998	10.02±0.29	2.62±0.13	3.83±0.23	25.9±1.0	167.8±38.2	126.0±30.4	75.4±6.6	24.0±0.8	10.0±2.0	12.5±2.8	128.
GM1A/G715	9.96±0.26	2.66±0.06	3.74±0.13	26.2±1.1	193.0±48.3	149.8±47.7	76.8±7.1	22.2±1.0	8.9±1.6	10.3±1.7	131.
GM2A/G715	11.08±0.31	2.64±0.11	4.21±0.28	27.2±1.2	214.0±61.6	163.1±50.2	76.1±6.6	24.0±0.6	11.0±2.8	12.6±3.7	132.

Table 4 Genotypes of rice quality related genes in tested parents

Variety	Fluorescence value and Genotype of *GS3*	Fluorescence value and Genotype of *Wx*	Fluorescence value and Genotype of *ALK*	Fluorescence value and Genotype of *Chalk5*
GM1B(GM1A)	*GS3*(FAM V)	Wx^b(HEX V)	*ALK*(HEX V)	*Chalk5*(FAM V)
GM2B(GM2A)	*gs3*	Wx^b(HEX V)	*ALK*(HEX V)	*Chalk5*(FAM V)
GH998	*gs3*(HEX V)	Wx^b(HEX V)	*ALK*(HEX V)	*chalk5*(HEX V)
G715	*gs3* (HEX V)	Wx^b(HEX V)	*ALK*(HEX V)	*Chalk5*(FAM V)
GM1A/GH998	*GS3/ gs3*	Wx^b	*ALK*	*Chalk5/ chalk5*
GM1A/G715	*GS3/ gs3*	Wx^b	*ALK*	*Chalk5*
GM2A/GH998	*gs3*	Wx^b	*ALK*	*Chalk5/ chalk5*
GM2A/G715	*gs3*	Wx^b	*ALK*	*Chalk5*

Table 5 Analysis of rice quality for parents and their cross combinations

Variety	Brown rice rate(%)	Milled rice rate(%)	Head rice rate(%)	Rice length (mm)	Rice width (mm)	Ratio of rice length to width	Translucency	Chalky rice rate(%)	Chalkiness degree(%)	Alkali spreading value	Ge co (m
GM1B(GM1A)	79.0±0.2	71.2±0.7	68.7±0.7	6.16±0.15	1.85±0.08	3.32±0.16	2±0	7.0±2.0	2.4±0.3	2.3±0.1	87
GM2B(GM2A)	78.9±0.3	70.5±0.7	66.9±0.3	6.83±0.12	1.89±0.09	3.61±0.20	1±0	6.3±1.5	2.0±0.6	3.1±0.2	91
GH998	76.9±0.4	68.2±0.4	66.5±0.3	6.93±0.24	2.27±0.09	3.05±0.18	2±0	9.7±0.6	5.0±0.2	2.0±0.2	88
G715	80.0±1.1	71.8±1.0	64.0±0.4	7.38±0.21	2.19±0.10	3.37±0.16	1±0	8.7±0.6	2.0±0.2	2.0±0.1	66
GM1A/GH998	80.3±0.9	72.4±0.8	68.8±0.4	6.63±0.25	2.26±0.06	2.94±0.10	2±0	10.0±1.0	3.1±0.2	2.7±0.1	78
GM2A/GH998	80.2±0.6	72.4±0.4	68.3±0.6	7.10±0.21	2.21±0.13	3.21±0.19	2±0	8.3±1.5	3.6±0.4	3.7±0.4	84
GM1A/G715	77.9±0.6	70.7±1.0	68.4±0.6	6.97±0.19	2.10±0.16	3.34±0.22	1±0	3.0±1.0	2.0±0.3	3.3±0.3	67
GM2A/G715	77.9±0.3	70.1±0.4	64.9±0.7	7.31±0.17	2.08±0.06	3.52±0.11	1±0	2.0±0	1.1±0.1	3.8±0.5	89

Figures

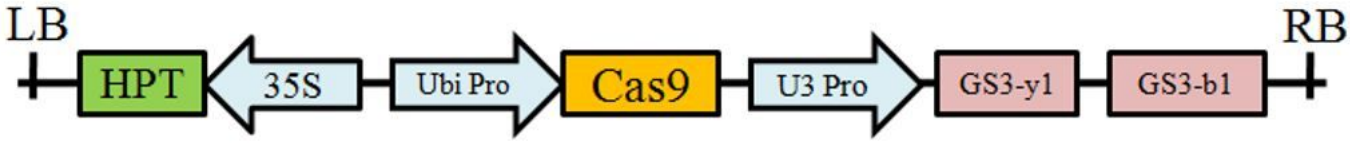

Figure 1

Schematic map of the recombinant plasmid CRISPR-Cas9 -GS3. The inserted fragment region includes knockout targets GS3-y1 and GS3-b1 activated by U3 promoter, Cas9 gene activated by ubiquitin promoter and hygromycin phosphotransferase gene activated by 35S promoter. LB, T-DNA left border sequence; RB, T-DNA right border sequence.

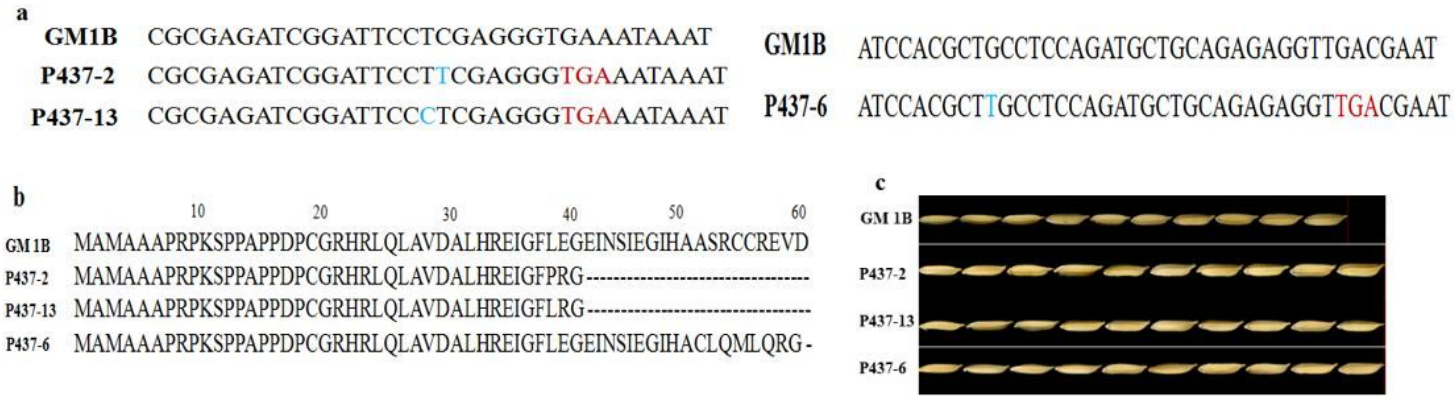

Figure 2

sequencing and amino acid analysis of 3 homozygous mutation types of GS3 and their corresponding grain shape. a sequencing analysis of three individuals. Mutations with 1bp insertion are represented by blue letters and stop codes are in red. b GS3 amino acid alignment of three mutants and wild type. c photograph of grain length comparison between WT and mutants. GM1B, WT maintainer line with GS3 allele; P437-2, -13 and -6, mutant maintainer lines with edited gs3 allele.

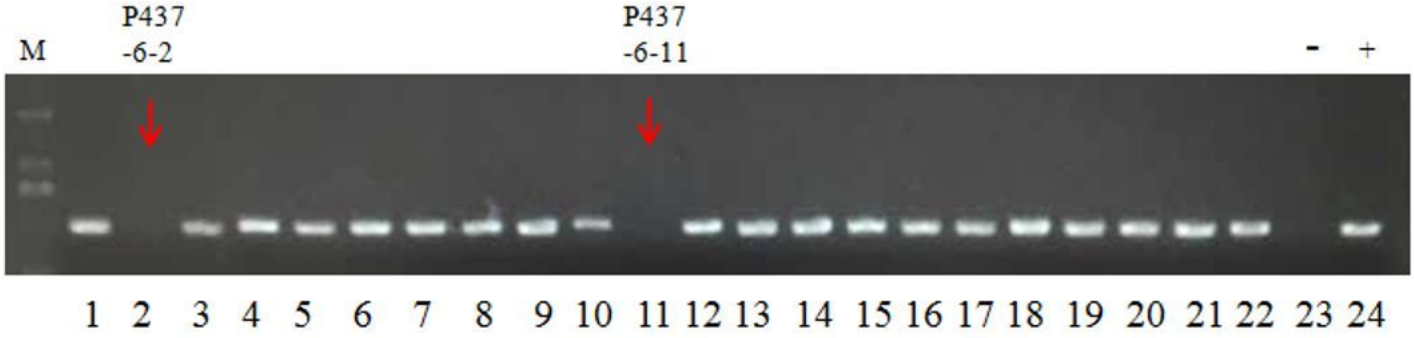

Figure 3

PCR identification of the transgene-free transgenic plants. Primers Hyg-R and Hyg-F were used to amplify a fragment from HPT (hygromin phosphotransferase) gene. Lane 1-22, individual seedlings of P437-6 mutant; Lane23, NIP, Negative control(Nipponbare); Lane, 24, +, positive control of transgenic line; M, Marker 2000. Lanes with amplified PCR fragment indicated transgene positive. Lanes without amplified PCR fragment indicated transgene-free. Two transgene-free mutants P437-6-2 and P437-6-11 were pointed with a red arrow.

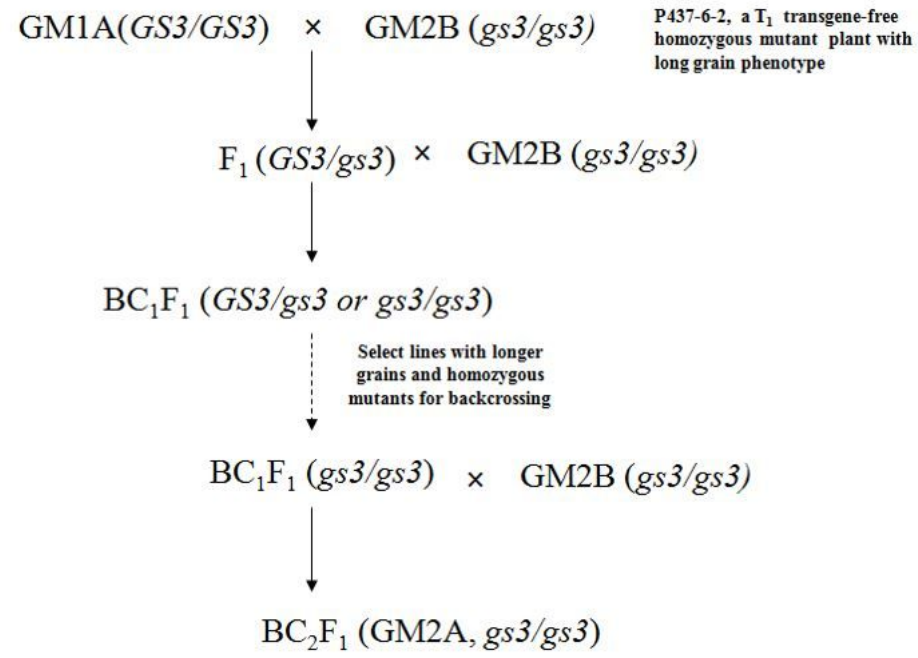

Figure 4

Breeding process of converting mutant maintainer line GM2B to mutant male sterile line GB2A.

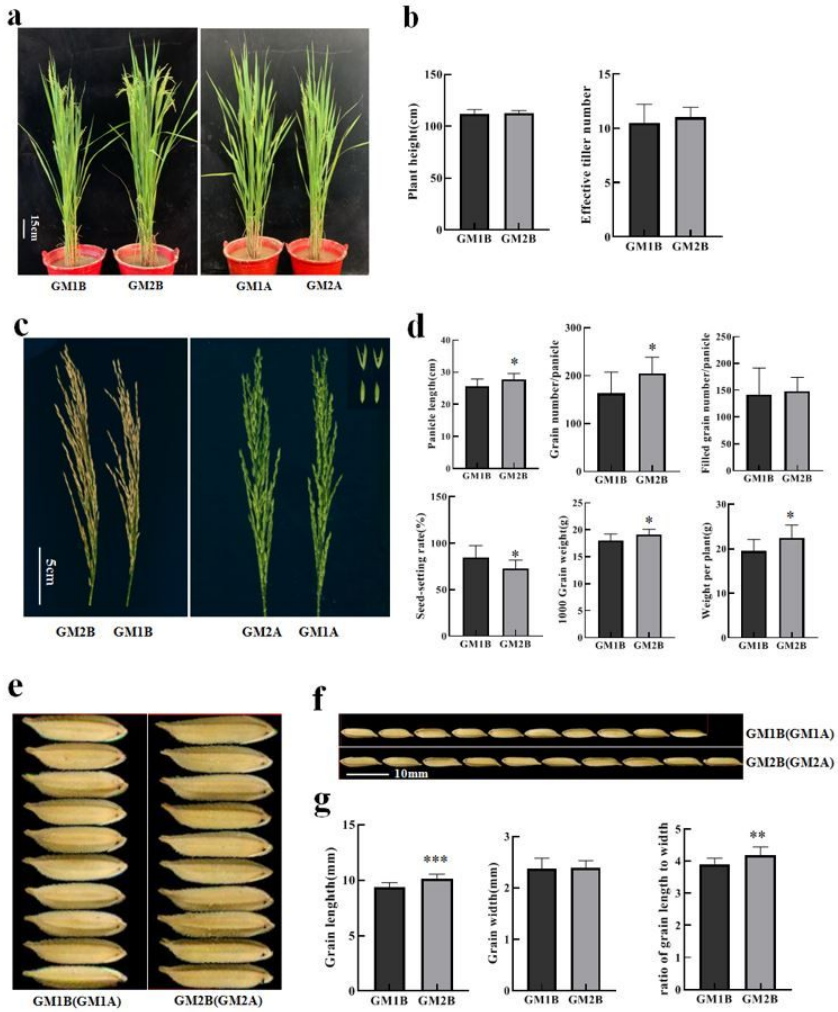

Figure 5

Comparison of traits of parents and their improved progenies. a, c, e, f shows the grass morphology, panicle and grain of the parent and its improvement. b, d, g performances of main agronomic traits of the parent and its improvement. *, **,*** indicate Significant difference at 0.05, 0.01and 0.001levels.

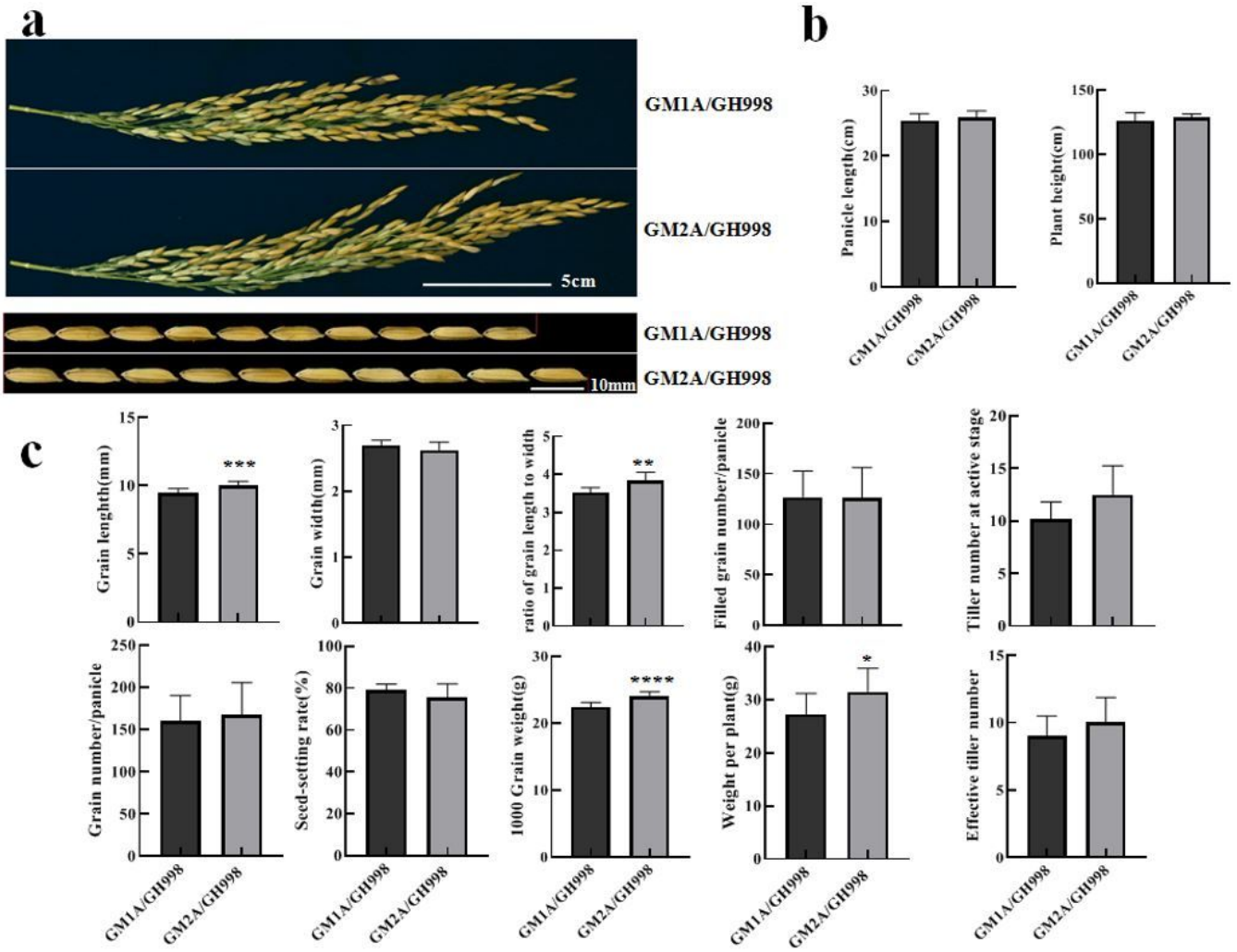

Figure 6

Statistical comparison of traits in GM2A/GH998 andGM1A/GH998. a panicle and grain length of combinations GM1A/GH998 and GM2A/GH998. b, c performances of main agronomic traits of the combination. *, **,***,**** indicate significant difference at 0.05, 0.01, 0.001and 0.0001levels.

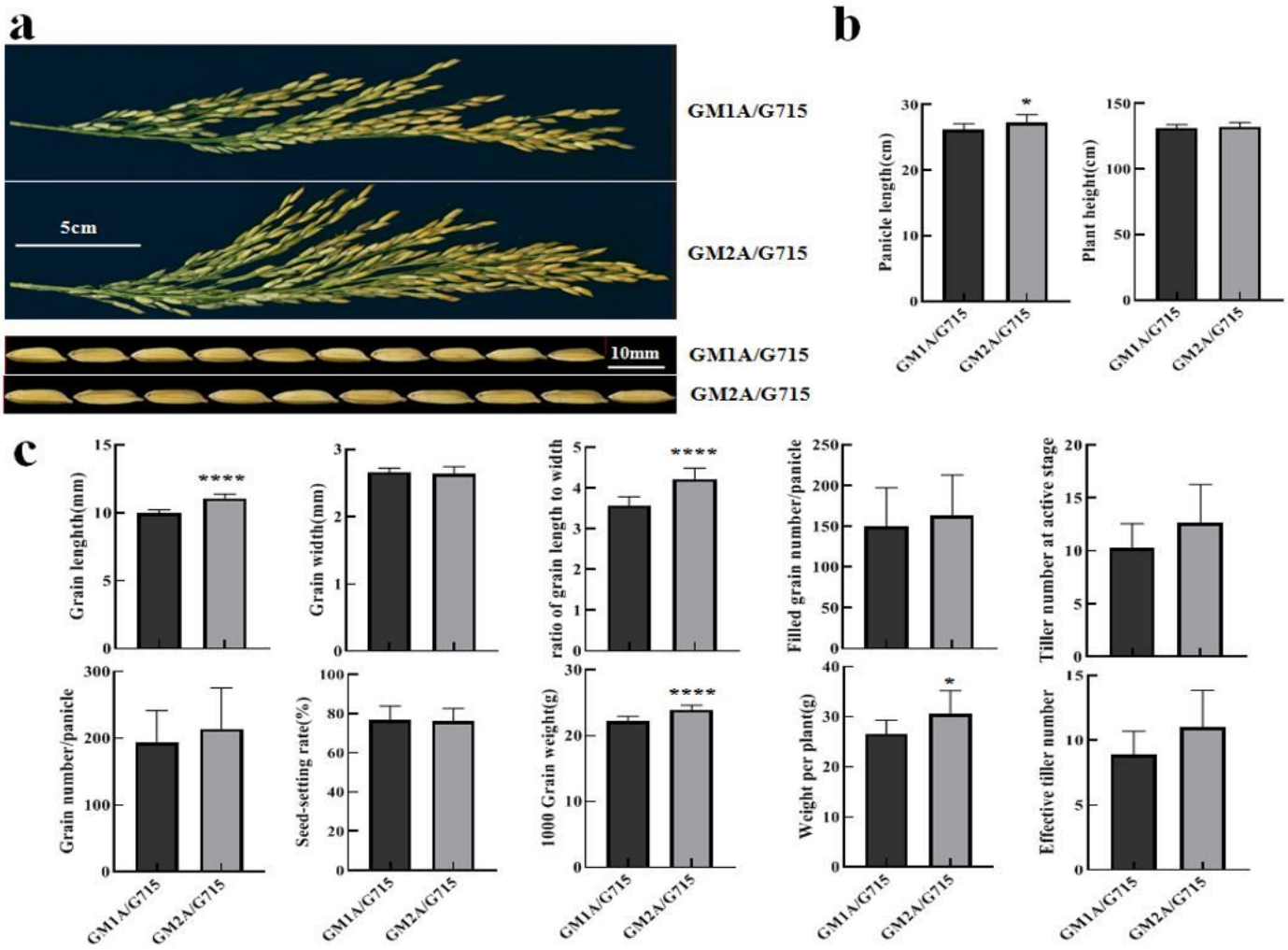

Figure 7

Statistical comparison of traits in GM2A/G715 and GM1A/G715.a panicle and grain length of combinations GM1A/G715 and GM2A/G715. b, c performances of main agronomic traits of the combination.*, **** indicate significant difference at 0.05 and 0.0001levels.

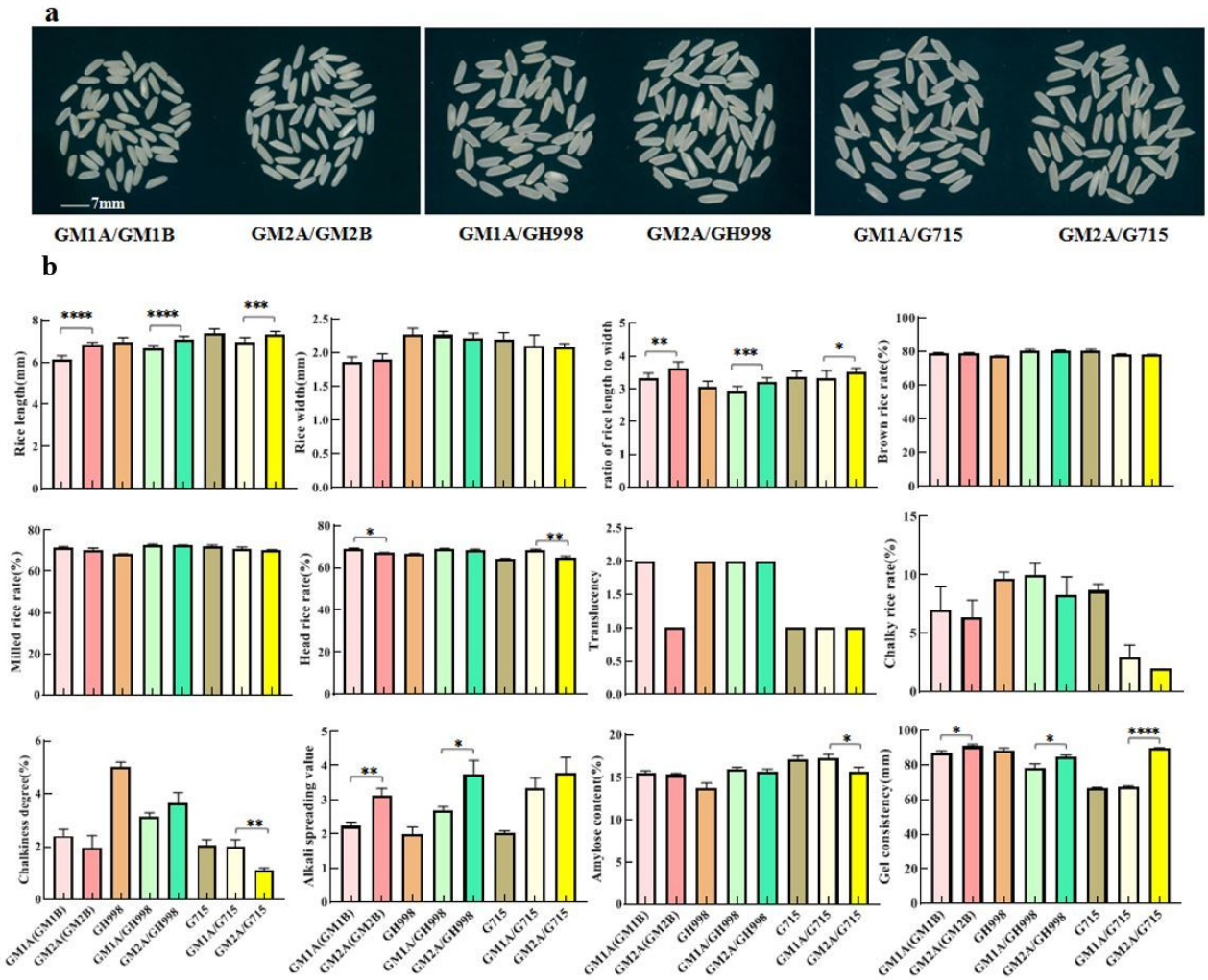

Figure 8

Rice grain quality comparison between parents and their combinations. a Photographs of polished rice grains. b Comparison of grain quality related parameters. GM1B, GM1A, WT maintainer and male sterile line; GM2B, GM2A, mutant maintainer and male sterile line. GH998, G715, restorer line.*, **,***,**** indicate Significant difference at 0.05, 0.01, 0.001and 0.0001levels.