FLOURY ENDOSPERM19 encoding a class I glutamine amidotransferase affects grain quality in rice

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Research Article

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Abstract

As a staple food for more than half of the world's population, the importance of rice is self-evident. Compared with ordinary rice, rice cultivars with superior eating quality and appearance quality are more popular with consumers due to its unique taste and ornamental value, even if their price is much higher. Appearance quality and CEQ (cooking and eating quality) are two very important aspects in the evaluation of rice quality. Here, we performed a genome-wide association study on chalkiness rate in a diverse panel of 533 cultivated rice accessions. We identified a batch of potential chalky genes and prioritize one (*LOC_Os03g48060*) for functional analyses. Two floury outer endosperm mutants (*flo19-1* and *flo19-2*) were generated through editing *LOC_Os03g48060* (named as *FLO19* in this study), which encodes a class I glutamine amidotransferase. The different performance of the two mutants in various storage substances directly led to completely different changes in CEQ. The mutation of *FLO19* gene caused the damage of carbon and nitrogen metabolism in rice, which affected the normal growth and development of rice, including decreased plant height and yield loss by decreased grain filling rate. Through haplotype analysis, we identified a haplotype of *FLO19* that can improve both CEQ and appearance quality of rice, Hap2, which provides a selection target for rice quality improvement, especially for high-yield *indica* rice varieties.

Introduction

As one of the three staple foods in the world, the yield and quality of rice have always been the focus of scientists and breeders (Liu et al. 2018). After the tide of green revolution, rice yield has been greatly improved (Li et al. 2018), but the development of quality breeding was relatively stunted (Zhang et al. 2020a). The CEQ and appearance quality of rice directly determine its market price and consumer preference. Therefore, it is urgent for breeders to improve the quality of rice, whether it is to increase farmers' income or meet consumers' higher demand, and the prerequisite for this is that there are abundant genetic resources available for the improvement of rice quality.

Starch and protein, as the two most important components in endosperm, are also the two most important factors affecting the CEQ (Hori et al. 2016). Amylose (AC), gel consistency (GC), and gelatinization temperature (GT) are three main indicators for physical and chemical properties of the starch in rice endosperm (Sun et al. 2006), which directly affected cooking and eating quality. Since *Wx* gene was responsible for the synthesis of amylose (AC), and gel consistency (GC) was negatively correlated with amylose (AC), both GC and AC were controlled by *Wx* (Zhang et al. 2020a). The synthesis of amylopectin is more complex than amylose, which mainly involves soluble starch synthase (SSS), starch branching enzyme (SBE) and starch debranching enzyme (DBE) (Yang et al. 2018a). The gelatinization temperature (GC) was mainly controlled by *ALK* gene, which encodes SSSII-3 (Gao et al. 2011). The base substitutions in *ALK* cause amino acid changes in SSSII-3 and result in the alteration of enzymatic activity, and thereafter alter the fine structure of crystalline lamellae of amylopectin, and are finally reflected in GT (Gao et al. 2011). So far, most of the researches on CEQ have focused on the abundant *Wx* allelic variations and *ALK* gene (Zhang et al. 2019; Zhang et al. 2020b; Hori et al. 2021;

Zhou et al. 2020), or the interaction between them(Yang et al. 2018a). Protein content is also an important factor affecting CEQ. Previous reports have revealed a significantly negative correlation between the protein content and CEQ (Okadome 2005; Martin and Fitzgerald 2002). Although many QTLs affecting protein content have been reported (Yano et al. 2016; Bhattarai and Subudhi 2018; Kashiwagi and Munakata 2018; Zhao et al. 2011), only *OsAAP6* (Peng et al. 2014)and *OsGluA2* (Yang et al. 2019), both are positive regulators of protein content, have been cloned by forward genetic methods so far. *Chalk5* (Li et al. 2014), a major chalky gene previously reported, is a negative regulator of protein content. These genes regulate protein content and also affect amylose and gel consistency (Li et al. 2014; Peng et al. 2014; Yang et al. 2019). Floury mutants are valuable genetic resources for dissecting the synthesis mechanisms of storage substances. So far, many genes responsible for floury phenotype have been identified. Some genes are directly involved in starch synthesis and storage protein transport (including *OsAPL2* (Wei et al. 2016), *FLO5* (Ryoo et al. 2007), *OsBEIIb* (Tanaka et al. 2004), *OsRab5a* (Wang et al. 2010), *OsVPS9a* (Liu et al. 2013), *GPA3* (Ren et al. 2014), *GOT1B* (Wang et al. 2016), *GPA5* (Ren et al. 2020)). Several transcription factors regulate the expression of starch or protein synthesis-related genes (including *RISBZ1*/ *OsbZIP58* (Wang et al. 2013), *RPBF* (Kawakatsu et al. 2009), *bHLH144* (Bello et al. 2018), *RSR1* (Fu and Xue 2010)), and there are more genes that indirectly affect starch and protein (including *PPR5* (Zhang et al. 2020c), *FLO8* (Long et al. 2017), *FLO16* (Teng et al. 2019), *FLO14* (Xue et al. 2019), *FLO15* (You et al. 2019), *FLO18* (Yu et al. 2020), etc.).

Chalkiness is a very important evaluation criterion for the appearance quality of rice, and it is also a highly undesirable trait for human food, due to its negative impact on CEQ and apperrance quality (Li et al. 2014). In the past 20 years, only *Chalk5* (Li et al. 2014) has been cloned from natural variants, while many more chalky genes have been identified from mutants, including *FLO2* (Wu et al. 2014), *FLO4* (Kang et al. 2005), *FLO5* (Ryoo et al. 2007), *FLO6* (Cheng et al. 2014) and *FLO7* (Zhang et al. 2015).

Transglutaminases (protein-glutamine c-glutamyl transferase, EC 2.3.2.13, TG) are a class of enzymes catalyzing an acyl-transfer reaction between the γ-carboxamide group of a peptide-bound glutamine and the ε-amino group of a lysine (Büttner et al. 2011). TGs were found to be widely distributed in microorganisms, plants, invertebrates, amphibians, fish and birds (Beninati and Piacentini 2004). Although microbial transglutaminase has been widely used in food (Kieliszek and Misiewicz 2014), the transglutaminase genes from animals and plants are rarely studied (Beninati and Piacentini 2004), especially in plants, only one gene *GAT1_2.1 gene* (*At1g15040*) has been explored in *Arabidopsis thaliana* (Zhu and Kranz 2012), without any related report on transglutaminase genes in rice.

In this study, we carried out GWAS with a population consisting of 533 rice varieties and further functional analysis of one predicted chalky gene *FLO19*. We showed that *FLO19* gene plays an important role in regulating rice quality and maintaining normal carbon and nitrogen metabolism in rice. Haplotype analysis provides a valuable elite haplotype of *FLO19* gene for breeders seeking to improve the quality of high-yield indica rice varieties.

Materials And Methods

Plant materials and growth conditions

Rice plants were grown under natural field conditions at the Experimental Stations of Huazhong Agricultural University, Wuhan and Hainan, China. Plants were separated by 16.5 cm in each row, and rows were 26 cm apart. Field management followed normal agricultural practices.

Vector construction and transformation

The CRISPR/Cas9 system was used to generate knock-out mutants (Wang et al. 2015). The target sgRNAs GGGCCGATCGAAGAAGGGCT for *FLO19* (LOC_Os03g48060) was inserted into intermediate vectors pER8-Cas9-U6 or pER8-Cas9-U3 and finally cloned them into pCXUN-Cas9. The correct construct confirmed by sequencing was introduced to *Agrobacterium tumefaciens* EHA105 and transformed into rice cultivar ZH11. A pair of specific SSR primer D5 was used to confirm T_0 transgenic-positive plants. DC5-F/DC5-R was applied to amplify the target sites, which were sequenced to validate the mutation in T_0 and further confirmed in the T_1 generation. The relevant primers are listed in Table S1.

Microscopy

For scanning electron microscopy, brown rice grains were transversely broken by two forceps, coated with gold under vacuum conditions, and examined with a scanning electron microscope (JSM-6390LV, JEOL) at an accelerating voltage of 10 kV and a spot size of 30 nm. Scanning electron microscopy analysis was based on at least three biological replications of the mounted specimens. All procedures were carried out according to the manufacturer's protocol.

For transmission electron microscopy, the sections of endosperm at 15 DAF were examined with a transmission electron microscope (H-7650, HITACHI).

Measurement of various quality traits

The determination of four main storage proteins, amylose content, alkali spreading value, gel consistency and brown rice protein content was performed as previously described (Chen et al. 2018; Tan et al. 1999; Mariotti et al. 2010; Wu et al. 2020). Content of sucrose, glucose and fructose was detected using a kit purchased from Grace Biotechnology Company (Suzhou). Developing seeds were freshly harvested at 7 days after flowering. After removing the glumes, husks and embryos, the endosperms were ground into powder in liquid nitrogen. The powder was placed in a 1.5-mL centrifuge tube and analyzed according to the manufacturer's instructions.

Hydroponic culture assay and enzyme activity assay

A standard rice culture solution for hydroponic experiments was used as previously described (Yang et al. 2018b). 0N, 0.5N, 1N, 2N and 4N represent nitrogen-deficient, 0.5-fold nitrogen, 1-fold nitrogen, 2-fold nitrogen and 4-fold nitrogen nutrient solution, respectively. The length of shoot and root was determined using ImageJ software (US National Institutes of Health). Plant tissues were immediately frozen in liquid

nitrogen after sampling and stored at -80 ℃ before use. Activities of glutamine synthase, glutamate synthase, asparagine synthetase and glutamine amidotransferase were determined using kits purchased from Grace Biotechnology Company (Suzhou), and analyzed according to the manufacturer's instructions.

RNA preparation and qRT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's instructions from flag leaf, root, sheath, stem, young panicle, pulvinus, seedlings and endosperm at 7 DAF of ZH11 and *flo19* mutants. qRT-PCR assay was performed as previously described (Zhou et al. 2020). The relevant primers are listed in Table S1.

Haplotype analysis

The SNP and InDel variation data for *FLO19* (*LOC_Os03g48060*) in all 533 accessions are available at RiceVarMap (http://ricevarmap.ncpgr.cn/). Subpopulation identities were inferred using ADMIXTURE (Alexander et al. 2009), and were also queried from RiceVarMap.

Genome-wide association analyses

GWAS analysis was performed as previously described (Zhou et al. 2017).

Statistical analysis

Statistical analyses were performed with SPSS 16.0 and Microsoft Excel 2016.

Results

Artificial creation of the flo19 mutants

In order to reveal the genetic basis of grain chalkiness in rice, we evaluated chalkiness rate of 533 *Oryza sativa* accessions, which contains worldwide landraces and elite varieties (Xie et al. 2015). Chalkiness rate displayed continuous and extensive variation, ranging from 1.1%-99.6% in the whole population (Fig. 1a, Data S1). The *indica* subgroup had the highest chalkiness rate and the *aus* subgroup had the lowest chalkiness rate, whereas the *japonica* subgroup had the intermediate value (Fig. 1b). Subsequently, we performed a genome-wide association study on chalkiness rate using 6.5 million SNPs characterized in the whole population, and detected 343 significant loci on 12 chromosomes (Fig. S1, Data S2). In this study, we focused our attention on the loci on chromosome 3. A total of 28 genes were annotated in the candidate region from the reference genome of Nipponbare (Data S2). Among those genes, *LOC_Os03g48060* encoding the class I glutamine amidotransferase was located at 70 kb downstream of the peak SNP (Data S2). To investigate the function of *LOC_Os03g48060*, we produced two mutants using a CRISPR/Cas9 vector targeting the second exon (Fig. 1c), of which both showed

consistent floury endosperm phenotype (Fig. 1d). Therefore, *LOC_Os03g48060* was named as *FLO19* and the two mutants were marked as *flo19-1* and *flo19-2*, respectively.

Phenotypic characterization of the flo19 mutants

Compared with wild ZH11, the endosperm of the two mutants was opaque and white (Fig. 1d). Interestingly, cross-section analysis showed that the peripheral region of *flo19* mutant grain appeared floury-white, while the inner endosperm was translucent, as in wild-type endosperm (Fig. 1e). Scanning electron microscope analysis revealed that the outer endosperm cells of *flo19* mutant grain were packed with loosely arranged composite starch granules, which was quite different from the dense irregular polyhedral starch granules in wild-type ZH11 (Fig. 1f, g). To better dissect developmental defects in the endosperm of *flo19* mutants, sections made from endosperm at 15 DAF (15 days after flowering) were used for transmission electron microscopy analysis. The amyloplasts in peripheral endosperm cells of ZH11 were filled with regular polyhedral starch granules, while the amyloplasts in *flo19* mutant showed a fragmented state and starch granules were scattered in the matrix between the amyloplasts (Fig. 1h, i).

Both the two mutants showed reduced height (Fig. 2a, b), but no difference on tiller number and grain number per spike, relative to wild ZH11 (Fig. S2a, b). By monitoring the dynamic filling process of ZH11 and mutants, we found that the endosperms of the mutants were flatter than ZH11 from 11 DAF, and this difference in fullness became more and more obvious with the passage of time (Fig. 2c). Correspondingly, the filling rate of *flo19* mutants was gradually lower than that of ZH11 from 7 DAF with the difference reached the maximum at 23 DAF, and then decreased slightly (Fig. 2d), which finally resulted in the decrease of 1000 grain weight (Fig. 2e). Both the two mutants showed reduced grain thickness, while only *flo19-2* showed increased grain length (Fig. 2f). In addition, TTC staining assays showed that the seed viability of *flo19-1* was significantly lower than that of the control, while *flo19-2* was not affected (Fig. S3a, b).

Effect of FLO19 gene mutation on rice quality

The occurrence of floury endosperm phenotype is often closely related to the changes of storage substances in endosperm. Next, we evaluated the content of starch and protein, the two major components in grain endosperm. Starch consists of two members, amylose and amylopectin. Due to the lack of a reliable method to evaluate amylopectin content, only the amylose content was measured. Compared with ZH11, *flo19-1* showed a similar value of amylose content, while *flo19-2* displayed a significantly lower value (Fig. 3a). In order to reflect the change of starch more accurately, the contents of three sugars, sucrose, glucose and fructose, were measured. Compared with ZH11, *flo19-1* showed significantly lower values in all three sugars, while *flo19-2* only had higher value in fructose (Fig. S4a-c). The majority of protein in rice endosperm is storage protein, including glutelin, prolamin, globulin and albumin. Compared with ZH11, *flo19-1* showed a significantly higher value in glutelin, but lower values in prolamin and albumin (Fig. S4d). In contrast, *flo19-2* displayed lower values in prolamin, albumin and globulin, but not in glutelin (Fig. S4d). The total amount of storage protein in *flo19-1* seeds increased significantly while that in *flo19-2* seeds remained basically unchanged (Fig. S4d).

To determine whether changes in starch and grain storage proteins accumulation were reflected by altered messenger RNA levels, we tested the expression of 81 key genes involved in grain storage materials (Peng et al. 2014). Compared with ZH11, the expression levels of 39 genes related to starch biosynthesis and metabolism in mutant *flo19* changed: 16 genes were down-regulated and 23 genes were up-regulated in *flo19-1*, while 19 genes were down-regulated and 20 genes were up-regulated in *flo19-2* (Fig. 3b). The expression levels of 17 genes related to storage protein biosynthesis and metabolism changed: 4 genes were down-regulated and 13 genes were up-regulated in both *flo19-1* and *flo19-2* (Fig. 3c).

The changes of storage substances in endosperm are likely to affect cooking and eating quality. Thus, we examined the gelatinization properties of milled rice flour. Rice flours from *flo19-2* yielded a significantly better RVA curve pattern than that from ZH11 and *flo19-1*, reflected by a significantly higher breakdown value (Fig. 3d). Similarly, *flo19-2* had a higher value than ZH11 and *flo19-1* in gel consistency (Fig. 3e). In addition, there was no difference in gelatinization temperature between ZH11 and *flo19* mutants (Fig. S4e).

Possible causes of phenotypic differences between mutants and phylogenetic tree analysis

The fact that editing the same gene produces rice lines with different cooking and eating qualities is an interesting point worthy of further exploration. We next examined temporal and spatial expression patterns of *FLO19* using eight tissues from ZH11 and *flo19* mutants (Fig. 4a). *FLO19* was expressed constitutively in all examined tissues and showed the highest expression in the root (Fig. S5). The expression level of *FLO19* was abnormally higher in the root and stem of *flo19-1*, and lower in other tissues than that of ZH11 (Fig. 4a). Similarly, higher expression level of *FLO19* was only observed in the root and seedling of *flo19-2* (Fig. 4a).

Comparative sequencing revealed that the allele from *flo19-1* had a 1-bp deletion and that from *flo19-2* had a 2-bp deletion (Fig. 1c), and these mutations directly led to varying degrees of shifting mutations and premature termination of the *FLO19* coding products (Fig. S6). The normal *FLO19* gene was predicted to encode a protein consisting of 293 amino acids with a GATase domain, a HTS domain, a Peptidase_C26 domain and two unknown-function LCR domains (http://smart.embl-heidelberg.de) (Fig. S7). It is worth noting that both of the mutated FLO19 proteins lost HTS domain and Peptidase_C26 domain, and the FLO19 protein of *flo19-1* mutant even lost the LCR domain in the C-terminal (Fig. S7). Considering the phenotypic differences between the two mutants, we hypothesized that the LCR domain in the C-terminal may be critical for FLO19 protein function in CEQ regulation.

In order to further understand the phylogenetic relationship between FLO19-related proteins in plants and eukaryotes, we searched and compared the predicted protein sequences of 13 different phylogenetic organisms. Based on the phylogenetic analysis, *FLO19* seems to be a green-plant-unique gene and rice *FLO19* may be an evolutionary product of fungi *FLO19* (Fig. S8). However, these genes have not been functionally identified even in the model plant *Arabidopsis thaliana*.

The effect of FLO19 on carbon and nitrogen metabolism

The *FLO19* gene was predicted to encode a class I glutamine amidotransferase (GAT1), and showed the highest expression in the root (Fig. S5), implying that this gene may be involved in the nitrogen assimilation process of rice. Thus, we conducted a two-week hydroponic nitrogen treatment with different nitrogen level for *flo19* mutants and ZH11 in the greenhouse. In contrast to ZH11, both mutants showed decreased shoot length accompanied by an increase in root length, and this change could not be rescued by increasing nitrogen supply levels (Fig. 4b-d). Subsequently, we determined the activities of key nitrogen assimilation-related enzymes and glutamine amidotransferase for seedlings. Interestingly, just as the two mutants of *flo19* differ in quality traits, the two mutants also differ in enzyme activities. Compared with ZH11, the enzyme activity of glutamine synthase (GS) in *flo19-1* showed no difference in shoot under all nitrogen conditions except for 0N nitrogen level, but was significantly reduced in root (Fig. 4e, f). In contrast, the activity of GS in *flo19-2* was significantly lower in both shoot and root except for 2N in the shoot (Fig. 4e, f). The glutamate synthase (GOGAT) activity of *flo19-1* was significantly lower under all nitrogen conditions in both shoot and root, while it was just the opposite in *flo19-2* (Fig. 4g, h). The asparagine synthetase (AS) activity of the two *flo19* mutants was higher than that of ZH11 in shoot, with the exception of 0N and 4N nitrogen levels (Fig. S9a). Meanwhile, the AS activity of *flo19-2* was only different from ZH11 under partial nitrogen conditions (0N, 1N and 2N) in the root, while no significant difference was observed in *flo19-1* under all nitrogen conditions (Fig. S9b). Glutamine amidotransferase (GAT) activity result showed that *flo19-1* had no difference with ZH11 under almost all nitrogen levels in the shoot except 0.5N nitrogen level, while *flo19-2* had lower values under almost all nitrogen levels except 2N nitrogen condition (Fig. 4i). Meanwhile, *flo19-1* showed no difference under almost all nitrogen conditions in the root except 1N nitrogen level, and conversely *flo19-2* exhibited lower activity under almost all nitrogen conditions, except 4N nitrogen level (Fig. 4j). The above results indicated that the nitrogen assimilation process in *flo19-1* was obviously suppressed, while the inhibition of nitrogen assimilation in *flo19-2* seemed to be improved to some extent.

Carbon and nitrogen metabolism is crucial for plant growth and development. Thus, we also determined the activity of Rubisco (Ribulose bisphosphate carboxylase oxygenase), a key enzyme for CO_2 assimilation into the biosphere, in *flo19* and ZH11 seedlings. Compared with the wild-type ZH11, Rubisco activities in these two *flo19* mutants were significantly reduced (Fig. S10a), demonstrating that the *FLO19* gene mutation caused damage to the carbon assimilation of rice. As an important metabolic intermediate, the concentration of acetyl-CoA not only reflects the general energy state of cells (Shurubor et al. 2017), but also affects the specificity and activity of a variety of enzymes (Pietrocola et al. 2015). The concentration of acetyl-CoA in *flo19-1* seedlings varied with nitrogen supply levels, which seemed to be irregular, while *flo19-2* showed significantly higher acetyl-CoA levels at all nitrogen levels (Fig. S10b). This indicated that the energy metabolism disorder in the *flo19* mutants or the metabolic synthesis of organic matter was blocked, thus the normal growth and development was disturbed.

Haplotype analysis of FLO19

In order to investigate natural variations in *FLO19*, we analyzed variations in the coding region of *FLO19* from the association population, and classified it into 11 haplotypes, of which Hap 1–4 were the main haplotypes (Fig. 5a, b and Data S1) and were used for further analysis. Hap 2 was mainly distributed in *japonica* rice subgroup, while the other three were primarily distributed in *indica* rice subgroup. Based on the phenotypic data of 533 varieties investigated previously(Chen et al. 2018; Zhou et al. 2020) and recently (Data S1), we conducted a comprehensive analysis of quality and yield traits of these four haplotypes. Notably, Hap2 had the lowest chalkiness rate, amylose content and gelatinization temperature, whereas no significant differences in chalkiness rate and gelatinization temperature were found between Hap2 and Hap4 (Fig. 5c, e). For protein content, there was no statistical difference among the four haplotypes (Fig. S11a). Hap2 had higher values in gel consistency and taste score than the remaining three haplotypes (Fig. 5f, g). Although Hap2 had the highest 1000-grain weight, its spikelet number, seed setting rate, and yield were similar to or lower than those of the other three haplotypes, and there was no difference in heading dates among the four haplotypes (Fig. 5h and Fig. S11b-e).

Discussion

Rice endosperm is known to be an excellent system for elucidating how gene networks regulate starch synthesis and amyloplast development (Nelson and Pan 2003; Satoh and Omura 1981), so various mutants with defective endosperm have been screened for further research. In terms of appearance, floury endosperm mutants can generally be divided into two types: completely floury endosperm mutant (such as *FLO13* (Hu et al. 2018), *FLO14* (Xue et al. 2019), *FLO16* (Teng et al. 2019) and *FLO18* (Yu et al. 2020)) and partially floury endosperm mutant (such as *FLO4* (Kang et al. 2005), *FLO7* (Zhang et al. 2015)and *FLO15* (You et al. 2019)). Interestingly, similar to previously reported *flo7* mutant, the peripheral region of *flo19* mutant grain also appeared floury-white (Fig. 1e). Differently, a growth arrest of amyloplasts occurred in the *flo7* mutant, but at least the shape of amyloplasts was intact, unlike the broken amyloplasts in *flo19* mutant (Fig. 1h, i), suggesting that the damage caused by *FLO19* gene mutation may be more serious. Notably, the expression level of *FLO7* was the highest in the developing endosperm, while *FLO19* was the highest in the root (Fig. S5), indicating that these two genes had different mechanisms of action affecting quality traits. The qRT-PCR results showed that among the detected genes related to starch and protein synthesis, these five genes (*TPS*, *MAPK4*, *Wx*, *ABP*, and *DRP*) had the most significant changes in expression (Fig. 3b, c). Except for the *Wx* gene encoding granule-bound starch synthase (GBSS), which directly controls the synthesis of amylose, the remaining four are not directly involved in the synthesis of starch or protein. Although two mutants, *flo19-1* and *flo19-2*, were produced by editing the same gene, the characterization results of various phenotypes were different. *flo19-1* significantly increased storage protein content and reduced all three sugar contents with no difference observed in amylose content, while *flo19-2* decreased amylose content and remarkably improved fructose content without difference in protein, sucrose and glucose content (Fig. 3a and Fig. S4a-d). An increase in fructose content and a decrease in amylose content of *flo19-2* mutant may indicate that the conversion from sugar to starch is impeded. The decrease of three sugar contents in *flo19-1* mutant may be related to the transformation between sugar and protein. These differences in

biochemical parameters also led to the differences in gel consistency and gelatinization characteristics between the two mutants (Fig. 3d, e).

The key nitrogen assimilation enzyme glutamate synthase (GOGAT) displayed significantly lower activity in *flo19-1* than that in ZH11, under all nitrogen concentrations in both shoots and roots, while it was just opposite in *flo19-2* (Fig. 4g, h). The activity of glutamine synthase (GS), another key enzyme for nitrogen assimilation, was significantly inhibited in shoots and roots of both *flo19* mutants (Fig. 4e, f). Although there was no statistically significant difference in the shoot of *flo19-1* mutant, the values were lower than those of ZH11 at almost all nitrogen concentrations except 0.5N nitrogen concentration (Fig. 4e). This may indicate that the main process of nitrogen assimilation in *flo19-1* mutant was completely inhibited, while in *flo19-2* mutant, although the enzyme activity of GS was also inhibited, the improved enzyme activity of GOGAT possibly alleviate the negative effect of suppressed nitrogen assimilation to some extent, which can be reflected in the differences in quality traits (Fig. 3a, d, e and Fig. S4), plant height (Fig. 2a, b) and seed vigor (Fig. S3a, b) between the two mutants.

In essence, the differences in editing sites result in completely different coding products of the *FLO19* gene in these two mutants. Compared with ZH11, the FLO19 protein in the *flo19-1* mutant terminated prematurely after changing five amino acids, while the FLO19 protein in the *flo19-2* mutant continued to translate a fragment containing 35 aa from the mutation site (Fig. S6). It was this extra amino acid sequence that allowed the mutated FLO19 protein to regain an LCR domain (although it may not be exactly the same as the original) (Fig. S7), which we hypothesized might be responsible for the functional differences between the two proteins. But the significant decrease of GAT activity in *flo19-2* seemed to argue that the newly generated LCR domain has caused more serious consequences (Fig. 4i, j), certainly more assays are needed to prove it.

Indica usually has higher nitrogen uptake capacity and nitrogen use efficiency than *japonica* (Hu et al. 2015; Islam et al. 2021), thus the yield per plant of *japonica* is often lower than that of *indica*. Haplotype analysis revealed that Hap2 could improve both appearance quality and CEQ of rice, but the yield per plant of Hap2 was also the lowest among the four main haplotypes (Fig. 5h). Considering that Hap2 mainly exists in *japonica* rice varieties, while the other three haplotypes mainly exist in *indica* rice varieties (Fig. 5b and Data S1), the disadvantage of Hap2 in yield is more likely to be caused by the differentiation of *indica-japonica* subspecies than the *FLO19* gene itself. In consequence, we advocate haplotype Hap2 as having great potential for rice quality improvement, especially for high-yield *indica* rice varieties.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The variation information of 533 rice accessions can be obtained through the website RiceVarMap (http://ricevarmap.ncpgr.cn/). The phenotypic data of 533 rice accessions can be obtained from the references mentioned in the main text or the Supplementary Data part of this study. For materials, please contact the corresponding author's email address.

Competing interests

Authors declare that there are no conflicts of interest.

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Author contributions

Guangming Lou and Pingli Chen performed most of the experiments; Jiawang Xiong, Shanshan Wan, Yuanyuan Zheng, Mufid Alam, Rongjia Liu, Yin Zhou, Hanyuan Yang, Yahong Tian, Jingjing Bai, Wenting Rao, Xuan Tan and Haozhou Gao participated in part of phenotyping, genotyping and biochemical experiments; Guangming Lou, Pingli Chen and Hao Zhou performed various data analysis; Guanjun Gao and Qinglu Zhang participated in partial field experiments. Hao Zhou, Yanhua Li, Xianghua Li and Chuanguang Liu provided guidance for the determination of partial quality traits. Guangming Lou, Pingli Chen and Yuqing He designed experiments. Guangming Lou wrote the manuscript and Pingbo Li improved it. All authors discussed and commented on the manuscript.

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Figures

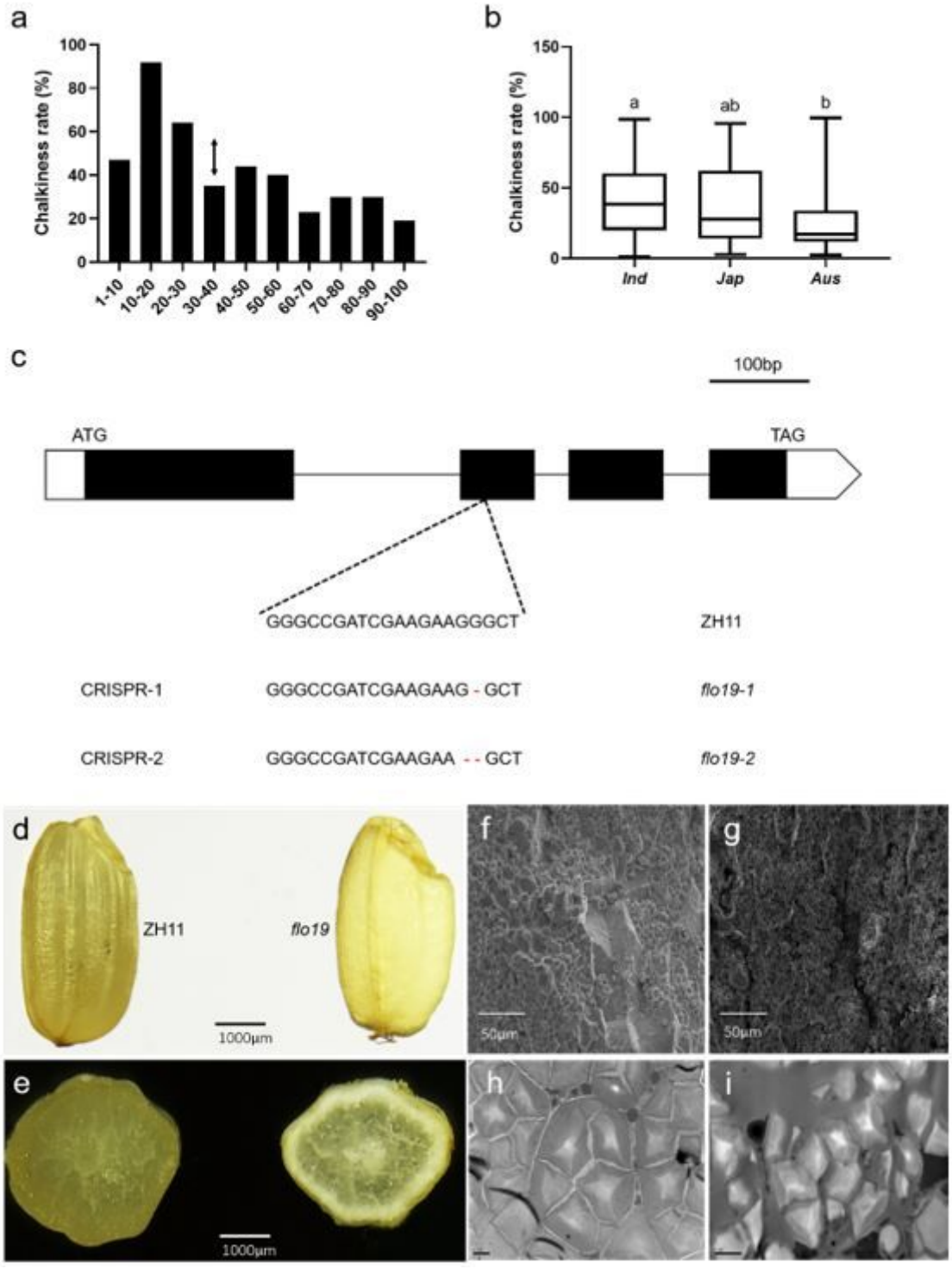

Figure 1

Distribution of chalkiness rate and CRISPR/Cas9–induced flo19 mutants. (a) Phenotypic distribution of chalkiness rate of milled rice in the whole population. Double sided arrowhead indicates mean value. (b) Comparison of chalkiness rates among different subgroups. (c) Sequence alignment of the sgRNA target region. The short red lines represent the missing bases. (d, e) Appearance and transverse sections of representative wild-type ZH11 and flo19 mutant dry seeds. Scale bars, 1000 μm. Scanning electron microscopy images of outer endosperm of the wild-type ZH11 (f) and flo19 mutant (g) grains. Scale bars,

50 μm. Transmission electron microscope analysis of the compound starch granules of the wild type ZH11 (h) and flo19 mutant (i) at 15 DAF. Scale bars, 2 μm.

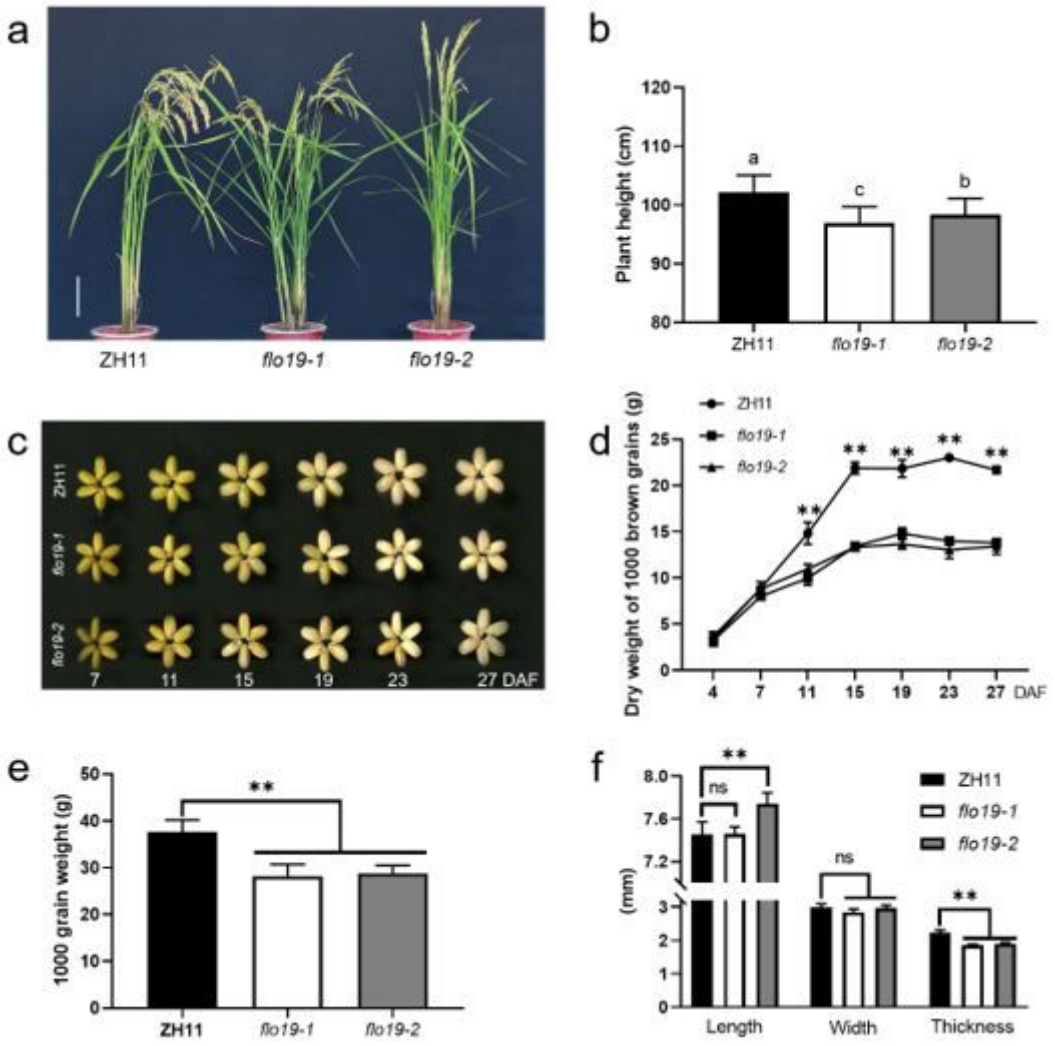

Figure 2

Phenotypic analysis of the flo19 mutant. (a) Comparison of plant architecture between ZH11 and mutants. Scale bars, 10 cm. (b) Plant height statistics of ZH11 and mutants ($n \geq 41$). Different letters represent significant differences at $P < 0.05$, Duncan's multiple range test. (c) Endosperm of ZH11 and flo19 mutants at different filling stages. (d) The accumulation of dry weight of ZH11 and flo19 mutants at dynamic grain filling stage. Asterisks indicate statistical significance compared with the wild-type (n=3). (e) 1000 grain weight of two mutants and ZH11. (f) Measurement of seed length, seed width, and seed thickness of ZH11 and flo19 mutant grains.

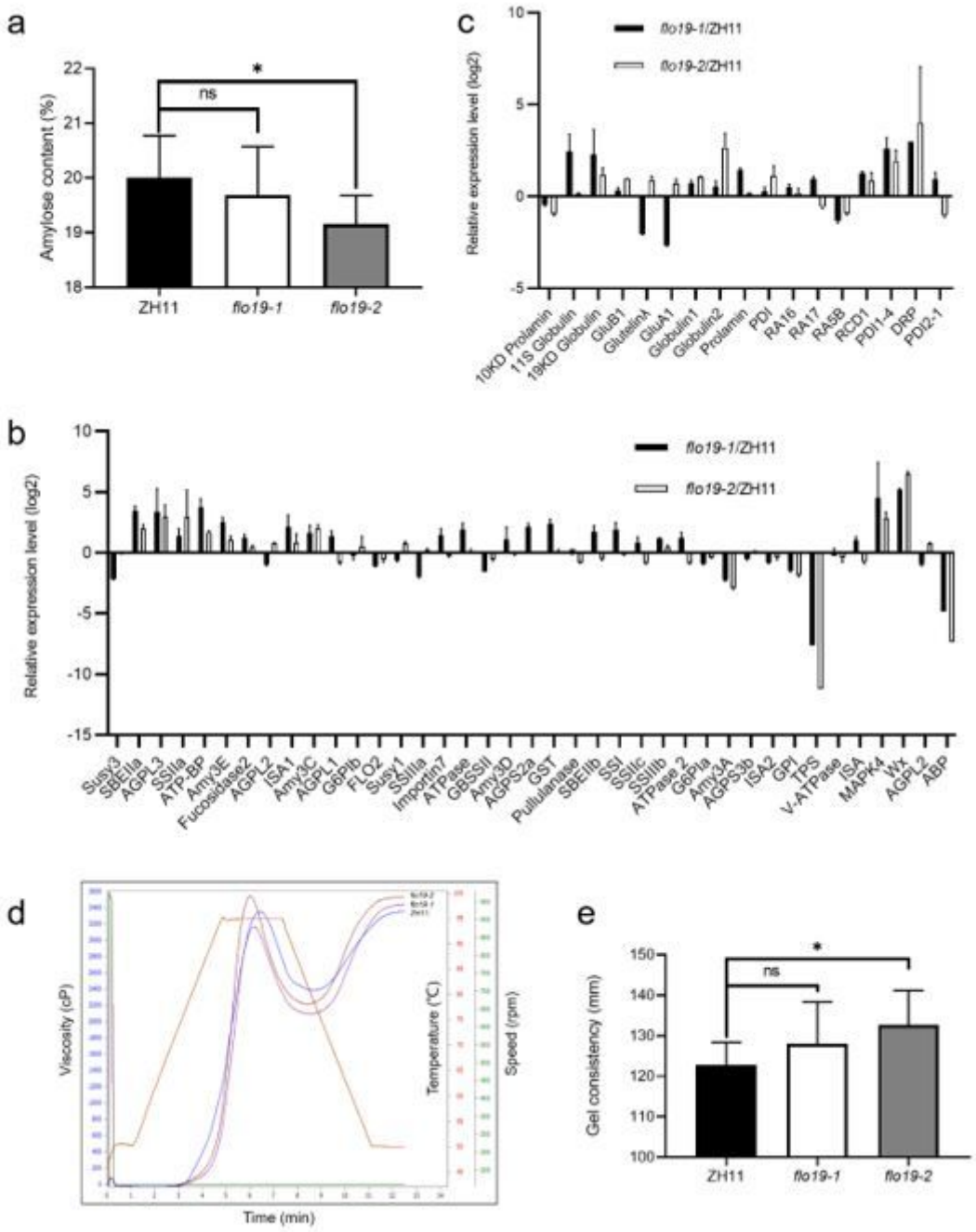

Figure 3

Effect of FLO19 on quality traits. (a) Amylose content in milled rice flour (n=10). (b) Expression levels of 39 genes involved in synthesis of storage starch in endosperm of flo19 mutant lines. (c) Expression levels of 17 genes involved in synthesis of storage protein in endosperm of flo19 mutant lines. (d) RVA profile. (e) Gel consistency (n=10). Data are means ± SD (Student's t-tests, *, $P < 0.05$; ns, no significant difference). In (b) and (c), only genes with significant differences are shown here. Expression levels were determined by qRT-PCR using RNA samples from endosperm at 7 DAF with three biological replications.

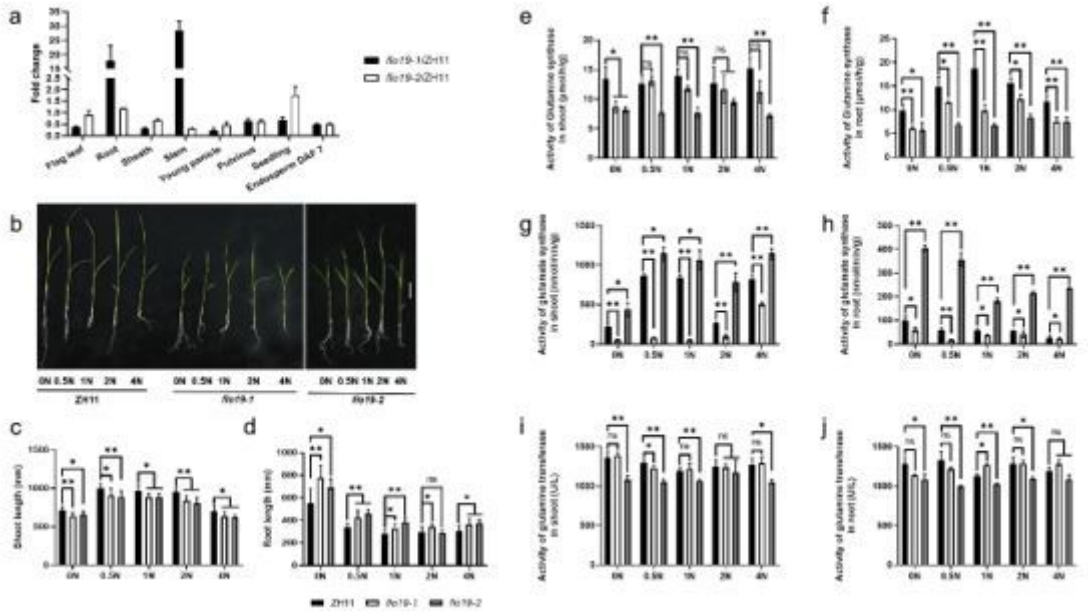

Figure 4

FLO19 gene expression profile and its effect on carbon and nitrogen metabolism. (a) Expression levels of FLO19 in the various tissues of two mutants. Actin1 was used as an internal control. Values are means ± SD. DAF, days after flowering. (b) Two-week nitrogen treatment experiment for mutants and ZH11 at seedling stage. Scale bars, 100 mm. (c, d) Shoot length and root length of two mutants and ZH11. Activities of glutamine synthase (GS) (e, f), glutamate synthase (GOGAT) (g, h) and glutamine amidotransferase (GAT) (i, j) in shoots and roots of mutants and ZH11. Data are means ± SD, n≥3 (Student's t-tests, *, P < 0.05; **, P < 0.01; ns, no significant difference).

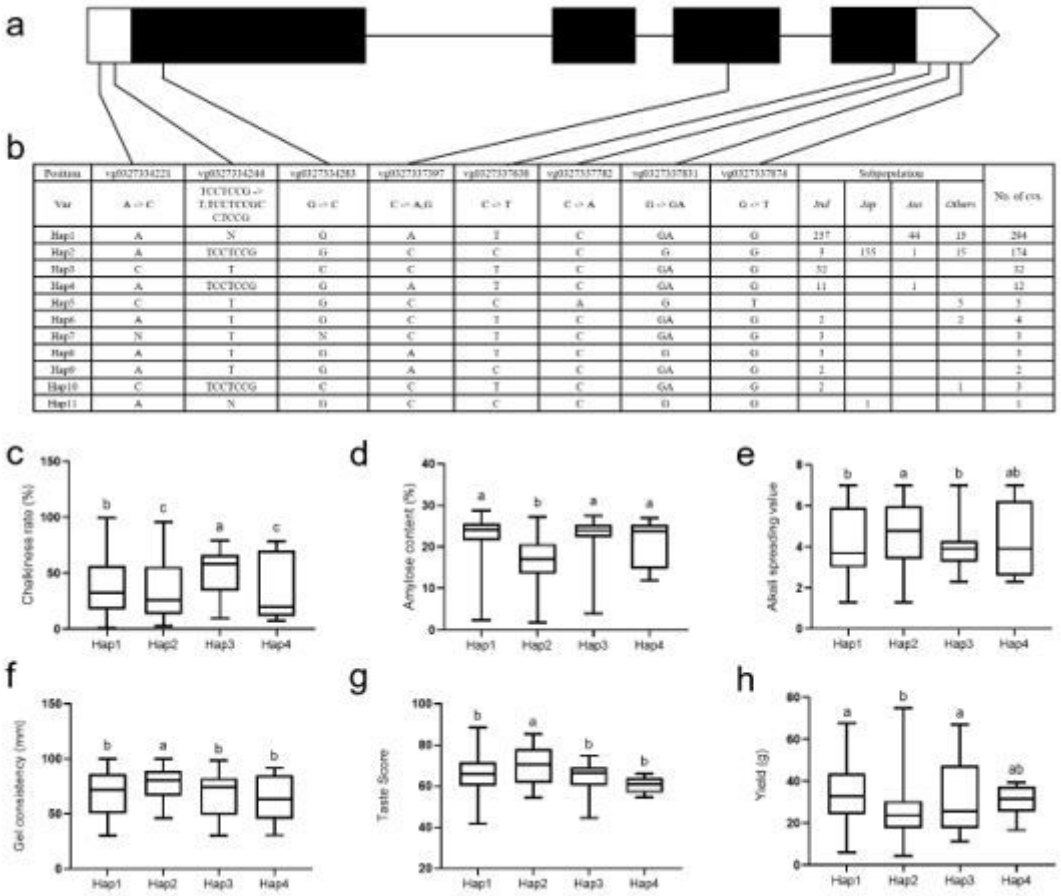

Figure 5

Haplotype analysis of FLO19. (a) Schematic of FLO19 gene structure. (b) Haplotype analysis of the FLO19 gene coding region from 533 rice cultivars. Ind, Jap and Aus represent indica population, japonica population and aus population respectively. (c) Chalkiness rate of milled rice. (d) Amylose content in milled rice flour. (e) Grading standards of alkali spreading value by visual assessment. (f) Gel consistency. (g) Taste score. (h) Yield per plant. Data are means ± SD (Hap1, n=294; Hap2, n=174; Hap3, n=32; Hap4, n=12). Different letters represent significant differences at P <0.05, Duncan's multiple range test.

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