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RESEARCH

Natural variation of *Grain size 3* allele differentially functions in regulating grain length in xian/indica and geng/japonica rice

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Abstract Grain yield in rice is largely determined by grain size. *Grain Size 3* (*GS3*) is a major quantitative trait locus for grain size. The C–A natural variation in the second exon of *GS3* was reported to play an important role in regulating grain length in rice. Here we evaluate *GS3* alleles among 303 germplasm accessions. The $GS3^{A}$ allele was predominant in *xian/indica* (XI) accessions, whereas *geng/japonica* (GJ) accessions mainly carried $GS3^{C}$. The *GS3* allele affected the grain length significantly in XI, while its function was minimal in GJ, indicating that introduction of *GS3* alleles might be useful to modify grain length in XI breeding programs, but not in GJ breeding. The association between *GS3* alleles and seed weight was not significant in any of the individual subpopulations, suggesting that the contribution of *GS3* to grain weight could be slight in terms of different subspecies. To develop an effective marker for *GS3*, a penta-primer amplification-refractory mutation system (PARMS) marker exploiting a single-base mutation (C–A) was developed, which entailed lower cost and less time than other available markers, and should be useful for fine marker-assisted selection of grain length in XI accessions breeding.

Keywords Xian · *Indica* subspecies · Grain size 3 · Grain length · Alleles · PARMS

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Introduction

Grain size, which is specified by grain length, width and length-to-width ratio, is a highly important quality trait and an important target for selection during domestication and breeding in rice (Fitzgerald et al. 2009; Xing and Zhang 2010; Sun et al. 2018). Long and slender grain is preferred in China for *xian/indica* rice because of its desirable appearance (Xu and Chen 2016; Huang and Qian 2017; Chen et al. 2023). *GRAIN SIZE 3* (*GS3*), encoding a Gγ subunit (Group III) of the heterotrimeric G protein, has been identified as a major QTL for grain length. In addition, *GS3* participates in stigma exsertion (Takano-Kai et al. 2011) and alkaline tolerance (Zhang et al. 2023). Natural variants of *GS3* have been shown to boost grain yield (Fan et al. 2006), in particular, with a single nucleotide polymorphism (SNP) between C and A in the second exon (Fan et al. 2009; Takano-Kai et al. 2009). With technological advances in functional genomics, great progress has been achieved in clarifying the molecular mechanisms that determine seed size (Song et al. 2007; Weng et al. 2008; Li et al. 2011; Qi et al. 2012; Wang et al. 2012, 2015, 2019; Zhang et al. 2012; Duan et al. 2015; Hu et al. 2015; Xu et al. 2018; Zhao et al. 2018). Among them, the *GS3* locus also provides an informative system for studying the evolutionary processes underlying rice domestication and breeding (Mao et al. 2010). However, the functional effect of natural variants of *GS3* has been described only in particular rice cultivars; its role in determining the grain size phenotype in different genetic backgrounds remains be confirmed.

The *GS3* gene has five exons and encodes 232 amino acids with a specific organ-size regulation (OSR) domain near the N terminus, a tumor necrosis factor receptor/nerve growth factor receptor-family cysteine-rich domain, and a von Willebrand factor type C module (Fan et al. 2006). The *GS3* gene functions as a negative regulator of grain size: the OSR domain is both necessary and sufficient for functioning as a negative regulator (Mao et al. 2010). A nonsense mutation (C–A) is found in the second exon of *GS3* shared among the long length-grain varieties but not in varieties with smaller grains. The C–A mutation causes a 178-amino acid truncation at the C terminus, resulting in part of the OSR domain and all the other three domains deleted (Mao et al. 2010). Besides the C–A mutation, a further three polymorphic loci were discovered in the second intron, the last intron and the final exon of *GS3*. It is worth noting that the C–A mutation is a highly effective QTL that explains one third of the grain length variation in rice (Wang et al. 2010). In this study, we evaluated the natural variation of *GS3* alleles among 303 germplasm accessions, including 81 *xian/indica* (XI) rice types and 222 *geng/japonica* (GJ) types to investigate the correlation between *GS3* alleles and grain length and weight. We also developed a novel intragenic marker that provides a valuable tool for the evaluation and use of the *GS3* gene in screening germplasm accessions and in breeding new varieties.

Materials and methods

Rice accessions and cultivars

We used 81 XI rice accessions and 222 GJ accessions in this study (Table S1). The XI accessions consisted of rice cultivars approved in the southern provinces of China, including Chinese landraces and cultivated varieties. The GJ accessions consisted of typical cultivated varieties, a small number of rice strains and 31 varieties approved by Shandong Crop Varieties Examination and Approval Committee in the last five years. In addition, the Indian cultivars Pokkali, Kasalath, and Dular and seven *aus* accessions were included in this study (Table S2).

Marker design and analysis

The penta-primer amplification-refractory mutation system (PARMS) marker is designed based on the C–A polymorphism in the second codon of *GS3*, as described in previously (Fan et al. 2009), and comprised the primer pair PARMS-GS3-F1 (GAAGGTGACCAAGTTCATGCTCTGCCTCCAGATGCTGA) and PARMS-GS3-F2 (GAAGGTCGGAGTCAACGGATTCTGCCTCCAGATGCTGC) plus PARMS-GS3-R-Common (TGCATGGTAAGAGTAAAGACGAGA). The primers GS3-F (AAATATCCCTCAGACATCAC) and GS3-R (CACTCAAAAAGCTTGCAC) amplified the C–A mutation region and were designed from the two allelic sequences of rice varieties '9311' and 'Nipponbare'.

We carried out PARMS amplification PCR in a total volume of 10 μL containing 1 μL genomic

DNA, 0.15 µL F primers, 0.4 µL R primers and 5 µL PARMS mix using an Applied Biosystems Q5 Real-Time PCR System according to the manufacturer's instructions. The PCR conditions were: 15 min at 94 °C for denaturation, followed by 10 cycles of 94 °C for 20 s and 65 °C (with the temperature for each cycle decreased by 0.8 °C) for 1 min, then 28 cycles of 94 °C for 20 s and 57 °C for 1 min, and finally by 1 min at ambient temperature. For the developed marker, the PCR reaction contained 2 µL (40 ng) genomic DNA, 1 µL each primer (10 µM), 10 µL 2×Taq PCR MasterMix and 6 µL ddH_2O. The PCR profile was 3 min at 94 °C for denaturation, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, then 3 min at 72 °C for extension. The products were detected by agarose gel electrophoresis and sequenced by the Qingke Biological Technology Co. Ltd.

Measurements of grain traits

Harvested paddy rice was air-dried and stored at room temperature before testing. Ten fully-filled grains were randomly chosen from each plant were divided into three equal groups. Each group was lined up length-wise along a vernier caliper to measure total grain length. Grain weight was calculated on the basis of 1000 grains. As described in the textbook 'The Principle and Method of Testing New Rice Varieties', the grain length and weight were selected as per the following table (Tables 1 and 2):

Statistical analysis

Duncan's test was performed to compare the means of seed traits for different allelic groups or cultivars/lines using SPSS 19.0. *T*-tests were performed or *F* statistic estimated for each allelic group using Excel 2007.

Results

Distribution of *GS3* alleles in rice subspecies from China

As mentioned above, the nonsense mutation (C–A) causes a truncated protein with part of the OSR domain and all the other three domains are deleted (Fig. 1A). To facilitate the genetic improvement of *GS3* in XI and GJ accessions in China, alleles of *GS3* were identified in 303 germplasm accessions using Fan's CAPS marker (Fan et al. 2009). We firstly analyzed the distribution of *GS3* in 81 XI accessions. The $GS3^A$ allele was found in 59 out of the 81 XI accessions (73%), and the $GS3^C$ allele in 22 out of 81 accessions (27%) (Fig. 1B). We then analyzed the distribution of *GS3* in GJ accessions: 176 out of the 222 GJ accessions (80%) carried the $GS3^C$ allele, and only 46 accessions (20%) carried the $GS3^A$ allele (Fig. 1C). These results indicate that *GS3* was selected independently of subspecies. It was apparent that the distribution of *GS3* alleles was opposite between XI and GJ

Table 1 The classification standard for rice grain length

Decsription	Extremely short	Extremely short to short	Short	Short to moderately short	Moderately short	Moderately short to long	Long	Long to estremely long	Estremely long
AC/mm	< 3.0	3.0 ~ 4.00	4.0 ~ 5.0	5.0 ~ 6.0	6.0 ~ 7.0	7.0 ~ 8.0	8.0 ~ 9.0	9.0 ~ 10.0	> 10.0
Code	1	2	3	4	5	6	7	8	9

Table 2 The classification standard for rice grain weight

Decsription	Extremely low	Extremely low to low	Low	Low to moderately low	Moderately low	Moderately low to high	High	High to estremely high	Estremely high
AC/mm	< 11.0	11.0 ~ 16.0	16.0 ~ 20.0	20.0 ~ 24.0	24.0 ~ 28.0	28.0 ~ 32.0	32.0 ~ 36.0	36.0 ~ 40.0	> 40.0
Code	1	2	3	4	5	6	7	8	9

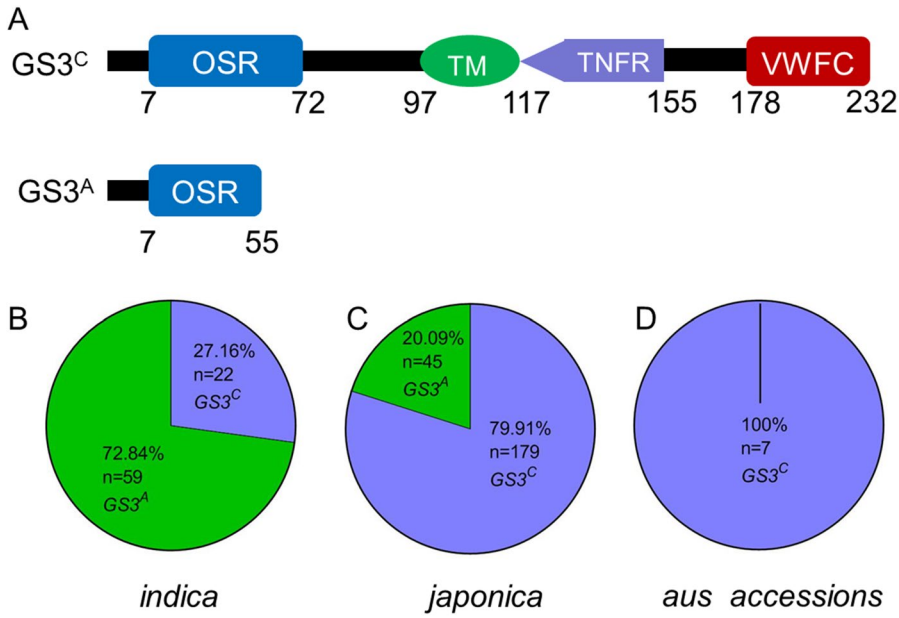

Fig. 1 the structure and distribution of the *GS3* haplotypes. **A** Protein structures of $GS3^C$ and $GS3^A$. **B–D** The distribution of XI, GJ and aus accessions

accessions. The distribution was broadly in keeping with the phenotype of grain length, in that long and slender grain is prevalent in XI rice and short round grain in GJ cultivars. Interestingly, all the *aus* accessions carried the $GS3^C$ allele (Fig. 1D). In view of the *aus* rice is mainly distributed across South Asia, mainly concentrated in the Indian subcontinent. It seems likely that $GS3^A$ allele was the result of later artificial selection in *indica* rice breeding in China.

Correlation between *GS3* alleles and grain length

To investigate whether there was a functional difference between XI and GJ accessions, caused by the *GS3* genotype, We measured their ten-seed length in all the 303 accessionsare (Fig. 2A,B; Supplementary Table 1). In XI accessions, the grain lengths of the $GS3^C$ allele mainly fell into the range of 5- to 7-class and most of them were below 6-class. In contrast, the grain lengths of $GS3^A$ accessions fell into the 6- to 9-class and most of them were 7-class and above (Fig. 2C). The average grain lengths of the 59 accessions carrying $GS3^A$ were significantly longer than that 22 accessions that carried $GS3^C$ (Fig. 2D). However, in GJ accessions, there was no significant difference in average grain length between $GS3^C$ and $GS3^A$ alleles (Fig. 2D), as shown by that the grain lengths of accessions carrying either the $GS3^C$ allele (176 accessions) or the $GS3^A$ allele (46 accessions) mainly fell into 5- to 6-class (Fig. 2E). As a result, the grain lengths of accessions carrying the $GS3^A$ allele in GJ accessions were significantly shorter than those in XI accessions (Fig. 2E). We deduced that the function of the $GS3^A$ allele in regulating grain length was less pronounced in GJ than in XI accessions.

In conclusion, the SNP (C–A) in the second exon of *GS3* was confirmed to be highly associated with grain length in Chinese accessions, consistent with the previous report that the coding sequence TGC occurred in almost all the short-grain group and the TGA (premature coding stop) in the long-grain group (Fan et al. 2009; Wang et al. 2010). Interestingly, this association was different between XI and GJ accessions. The functional effect of the *GS3* alleles was significant in XI but unproductive in GJ accessions.

To further confirm the effects of the SNP variation on *indica–japonica* differentiation, statistical analysis was carried out. There was a highly significant strong correlation between grain length and allele (C–A) in XI cultivars ($P < 0.01$), whereas the correlation in GJ accessions was weaker (correlation coefficient 0.068; Table 3). These results further confirmed that the natural variation (C–A) was subjected to artificial selection in XI but not in GJ.

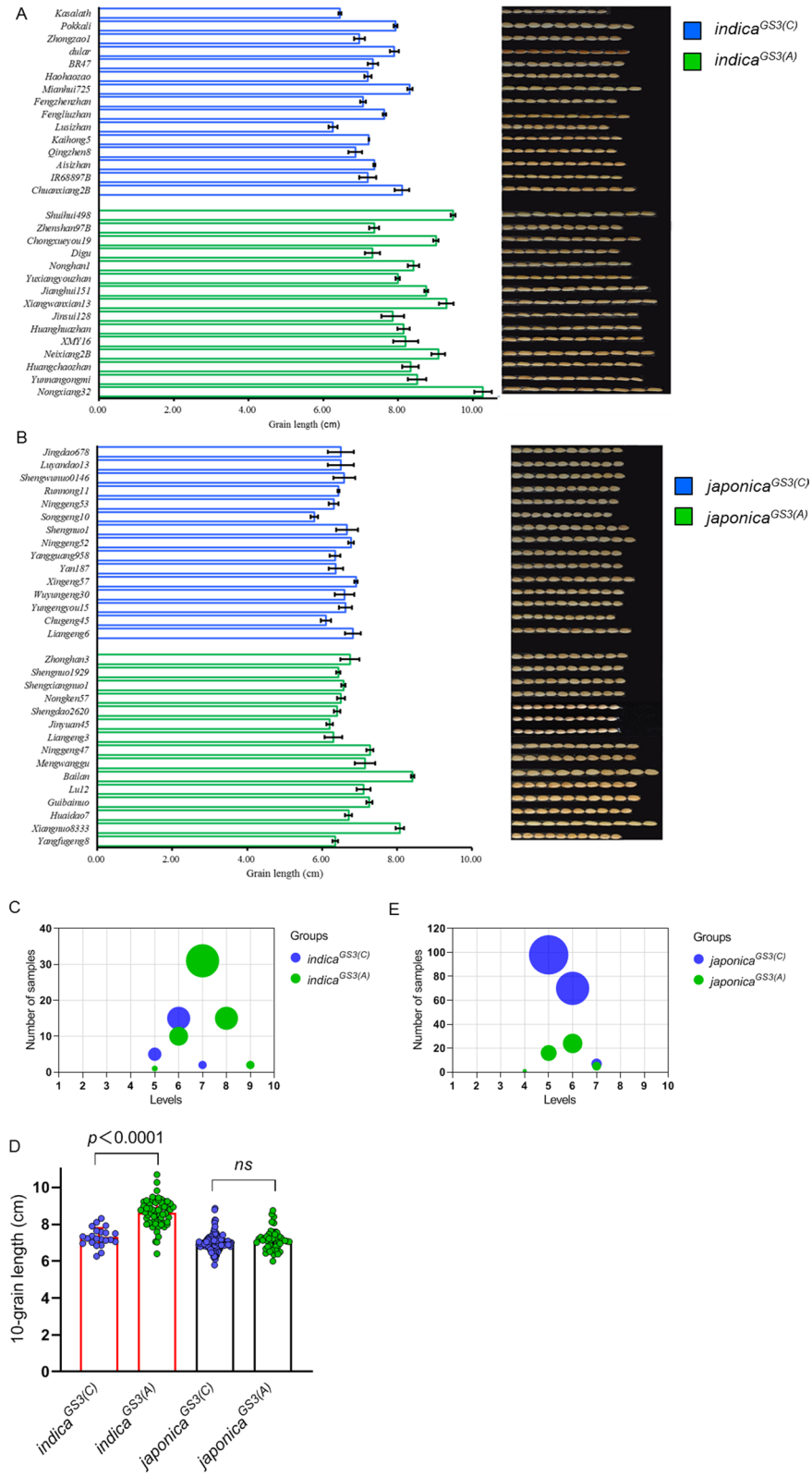

Fig. 2 the grain length levels between XI and GJ accessions. **A**–**B** The overall trend of the grain length in random accessions: **A** XI accessions; **B** GJ accessions; **C**–**D** The situation of grain length levels: **C** *indica* cultivars; **D** *japonica* cultivars; **E** The average 10-grain length in 303 accessions. Student's *t* test was used to generate the *P* values

Table 3 Correlation analysis of grain length with *GS3* in *indica* and *japonica* subspecies

Correlation analysis		*Indica*	*Japonica*
Grain length	Pearson correlation	0.619**	0.324
	Significance (two-tailed)	0.000	0.077
	N	30	30

Student's *t* test was used to generate the *P* values and '**' indicates $P < 0.01$

Correlation between *GS3* alleles and grain weight

Numerous studies have shown that the rice *GS3* gene is associated with not only grain length but also grain weight (Takano-Kai et al. 2009). The *GS3* gene has a significant negative effect on seed length and weight. However, previous studies were based on a single cultivar or transgenic lines, and it remains unclear whether the correlation between *GS3* alleles and grain weight is observed more widely in XI and GJ accessions. For this purpose, we selected all 303 accessions from XI and GJ, and measured their 1000-grain weight. The distribution of grain weights overlapped between accessions that carried $GS3^C$ and $GS3^A$ in either XI or GJ subspecies. The grain weight ranges of XI accessions carrying $GS3^A$ were concentrated at level 4–6, while those with $GS3^C$ were at level 3–5 (Fig. 3A, C). In GJ accessions, the grain weights were mostly at level 4–6, regardless of allele (Fig. 3B, D). There was no significant difference in average weight between $GS3^A$ and $GS3^C$, in either XI or in GJ (Fig. 3E). Further, statistical analysis showed that the correlation coefficient was 0.248 between $GS3^C$ and $GS3^A$ alleles in XI, and 0.214 in GJ, which meant there was only a weak correlation between grain weight and allele (C–A) in XI and GJ cultivars (Table 4).

Development of intragenic markers for *GS3* gene

The SNP (C–A) was confirmed to be highly associated with grain length in the Chinese rice accessions. Based on the two alleles, several markers have been developed, such as a CAPS marker (Fan et al. 2009), linked marker (Huang et al. 2013), and tetra-primer ARMS marker (Wu et al. 2019). However, both enzyme digestion and electrophoresis entail significant time and cost. To develop an effective marker for *GS3*, we chose to focus on the C–A mutation variation and designed a penta-primer amplification refractory mutation system (PARMS) marker, which would entail lower cost and take less time than would other available markers. Specifically, the sequence containing the C nucleobase (causing short grains) was matched and combined with HEX fluorescence and showed as red dots. The sequence with the A nucleobase is matched with FAM fluorescence and shown blue dots. Meanwhile, if the sample being tested is heterozygous, the result is shown fluorescent green (Fig. 4A, B). To confirm the specificity and accuracy of this primer, the *GS3* marker of Wu (Wu et al. 2019) was used to test 11 random samples from 303 accessions: the results confirmed exact agreement with our new PARMS marker (Fig. 4C). Further, next-generation sequencing was used to characterize and confirm the amplification fragment. Together, these results confirmed that this PARMS primer would be useful as a functional marker in selecting for improvement of rice grain length (Fig. 4D).

Discussion

In the previous study, the *GS3* gene had a major effect on rice grain size and the C–A mutation in the second exon of *GS3* was functionally associated with enhanced grain length and weight (Mao et al. 2010; Yan et al. 2011). However, this result was found based on a specific cultivar background. This study has evaluated the functional effect of different alleles of *GS3* on grain length and weight between between XI and GJ subspecies.

First, the distribution of the *GS3* gene alleles (C–A mutation) between XI and GJ accessions was not completely dependent on *indica–japonica* differentiation. Specifically, the XI accessions mostly carried the $GS3^A$ allele whereas the GJ accessions have largely the $GS3^C$ allele, which probably explains the phenotypic difference, namely that long and slender grain in XI while short round grain in GJ accessions (Fig. 1B, C). Rice is the largest cereal crop planted throughout the world. It is widely believed that *Oryza indica* originated in Southeast Asia and South Asia countries such as India (Khush 1997; Ikehashi 2009). In this study, we analyzed ten typical ancient accessions from India, including the cultivars Pokkali, Kasalath and Dular. None of them carried $GS3^A$ allele. However, the $GS3^A$ allele was predominant in

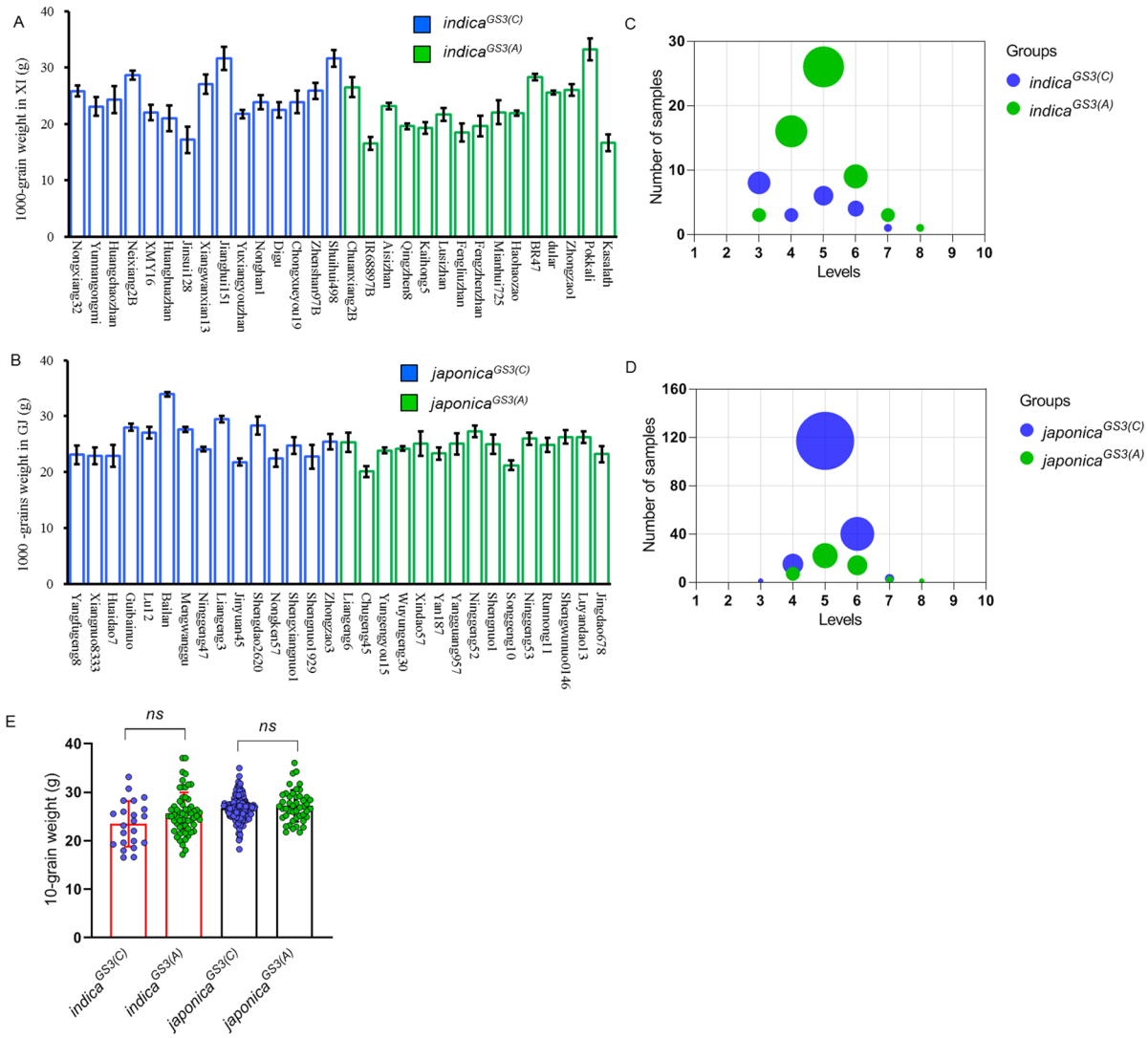

Fig. 3 the distribution of the *GS3* haplotypes and grain weight levels. **A**–**B** the overall trend of the grain weight in random accessions: **A** *indica* cultivars; **B** *japonica* cultivars; **C**–**D** the situation of grain weight levels: **C** *indica* cultivars; **D** *japonica* cultivars; **E** the average 1000-grain weight in 303 cultivars. Student's *t* test was used to generate the *P* values and there is no significant differences between *indica* $^{GS3(C)}$ and *indica* $^{GS3(A)}$, *japonica* $^{GS3(C)}$ and *joponica* $^{GS3(A)}$

Table 4 Correlation analysis of grain weight with *GS3* in *indica* and *japonica* subspecies

Correlation analysis		In *Indica*	In *Japonica*
Grain weight	Pearson correlation	0.356	0.417
	Significance (two-tailed)	0.071	0.396
	N	30	30

XI accessions from China. We therefore deduced that the $GS3^A$ genotype was the result of later artificial selection in XI subspecies (Fig. 1D).

Second, the $GS3^A$ allele affected the grain length significantly in XI accessions: the average grain length of accessions that carried $GS3^A$ was significantly larger than in lines that carried $GS3^C$ (Fig. 2E). Also, there was a strong and significant correlation between grain length and allele (C–A) in XI cultivars ($P < 0.001$; Table 3). Interestingly, the effect of

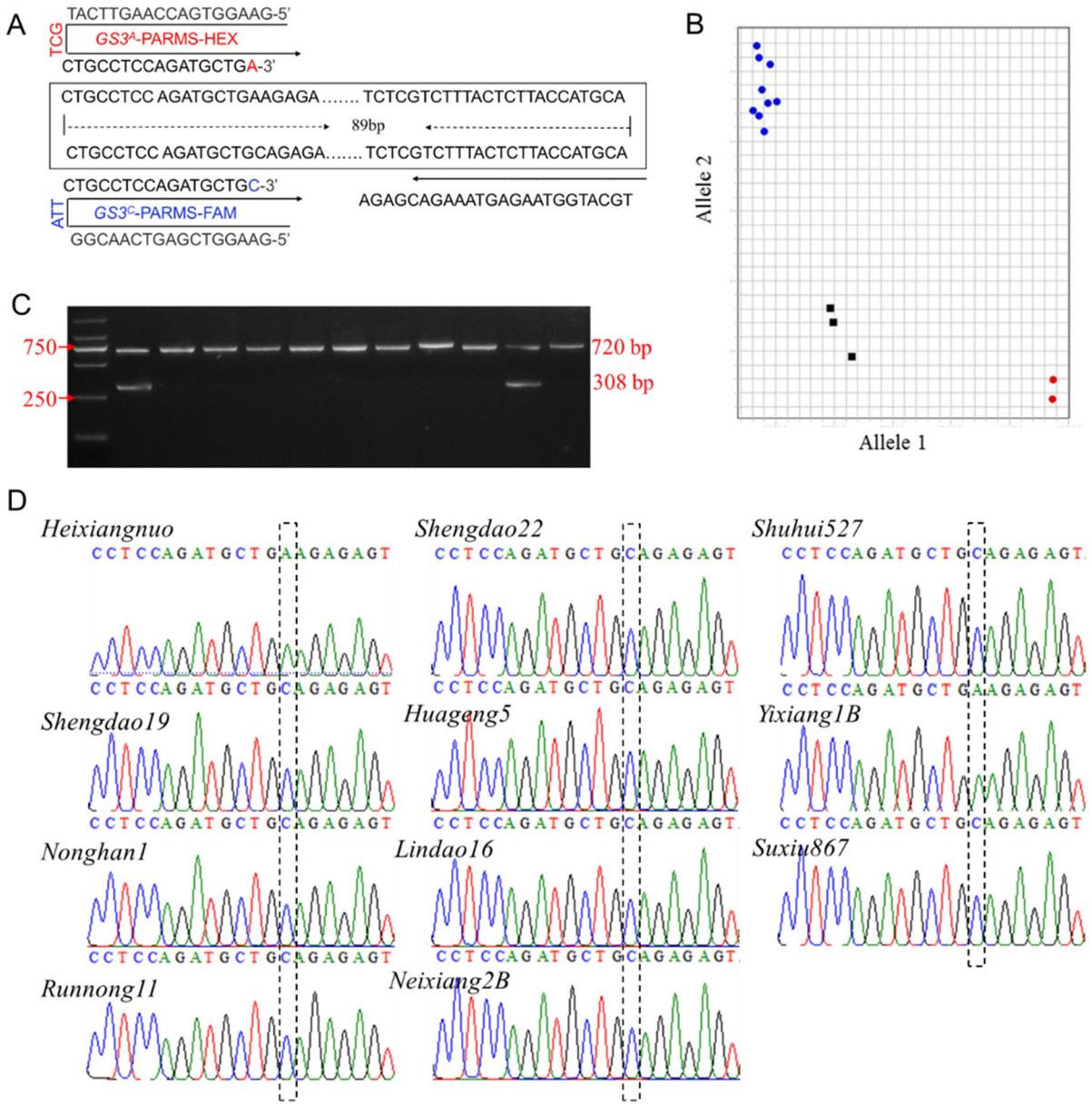

Fig. 4 The PARMS marker development and validation for *GS3*. **A** the schematic diagram of design principle about *GS3* PARMS primers; **B** The allelic discrimination plot of *GS3*. The homozygous Allele1 (red dots) indicated $GS3^A$ type, Allele2 (blue dots) indicated $GS3^C$ type. If fluorescent green dots are presented, these are heterozygous. The black dots indicated blank control; **C** the Tetra-primer ARMS marker (Wu et al. 2019). The electrophoretic bands of 720 bp and 308 bp indicate $GS3^A$ and $GS3^C$ type. Here are *Heixiangnuo*, *Shengdao19*, *Nonghan1*, *Runnong11*, *Shengdao22*, *Huageng5*, *Lindao16*, *Neixiang2B*, *Shuhui527*, *Yixiang1B* and *Suxiu867* from left to right; **D** The sequencing validation of natural variation

the $GS3^A$ allele on grain length was minimal in in GJ accessions (Fig. 2D, E). Why does $GS3^A$ alleles function differentially in regulating grain length between XI and GJ rice? As shown in the previous study, GS3, although having no direct function in promoting or inhibiting grain length, regulates grain length by blocking the interaction of DEP1 (Dense panicle 1) and GGC2 (G protein gamma subunit) with RGB1 (G protein beta subunit) (Sun et al. 2018). The GS3 and DEP1 proteins interact directly with the conserved

keratin-like domain of MADS transcription factors, and function as cofactors to enhance OsMADS1 (MADS-domain transcription factor) transcriptional activity, thereby regulating grain size (Liu et al. 2018). In addition, GL3.3 (Grain length and weight) protein interacts with GS3 and leads to extra-long grains in rice (Xia et al. 2018). The net outcome of the functional interactions among these genes thus determines grain size (Sun et al. 2018). Considering multiple factors interacting with GS3, we speculate that factors acting downstream of GS3 probably differentially function to regulate grain length between XI and GJ rice. In addition, it has been reported that grain size is determined by a complex regulatory network (Ren et al. 2023). It is possible that other regulatory pathways determining grain length have antagonistic effects with $GS3^A$ pathway on grain length in GJ accessions. However, our results suggest that introduction of *GS3* alleles would be directly useful to modify grain length in XI breeding programs, but would be of doubtful value in GJ breeding programs. Further research will be needed to understand the biological functions of GS3 more fully.

Third, numerous studies have identified the *GS3* gene as being associated with grain weight (Fan et al. 2006; Takano-Kai et al. 2009; Yan et al. 2011), which is different from our results in this study. We found the association between *GS3* alleles and seed weight was not significant in any of the individual subpopulations (Fig. 3; Table 4). Considering that grain weight is affected by grain length, grain width, grain thickness and grain filling, we speculate that it might be difficult to increase rice yield by genetic improvement using *GS3* alleles. Finally, the intragenic marker for the *GS3* gene that we developed in this study could be of great use in evaluating the genotype of germplasm accessions and guiding the improvement of grain length in XI cultivars in the future (Fig. 4).

Conclusion

We identified *GS3* alleles and analyzed the distribution of *GS3* in 81 XI and 222 GJ accessions. The results showed that *GS3* was selected independently of subspecies and the distribution was broadly in keeping with the phenotype of grain length, in that long and slender grain is prevalent in XI rice and short round grain in GJ cultivars. Perhaps $GS3^A$ allele was the result of later artificial selection in indica rice breeding in China. The average grain lengths of the 59 accessions carrying $GS3^A$ were significantly longer than that 22 accessions that carried $GS3^C$ in XI accessions, however, there was no significant difference in average grain length between $GS3^C$ and $GS3^A$ alleles in GJ accessions. The functional effect of the *GS3* alleles on grain length was significant in XI but unproductive in GJ accessions. For the grain weight, there was no significant difference in average weight between $GS3^A$ and $GS3^C$, in either XI or in GJ. To develop an effective marker for *GS3*, a PARMS marker was designed, which would entail lower cost and take less time than would other available markers. Taken together, the analysis of *GS3* alleles helps gain insight into the molecular mechanisms involved in grain size in rice, and $GS3^A$ alleles in XI accessions may provide a new opportunity to improve rice grain size breeding.

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Declarations

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

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