Identification, Pyramid and Candidate gene of QTL for Yield-related Traits Based on Rice CSSLs in *Indica* Xihui18 Background

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Abstract

Chromosome segment substitution line (CSSL) in rice is important for functional analysis and design breeding of target genes. Here, a novel rice CSSL-Z431 was identified from *indica* restorer line Xihui18 as recipient and Huhan3 as donor. Z431 contained six segments from Huhan3, with an average substitution length of 2.12 Mb. Compared with Xihui18, Z431 increased panicles per plant (PN) and displayed short-wide grains. The short-wide grain of Z431 were caused by reducing of cell length and increasing of cell width in the glume. Then, thirteen QTLs were identified in a secondary F_2 population derived from Xihui18/Z431. Among them, six QTLs (*qPN3*, *qGL3*, *qGW5*, *qRLW2*, *qRLW3*, *qGWT-5-2*) were validated by four single-segment substitution lines (SSSLs, S1-S4) developed in F_3. In addition, thirteen QTLs (*qPN1*, *qPN2*, *qPL1*, *qPL2*, *qGPP1*, *qGPP2*, *qGL2*, *qGW1*, *qGW2*, *qGW3*, *qRLW5-2*, *qRLW1*, *qGWT2*) were detected by these SSSLs, while not be identified in the F_2 population from Xihui18/Z431. Increase of panicles per plant in Z431 was controlled by *qPN3*, *qPN1* and *qPN2*. *OsIAGLU* should be the candidate gene for *qPN3* by DNA sequencing. The short-wide grain of Z431 was controlled by *qGL3*, *qGL2*, *qGW5*, *qGW2* and *qGW3*. By sequencing between Xihui18 and according SSSL, three candidate genes for *qGL3* and two candidate genes for *qGW5* were identified, respectively. In addition, pyramid of different QTLs (*qPN1*and *qPN3*; *qPN2* and *qPN3* etc.) yielded different epistatic effects. These results lay good foundation in molecular mechanism analysis of unreported genes and rice molecular design breeding.

Introduction

Rice (*Oryza sativa* L.) is one of the major food crops, providing a staple food source for more than half of the world's population (Ranjith et al. 2020). Breeding of high-yield hybrid rice is an effective way to solve the problem of food shortage. Since the 1960s, the utilization of heterosis has significantly increased rice yield and solved subsistence for the world population (Zhang et al. 2020a). However, with the growth of population and the decreasing of available field, improving rice yield is still main goal of rice breeding (Li et al. 2016). Yield of rice is mainly determined by the number of panicles per plant, number of grains per panicle and grain weight (Heng et al. 2018). However, these traits all belonged to quantitative traits, their inheritance are very complex.

In last 3 decades, quantitative trait locus (QTLs) for rice yield traits in rice have been identified on almost all 12 chromosomes using primary segregating populations, such as F_2 lines, recombinant inbred lines (RILs) and doubled haploid lines (DHs) (Xing et al. 2010; Hu et al. 2018). However, seldom of these QTLs were cloned due to non-accurate of QTL mapping caused by excessive genetic background noise in primary population (Liu et al. 2018). To resolve this problem, advanced populations such as chromosome segment substitution lines (CSSLs) has been used in identification of QTL for a wide range of traits in food and commercial crops (Wu et al. 2020). Chromosome segment substitution lines (CSSLs) are genetic stocks representing the complete genome of any genotype in the background of a cultivar as overlapping segments. Generally, each CSSL has a single or several specific marker-defined chromosome segment from the donor with a maximum recipient parent genome recovered in the background (Balakrishnan et al. 2019). When each line only carries single substitution segment, it can be known as single segment substitution lines (Zhang et al. 2019). Particularly, CSSLs are valuable prebreeding tools for broadening the genetic base of existing cultivars and a powerful platform for breeding by design (Balakrishnan and Zhang, 2019). So far, the efficiency of QTL cloning has been improved greatly using CSSLs. Such as, *qPPP2* for panicles per plant (Tao et al. 2016), *qSP1* for spikelets per panicle (Ma et al. 2019), *qGL3* for grain length (Kashif et al. 2020), *qKL3* for kernel length (Wang et al. 2020a), *qGW1-2*, *qGW3-2* and *qGW4-1* for grain width (Li et al. 2019), *qGWT5* and *qTGW11* for 1000-grain weight (Okada et al. 2018; Wang et al. 2021) et al. Although these genes revealed some molecular regulatory mechanisms of rice yield traits. However, due to separating of favorite alleles in different varieties (Zheng et al. 2020; Dhat et al. 2021), it is necessary to identify more QTLs for yield traits using novel CSSL developed from different elite cultivars.

Restorer line is key in hybrid rice breeding. Xihui18, an excellent rice restorer line, was bred by Rice Research Institute of Southwest University. It has the advantage as high general combining ability, good flowering habit and long panicles and multiple grains per panicle. However, the number of panicles per plant in Xihui18 is few and the grains are long and narrow. Huhan3 is characterized by multiple panicles, short and wide grains, and strong resistance to stress. Here, we identified a rice CSSL-Z431, which is mainly characterized by short-wide grain and multiple panicles per plant, derived from Xihui18 as recipient and Huhan3 as donor parent. Then, we will analyze Z431 systematically and map QTL using secondary F_2 population constructed by a cross between Xihui18 and Z431, as well as verify QITL with the developed SSSL and analyze the pyramid of QTLs using the developed DSSL. Finally, we also analyze candidate genes of major target QTL.

Materials And Methods

Experimental materials

Development of Z431

The rice multiple panicles and short-wide grain CSSL Z431 with six substitution segments was used in this experiment. Z431 was derived from Xihui18 as the recipient parent and Huhan3 as the donor parent. Firstly, 241 polymorphic markers between them were selected from 429 simple sequence repeat (SSR) markers that covered the whole rice genome. Then, molecular marker-associated selection (MAS) was applied to develop CSSL from BC_2F_1 to BC_3F_7. 20 plants each generation. Finally, a CSSL Z431 with six substitution segments, which displayed multiple panicles per plant and short-wide grain, was identified in the process of SSR marker screening. The identification of substitution segment in Z431 was conducted according to the method of Ma et al (2019). The substitution segment's estimated length was calculated by the method of Paterson et al (1991). The chromosome map was constructed with the Map chart 2.32 software (https://www.wur.nl/en/show/Mapchart.htm).

Material for QTL mapping

A secondary F_2 population with 150 individuals was used for QTL mapping, which derived from a cross between Xihui18 and Z431.

Materials for development of SSSL and DSSL

Nine individuals harboring target QTL and no or few heterozygous markers were selected from the F_2 generation to develop SSSL and DSSL in F_3 population.

Field planting

In July of 2018, Xihui18 was crossed with Z431 at experimental station of Southwest University of Chongqing, China to get hybrid. In September of the same year, the hybrid seeds were planted at Lingshui Experimental Base of Hainan Province to get F_1 seeds. In March 10 of 2019, Xihui18, Z431 and the F_2 population were planted at experimental station of Southwest University of Chongqing, China. In April 13, 30 plants of Xihui18 and Z431 and 150 plants of F_2 population were transplanted in the same field. The spacing between the hills and rows was 16.67 cm ×26.67 cm. In March 2020, Xihui18, Z431 and nine individuals for development of SSSL and DSSL were planted into the same experimental field and transplanted 30 plants for each material in April 15 of the same year. Conventional field management practices were applied.

Measurement of yield-related traits

Ten plants each from Xihui18 and Z431, together with 150 F_2 plants were harvested at the maturity stage. Ten yield-related traits were measured, including number of panicles per plant, panicle length, number of spikelets per panicle, number of grains per panicle, grain length, grain width, ratio of length to width ratio, 1000-grain weight, seed setting rate and yield per plant. The methods of measurement were the same as for Ma et al (2020). The mean phenotypic value and *t*-test of the above traits for Xihui18 and Z431 were calculated in Microsoft Excel 2016.

Scanning electron microscopy

At the completion of the booting stage and before the heading period, the phenotypic characteristics of the inner and outer epidermal cells of the glume in Xihui18 and Z431 were investigated using a Hitachi SU3500 scanning electron microscope (Hitachi, Tokyo, Japan) with a frozen stage (− 40°C) under a low-vacuum environment (Zhang et al. 2020b).

QTL Mapping

Total genomic DNA of Xihui18, Huhan3, Z431, and the 150 individuals from the F_2 population was extracted using the cetyl-trimethyl-ammonium-bromide (CTAB) method. PCR amplification, non-denaturing poly acrylamide gel electrophoresis, and rapid silver staining were performed as described previously (Zhao et al. 2016). Bands same with Xihui18 were scored as "− 1", bands same with Z431 were scored as "1", heterozygous bands were scored as "0", and the absence of marker bands was scored as ".". The marker assignments of all SSR markers on the substitution segments of Z431, together with the phenotypic values of each individual in the F_2 population, were used for QTL mapping. QTL mapping was performed using the restricted maximum likelihood method by mixed linear models (REML) implemented in the HPMIXED procedure of SAS (SAS Institute Inc, Cary, NC, USA), with significance determined at $\alpha = 0.05$.

Development of SSSLs, DSSLs and verification and pyramid of QTLs using SSSLs and DSSLs

According to the QTL mapping, nine individuals carrying target QTL and no or few heterozygous markers were selected and planted as line Z801 ~ Z809 in 2020, with 30 plants each. Then, DNA of 30 individuals was taken and extracted DNA from each line to further develop SSSLs and DSSLs with residual heterozygous markers by MAS.

In 2020, ten plants of Xihui18 and all plants of each SSSL and DSSL were sampled after maturity and ten yield related traits as above involved were measured. Since only one difference of single substitution segment was existed between each SSSL and its recipient Xihui18. Thus, under certain environment (same year and same experimental field and no replicate plot designed), the genetic model for Xihui18 and $SSSL_i$ carrying a specific QTL was: $P_0 = \mu + \varepsilon$, and $P_i = \mu + a_i + \varepsilon$, respectively, where P_0 and P_i represent the phenotype value of any plant in plot of Xihui18 and the $SSSL_i$ carrying the'i'substitution segment. μ represent the mean value of Xihui18 population, a_i represent additive effect of QTL, ε represent random error. Consequently, t-test was used to map QTL for a certain trait by analyzing the statistical differences between each SSSL and Xihui18, and when the P-value was less than 0.05, we think a QTL was considered to exist in $SSSL_i$. The additive effect of the QTL was calculated as half the difference between the mean phenotypic values of SSSL and Xihui18 (Zhang et al. 2020). All calculations were conducted in Microsoft Excel 2016.

The genetic model for DSSL was: $P_{ij} = \mu + a_i + a_j + l_{ij} + \varepsilon$, where P_{ij} represent the phenotype value of any plant in plot of the $DSSL_{ij}$, a_i and a_j represent the additive effect of QTL in substitution segment i, j, respectively. l_{ij} represent the a_ia_j epistatic effect between QTLs in substitution segment i and j. Thus, the epistatic effect between QTLs in DSSL could be checked using t-test by checking the difference of each trait between (Xihui18 + $DSSL_{ij}$) and ($SSSL_i$+$SSSL_j$), where $SSSL_i$, $SSSL_j$, $DSSL_{ij}$ and Xihui18 represent the phenotypic value of a trait corresponding to SSSL, DSSL, and Xihui18, respectively. When the *P*-value is less than 0.05, it is considered that there is an epistatic effect between QTLs in DSSL. The epistatic effects between two QTLs were estimated as half of the mean phenotypic values of (Xihui18 + $DSSL_{ij}$)-($SSSL_i$+$SSSL_j$) (Zhang et al. 2020).

Candidate gene prediction and DNA sequencing of *qPN3, qGL3* and *qGW5*

At the substitution interval of three major QTL, including *qPN3*, *qGL3* and *qGW5*, we analyzed the candidate gene information by the Rice Annotation Project (https:/rapdb.dna.affrc.go.jp/) and the China National Rice Database Center (http:/www.ricedata.cn/) and the Gramene (http:/www.gramene.org/). As for possible gene, the whole sequence was downloaded, and the primers were designed on Vector NTI to amplify the target fragments using DNA of Xihui18 and corresponding SSSL as templates, respectively. The PCR products were forwarded for sequencing to Tsingke Biological Technology Co., Ltd (Chongqing, China).

Results

Identification of substitution segments in Z431

Based on the development of Z431, we further identified the substitution segments and the genetic background of 10 plants of Z431 using all SSR markers in the six substitution segments of Z431 and 36 SSR markers outside of the substitution segments. The results showed that the substitution segments of 10 plants of Z431were identical and no other residual segments from Huhan3 were detected. Z431 contained six substitution segments from Xihui18, distributed on chromosomes 1, 2, 3, 5 and 12. The fifth chromosome contains two substitution segments and the other chromosomes each contain one substitution segment (Fig. 1). The total substitution segments length was 12.71 Mb, the longest substitution length was 3.76 Mb, the shortest substitution length was 0.95 Mb and the average substitution length was 2.12 Mb.

Phenotype of Z431

Compared with Xihui18, the panicle length, number of grains per panicle, spikelets per panicle, grain length and the ratio of length to width in Z431 decreased significantly by 5.05 cm, 77.86 grains, 86.42 spikelets, 0.14 mm and 1.13, respectively. The number of panicles per plant, grain width, 1000-grain weight and yield per plant of Z431 were significantly increasing than Xihui18 by 3.4 panicles, 0.9 mm, 4.80 g and 8.46 g, respectively (Fig. 2). There was no significant difference in seed setting rate between Z431 and Xihui18. Therefore, the increased yield per plant of Z431 was caused predominantly by increase in the number of panicles per plant and grain weight.

Cytological analysis of Z431

To examine the factors responsible for the decrease in grain length and increase in width of Z431, scanning electron microscopy was used to observe the cell morphology of glumes in Xihui18 and Z431 before the heading stage. The cell length of the glume in Z431 was 88.06 μm, shorter than that (178.60 μm) of Xihui18. While the cell width in Z431was 40.00 μm, wider than that (29.00 μm) of Xihui18 (Fig. 3a-d, and Fig. 3g-h). There were no significant difference in total cell number between Xihui18 and Z431 in the outer epidermis of the glume along the longitudinal axis (Fig. 3e-f and Fig. 3i). These results indicated that short-wide grain of Z431 was caused by increase of cell width and decrease of cell length and not by change of cell number of glume.

Identification of QTL for yield-related traits using secondary F_2 population from Xihui18/Z431

A total of thirteen QTLs were identified in the secondary F_2 population constructed from crosses between Xihui18 and Z431. These QTLs distributed on five substitution segments of Z431 and explained 2.10–30.75% of the phenotypic variation (Table 1). Among them, there is one QTL for panicle length, number of panicle per plant, number of spikelets per panicle, number of grains per panicle, grain length and grain width, respectively, and 3 QTLs for ratio of length to width and 100-grain weight, respectively. *qGL3* for grain length from Huhan3 reduced the grain length of Z431 by 0.27 mm, explained 30.75% of the variation in grain length. *qGW5* increased the grain width of Z431 by 0.09 mm, explained 2.10% of the phenotypic variation. *qPN3* increased the number of panicle per plant in Z431 by 0.51, explained 6.75% of the phenotypic variation. The 1000-grain weight of Z431 was mainly controlled by two major QTL (*qGWT3*, *qGWT-5-1*) and one minor QTL (*qGWT-5-2*), and the additive effect of the QTL from Huhan3 increased the 1000-grain weight of Z431 by 1.09 g, 0.84 g, and 0.76 g, respectively.

Table 1
QTL for yield-related traits identified in substitution segments of Z431

Trait	QTL	Chromosome	Linked marker	Additive effect	Variance (%)	P-value
Panicle length (cm)	*qPL3*	3	RM6266	-0.89	17.59	< 0.0001
Number of panicles per plant	*qPN3*	3	RM6266	0.51	6.75	0.0204
Number of grains per panicle	*qGPP12*	12	RM1261	-7.01	4.42	0.0143
Number of spikeletes per panicle	*qSPP12*	12	RM1261	-7.25	3.85	0.0206
Seed-set rate	*qSSR5*	5	RM3322	-1.19	13.30	0.0004
Grain length (mm)	*qGL3*	3	RM6266	-0.27	30.75	< 0.0001
Grain width (mm)	*qGW5*	5	RM169	0.09	2.10	< 0.0001
Ratio of length to width	*qRLW2*	2	RM2770	0.06	4.98	0.0259
	qRLW3	3	RM6266	-0.08	8.78	0.0018
	qRLW5	5	RM3322	-0.06	4.70	0.0390
1000-grain weight (g)	*qGWT3*	3	RM6266	-1.09	18.86	< 0.0001
	qGWT-5-1	5	RM3322	0.84	11.90	0.0022
	qGWT-5-2	5	RM169	0.76	9.38	0.0085

Development of SSSL and DSSL, as well as verification and pyramid analysis of QTLs using the SSSLs and DSSLs

Based on the QTL mapping, four SSSLs (S1 ~ S4) and two DSSLs (D1 ~ D2) were further developed in F_3 using MAS (Fig. 4a).

Among thirteen QTLs detected in 2019 in secondary F_2 population, six QTLs (*qPN3*, *qGL3*, *qGW5*, *qRLW2*, *qRLW3*, *qGWT-5-2*) were validated by four SSSLs, suggesting that these QTLs were genetically stable. Five QTLs (*qGPP12*, *qSPP12*, *qSSR5*, *qRLW5*, *qGWT-5-1*) could not be verified due to none corresponding SSSL. In addition, thirteen QTLs (*qPN1*, *qPN2*, *qPL1*, *qPL2*, *qGPP1*, *qGPP2*, *qGL2*, *qGW3*, *qGW2*, *qGW1*, *qRLW5-2*, *qRLW1*, *qGWT2*) could be detected by SSSLs (Fig. 4a), which were not detected in the secondary F_2 population, indicating that SSSL had higher efficiency of QTL detection.

The number of panicles per plant (6.50, 6.65 and 7.36) in S1, S2 and S3 carrying QTLs (*qPN1*, *qPN2* and *qPN3*) with increasing effect were significantly more than that (4.00) of Xihui18, while 5.00 panicles per plant in S4 without QTL showed no significant difference with Xihui18 (Fig. 4a and 4c). Grain length (9.87 and 9.36 mm) in S2 and S3 carrying QTLs (*qGL2* and *qGL3*) with decreasing effect were significantly shorter than that of Xihui18 (10.38 mm), while grain length (10.21 and 10.36 mm) of S1 and S4 without QTL displayed no significant difference with that of Xihui18 (Fig. 4a-b and Fig. 4d). Grain width (3.16, 3.40, 3.32 and 3.28 mm) in S1, S2, S3 and S4 harboring QTL with increasing effect were significantly wider than that (3.11 mm) in Xihui18 (Fig. 4a-b and Fig. 4e). Ratio of length to width in S1, S2, S3 and S4 with QTL decreasing the trait were significantly less than that (3.34) in Xihui18 (Fig. 4a-b and Fig. 4f). 1000-grain weight (32.00 and 33.50g) of S2 and S4 carrying QTLs (*qGWT2* and *qGWT5*) with increasing effect were significantly higher than that (29.89g) of Xihui18, while ones (28.80 and 29.11g) of S1 and S3 without QTL displayed no significant difference with that of Xihui18 (Fig. 4a-b and Fig. 4g).

Pyramid of *qPN1* (a = 1.03) and *qPN3* (a = 1.08) yielded an epistatic effect of 1.54, resulting in theoretically increasing 3.65 panicles per plant in D1. In fact, D1 had 9.63 panicles per plant, which increased significantly than that (4.00) of xihui18, which was consistent with the theoretical genetic model value (7.65) in D1. The result suggested that pyramid of *qPN1* and *qPN3* yielded more panicles than that (6.50) in S1 (containing *qPN1*) and S3 (7.36)(containing *qPN3*) (Fig. 4a and Fig. 4c). Pyramid of *qPN2* (a = 1.32) and *qPN3* (a = 1.08) yielded an epistatic effect of -2.00, thus increasing 0.40 panicles per plant in D2. The result suggested that pyramid of *qPN2* and *qPN3* yielded less panicles than that (6.60) of S2 (containing *qPN2*) and S3 (7.36) (containing *qPN3*)(Fig. 4a and Fig. 4c). Pyramid of *qGL3* (a=-0.51) and a substitution segment without QTL for grain length on chromosome1 yielded no epistatic effect in D1, whose phenotype was 9.52 mm, displayed no significant difference with that (9.36 mm) of S3 (Fig. 4a-b and Fig. 4d). Pyramid of *qGL2* (a=-0.25) and *qGL3* (a=-0.51) yielded an epistatic effect of 0.38, resulting in increasing 0.38 mm of grain length in D2, which indicated that pyramid of *qGL2* and *qGL3* yielded longer grain than S3 (containing *qGL3*)(Fig. 4a-b and Fig. 4d). Pyramid of *qGW1* (a = 0.14) and *qGW3* (a = 0.11) yielded an epistatic effect of -0.10mm, resulting in increase 0.15mm of grain width in D1, which showed that pyramid of *qGW1* and *qGW3* yielded wider grain than S1(containing *qGW1*)(Fig. 4e). Pyramid of *qGW2* (a = 0.15) and *qGW3* (a = 0.11) yielded an epistatic effect of -0.13 mm, which resulted in increase 0.13 mm of grain width in D2. The result suggested that pyramid of *qGW2* and *qGW3* yielded wider grain than S3 (containing *qGW3*)(Fig. 4e). Pyramid of *qRLW1* (a=-0.09) and *qRLW3* (a=-0.26) yielded an epistatic effect of 0.11 in D1 and pyramid of *qRLW2* (a=-0.22) and *qRLW3* (a=-0.26) yielded an epistatic effect of 0.35 in D2 (Fig. 4a-b and Fig. 4f). Pyramid of *qGWT2* (a = 1.06) and a substitution segment without QTL for 1000-grain weight on chromosome 3 yielded an epistatic effect of -2.84, which resulted in -1.78g of 1000-grain weight in D2. The result suggested that the pyramid of *qGWT2* and the segment on chromosome 3 yielded heavier grain than S3(containing this segment) (Fig. 4g). All the result showed that pyramid different QTLs yield various epistatic effect.

Candidate gene prediction and DNA sequencing of major QTLs- *qPN3, qGL3* and *qGW5*

Compared with Xihui18, the outstanding characteristic of Z431 showed multiple panicles and shorter and wider grain. Thus, *qPN3*, *qGL3* and *qGW5* deserved our priority attention, *qPN3* and *qGL3* were all located on S3 whose substitution length was 1.48 Mb (Fig. 5a and Fig. 5c). *qGW5* was located on S4 whose substitution length was 1.68 Mb (Fig. 5b).

About *qPN3*, we found in the substitution interval a reported gene (*OsIAGLU*), which affects the number of panicles per plant by negatively regulating IAA. By DNA sequencing of *OsIAGLU* between Xihui18 and S3, there were four single nucleotide polymorphisms (SNPs) in CDS between S3 and Xihui18, which all caused changes in amino acid (Fig. 5a). Thus, *OsIAGLU* should be the candidate gene for *qPN3*.

Concerning *qGL3*, we found 4 possible genes related to grain length with sequence difference between Xihui18 and S3 (Fig. 5b). For the candidate gene1 as an auxin-responsive protein, there were 2 SNPs and 2bp deletion in S3 compared with Xihui18, where the 175th base of CDS changed from C in Xihui18 to T in S3, resulting in Leu and of Xihui18 to Phe of S3, and another SNP changes (from C to T) in the 357th of CDS without resulted in amino acid changes. The 2bp deletion in 401th base of CDS in S3 caused delaying the termination of the translation (Fig. 5b). For the candidate gene 2 as serine/threonine protein kinase in ABA signal transduction, although there was 1 SNP differences, however no resulting in amino acid change (Fig. 5b). Thus, the gene should not be the candidate gene for *qGL3*. For candidate 3 also as an auxin-responsive protein, we found 4 SNPs difference between Xihui18 and S3, where the base C, T and C in 125th, 215th and 274th of the CDS in Xihui18 changed to A, G and A in S3, which caused amino acid mutation from Ala, Val and Ala in Xihui18 to Glu, Gly and Glu in S3. While another SNP deference did not cause amino acid change (Fig. 5b). For candidate gene 4 as a serine/threonine-protein kinase-like protein ACR4, there was a deletion of base T in 1900th of CDS in S3, which caused frame shift mutation of the amino acid. In addition, there were 3 SNP difference (Fig. 5b). In conclusion, candidate gene1, 3 or 4 could acted as the candidate genes of *qGL3*.

Regarding *qGW5*, we found 4 possible genes related to grain width in the substitution interval of S4. By DNA sequencing, candidate gene4 (*BGIOSGA019432*) for auxin-responsive protein did not exist any sequence difference between Xihui18 and S4. The other three were found to show differences between Xihui18 and S4. For the candidate gene1 as E3 ubiquitin-protein ligase, although displayed 3 SNP differences in the CDS between Xihui18 and S4. However, these base difference all belonged to nonsense mutation (Fig. 5c). Thus, two genes above mentioned should not be the candidate genes of *qGW5*. For the candidate 2 as eukaryotic translation initiation factor 3 subunit, there were 3 SNP differences in the CDS between Xihui18 and S4, where the 1500th base changed from G in xihui18 to C in S4, resulting amino acid mutation from Leu to Phe. While the other SNP did not cause amino acid changes (Fig. 5c). For the candidate 3 as RING-type E3 ubiquitin transferase, there were 2 SNP differences in the CDS between Xihui18 and S4, where the base G and T in 62th, 175th of the CDS in Xihui18 changed to C and C in S4, which caused amino acid mutation from Glu and Met in Xihui18 to Ala and Thr in S4. Thus, the candidate gene 2 for eukaryotic translation initiation factor 3 subunit or gene 3 for RING-type E3 ubiquitin transferase should be responsible for *qGW5*.

Discussion

Z431 and its secondary substitution line are potential to be used as novel rice restorer line in breeding novel hybrid rice

The successful breeding of hybrid rice is a significant breakthrough in rice breeding, which has dramatically improved crop genetics and breeding theory and breeding (Cui et al. 2020). To date, most of the hybrid rice varieties were interspecific hybrids (Li et al. 2020). Due to the close interspecies relationships and

relatively small genetic differences, the heterosis was not strong enough. Thus, the use of heterosis between subspecies and wild species will be imperative. However, direct subspecies crosses often result in sterility and a reducing seed-setting rate due to reproductive isolation (Nadir et al. 2018). Development of indica-japonica rice chromosome segment substitution lines can overcome the limitation (Ma and Singh 2020). Excellent restorer line is an essential part of heterosis utilization, which often contained the main fertility recovery genes *Rf-1* (Akagi et al. 2004), *Rf2* (Etsuko et al. 2011), *Rf3* (Cai et al. 2013), and *Rf4* (Tang et al. 2014). In this study, Xihui18 was an elite rice *indica* restorer line with strong combining ability, long panicle with multiple grains and long-narrow grain. A short-wide grain rice CSSL-Z431 containing six substitution segments, with Xihui18 as the recipient parent and Huhan3 as the donor parent, was identified by whole-genome SSR marker-assisted selection. Then, four SSSLs and two DSSLs were selected by MAS in F_3 generation. One of the notable findings of this study is that all the fertility-restoring genes *Rf-1 ~ Rf4* were all not substituted in Z431, SSSLs and DSSLs, but also to some extent these substitution lines make up for the deficiencies of Xihui18. For example, the number of panicle per plant of Z431 increased from 4.2 panicle to 7.6 panicle, and the grain width and 1000-grain weight also increased significantly. Accordingly, the secondary substitution lines as S1, S2, S3, D1 and D2 all increased significantly the panicles per plant. Therefore, Z431 and these secondary substitution lines are potential to be used as novel restorer lines for breeding novel rice hybrid varieties and crop improvement.

Comparison Of Qtls Identified With The Reported Genes

Since Z431 had still six chromosomal substitution segments from donor Huhan3 and significant differences in 10 agronomic traits with recipient parent Xihui18. Which QTLs are responsible for these different traits in these substitution segments? We further identified 13 QTLs for these traits using the secondary F_2 population from crosses between Xihui18 and Z431. Among them, six QTLs could be validated by the developed four SSSLs. In addition, thirteen QTLs could be detected by these SSSLs, while were not identified in the secondary F_2 population. Thus, there were 26 QTLs were identified in total. Among them, *qPN1*, *qPL1*, *qGPP1*, *qGW1* and *qRWL1* were all linked with RM283 (5.27Mb), indicating that some may belong to tight linkage QTLs or others have pleiotropism. *qPL1* and *qPN1* might be alleles with *CCP1* (7.15 MB), which were 1.92 Mb from RM283. *CCP1* encodes EMF1-like protein and negatively regulates *OsMADS58*. The number of panicles per plant in *ccp1* mutant increased, the panicle length and the seed setting rate decreased significantly (Yan et al. 2015). *qGW1* and *qRWL1* may be alleles of *OsOFP1* (7.01 Mb), which was 1.78 Mb from RM283. *OsOFP1* negatively regulates the BR signal transduction pathway, which regulates cell reproduction and elongation and the seeds of overexpressing *OsOFP1* in plants became shorter, wider and thicker (Yang et al. 2018). *qPN3*, *qGL3*, *qGW3*, *qRLW3* and *qGWT3* were all linked to RM6266 (27.64 Mb) on chromosome 3. *qPL3*, *qGL3, qRLW3* and *qGWT3* were also detected by Wang et al (2020b), which indicated that these QTLs could be stably inherited. *qPL3* and *qPN3* may be allelic to *OsIAGLU* (27.78 Mb). *OsIAGLU* encodes an auxin-binding enzyme, negatively regulating IAA and affects the number of panicles per plant (Choi et al. 2012). By DNA sequencing of *OsIAGLU*, there were 4 SNP differences between Xihui18 and single segment substation line 3 (S3) harboring *qPL3* and *qPN3* and resulted in 3 amino acid changes. Thus, *OsIAGLU* should be candidate gene of *qPL3* and *qPN3*. Although, *OsIAGLU* has been cloned, compared with its overexpression transgenic plants, S3 with increase the number of panicles per plant are favorable to be taken advantage directly in rice design breeding. *qGL3, qRLW3* and *qGWT3* maybe belonged to pleiotropy, in the substitution interval of S3, there were four possible genes related to grain size development, found, including candidate 1 for auxin-responsive protein, candidate 2 for serine/threonine protein kinase in ABA signal transduction, candidate 3 for auxin-responsive protein, and candidate 4 for serine/threonine-protein kinase-like protein ACR4. Several previous studies have showed that the phytohormone and some protein kinases involves the regulation of grain size. For example, *BG1* as a primary response gene for auxin is involved in regulating auxin transport, positively regulating cell division and cell elongation (Liu et al. 2015). *SMG11* encodes mitogen activated protein kinase, which is involved in the MAPK signaling pathway. The *smg11* mutant produces small and light grains due to a decreased cell number (Duan et al. 2014). However, by sequencing, the candidate 2 should not be the candidate genes of *qGL3* because there were no amino acid changes, although existing a SNP in CDS between Xihui18 and S4. Only candidate gene 1, 3 or gene 4 should be responsible for *qGL3* due to existing many sequence differences between Xihui18 and S3. What is interest, three genes are still not be cloned. *qRLW5* and *qGWT-5-1* were all linked to RM3322 (4.39 Mb). In the substitution interval, *GW5* (5.36 Mb) was existed. *GW5* encodes a novel BR-mediated positive regulator that inhibits the phosphorylation of *GSK2* and the phosphorylation of *OsBZR1* and *DLT* by *GSK2*, thus affecting the accumulation of non-phosphorylated *OsBZR1* and DLT proteins in the nucleus; therefore, the expression level and growth response of BR responsive gene were regulated (Liu et al. 2017). However, the sequence of *GW5* in Xihui18 and Z431 was found to be identical by DNA sequencing, indicating that *GW5* was not a candidate gene for *qRLW5* and *qGWT-5-1*. *qGW5, qRLW5-2* and *qGWT5-2* were all linked to RM169 (7.85 Mb) and they could also be detected by Zhang et al (Zhang et al. 2020), which indicated that these two QTLs could be stably inherited. In this substitution interval of S4, we found 4 genes possibly regulating development of grain size, including candidate 1 for E3 ubiquitin-protein ligase, candidate 2 for eukaryotic translation initiation factor 3 subunit, candidate 3 for RING-type E3 ubiquitin transferase and candidate 4 for auxin-responsive protein. That reports showed that transcription factors and ubiquitin also involves in the regulation of grain size (Li et al. 2016). *GW8* codes a SBP-domain transcription factor, regulates grain width as a positive regulator, can bound directly to the *GW7* promoter and repressed its expression (Wang et al. 2015). *GRAIN WEIGHT 2* (*GW2*) encodes a RING-type protein with E3 ubiquitin ligase activity. Loss of *GW2* increases cell number, which results in broader glumes and an accelerated rate of grain filling, thus increasing grain width, weight and yield (Song et al. 2007). However, the candidate 4 (*BGIOSGA019432)* for auxin-responsive protein and candidate 1 as E3 ubiquitin-protein ligase should not be the candidate genes of *qGW5* due to no sequence difference or amino acid changes between Xihui18 and S4. While gene 2 for eukaryotic translation initiation factor 3 subunit or gene 3 for RING-type E3 ubiquitin transferase should be acted as the candidate genes for *qGW5* due to many sequence differences between Xihui18 and S4. Interestingly, two genes are still not cloned. In conclusion, sixteen QTLs (*qPN2*, *qPL2*, *qGPP2*, *qSPP2*, *qGPP12*, *qSPP12*, *qGL2*, *qGL3*, *qGW2*, *qGW3*, *qGW5*, *qRLW2*, *qRLW3*, *qRLW5-2*, *qGWT3*, *qGWT-5-2*) were still unreported. These results will be important for both genetic analysis in theory and breeding hybrid varieties in application.

SSSLs and DSSLs can improve efficiency of QTL identification and epistatic effects detection between QTLs, thus are ideal materials for favorable gene pyramiding

Breeding by design refers to the breeding of varieties by crop design utilizing favorable alleles dispersed in different genetic resources in a genome (Zhang, 2019). Thus, SSSLs are ideal materials to realize the strategy due to each SSSL carrying only one single segment from donor parent in a good genome of recipient. At first. SSSLs can improve efficiency of QTL identification (Zhao et al, 2016; Balakrishnan et al, 2019; Wang et al. 2020a). In this study, we

developed four SSSLs (S1 ~ S4) from progenies of Xihui18/Z431 secondary F_2 polulation. And using these SSSLs we validated six QTLs (*qPN3*, *qGL3*, *qGW5*, *qRLW2*, *qRLW3*, *qGWT-5-2*) which were detected by the Xihui18/Z431 secondary F_2 polulation. In addition, we identified 13 QTLs (*qPN1*, *qPN2*, *qPL1*, *qPL2*, *qGPP1*, *qGPP2*, *qGL2*, *qGW3*, *qGW2*, *qGW1*, *qRLW5-2*, *qRLW1*, *qGWT2*) by these SSSLs, which were not detected in the above involved F_2 population. The results indicated that SSSLs are more sensitive for QTL identification. Many research also supported the conclusion (Zhao et al, 2016; Balakrishnan et al, 2019; Wang et al. 2020a). With the explicit genetic backgrounds of excellent recipient and known favorable alleles for breeding, SSSLs and DSSLs can be used together in the analysis of epistatic interaction and pyramiding effects (Zou et al. 2020). Here, we analyzed epistatic effects and pyramid performance of many pairs QTLs by 4 SSSLs and 2 DSSLs. The results showed that pyramiding different QTL pairs (*qPN1/qPN3*, *qPN2/qPN3*, *qGL2/qGL3*, *qGW1/qGW3*, *qGW2/qGW3*, *qRLW1/qRLW3* and *qRLW2/qRLW3*) yielded various epistatic effects and novel favorable genotypes. For example, pyramid of *qPN1* and *qPN3*, *qPN2* and *qPN3* produced 1.54 and − 2.00 of epistatic effects, however increased by 5.63, 0.40 panicles per plant, respectively than that (4.00) of Xihui18. Pyramid of *qGL2* (a=-0.25) and *qGL3* (a=-0.51) for two short grains yielded an epistatic effect of 0.38 and produced longer grain than S2(*qGL2*) and S3 (containing *qGL3*). Zhang et al (2020) showed that pyramid qGL5 and qGL6 for two long grain yielded positive epistatic effects and produced longer grain than that of its recipient parent Nipponbare and the responding SSSLs. Thus, these findings suggested that different genes pyramid yielded different epistatic interaction. We can design a needful genotype according to the prediction of additive effects and epistatic effects of target genes based on a certain genetic background.

Abbreviations

CSSL	Chromosome segment substitution line
SSSLs	Single segment substitution lines
DSSLs	Double segment substitution lines
QTL	Quantitative trait loci
MAS	Marker-associated selection
SSR	Simple sequence repeat markers
SNP	Single nucleotide polymorphism

Declarations

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Authors' contributions

FM Zhao proposed the structure and content. SF Sun completed QTL mapping and drafted the manuscript. FM Zhao, SF Sun, SQ Xiang, M Lv, K Zhou, J Li and PX Liang developed and identified the chromosome segment substitution line Z431 and development of SSSLs and DSSLs. RX Li, MM Li, GH He, YH Ling assessed the agronomic traits. All authors read and approved the final version.

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Consent for publication

Informed consent for publication was obtained from all participants.

Conflict of interest

The authors declare that they have no conflict of interest.

Availability of data and material

All data generated or analysed during this study are included in this published article.

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Figures

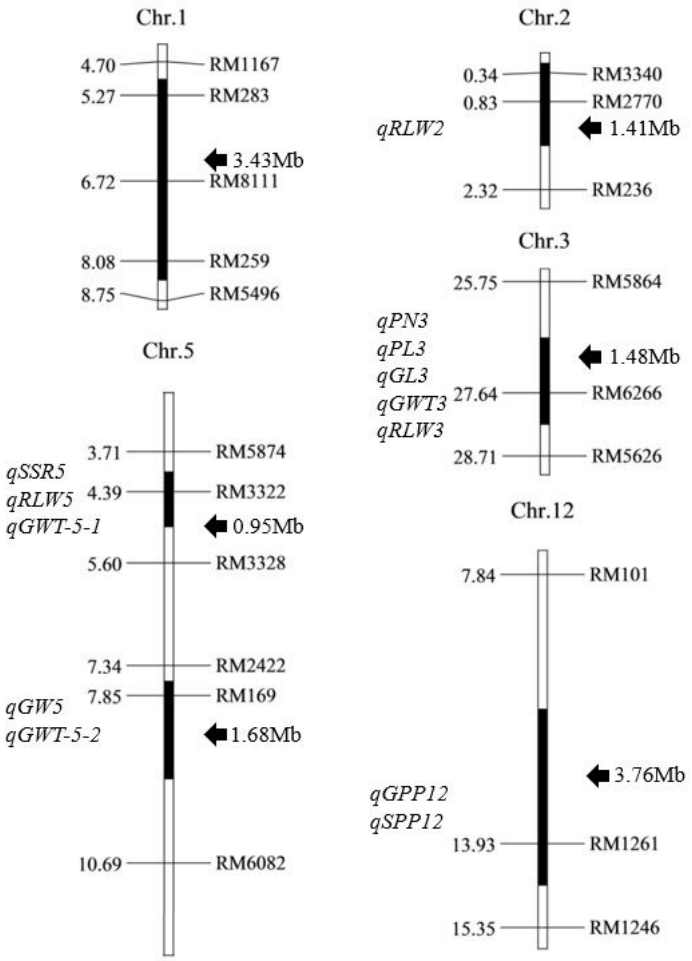

Figure 1

Chromosome substitution segments and harboring QTLs for yield traits of Z431 Physical distance (Mb) is specified on the left of each chromosome and markers are specified on the right. The solid black segment is the substitution segment segment from the donor Huhan3 and the identified QTLs are listed on the left of each chromosome in italics. PN, the number of panicles per plant; PL, panicle length; GPP, number of grains per plant; SPP, number of spikelets per panicle; SSR, seed-set rate; GL, grain length; GW, grain width; RLW, ratio of length to width; GWT, 1000-grain weight.

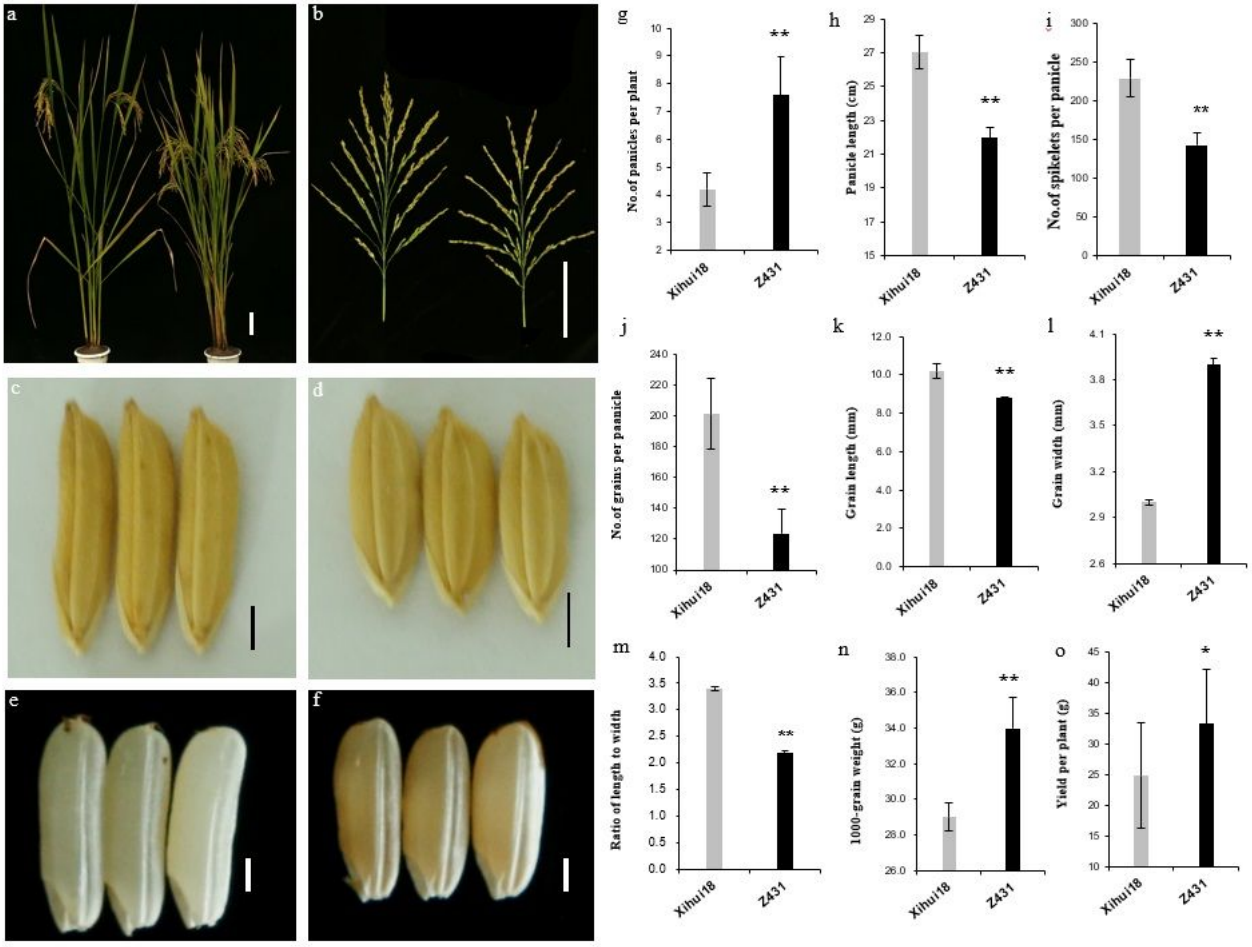

Figure 2

Phenotype of Xihui18 and Z431 a, Plant type of Xihui18 (left) and Z431 (right). b, Main panicle of Xihui18 (left) and Z431 (right). c, d, Grains of Xihui18 (c) and Z431 (d). e, f, Brown grains of Xihui18 (e) and Z431 (f). g-o, no.of panicles per plant (g), panicle length (h), no.of spikelets per panicle (i), no.of grains per panicle(j), grain length(k), grain width(l), ratio of length to width(m), 1000-grain weight(n), yield per plant(o) of Xihui18 and Z431. Data are given as the mean and SE (n = 20). * and ** indicate significant differences of traits between Xihui18 and Z431 at $P < 0.05$ and $P < 0.01$, respectively. Bars in a and b, 10 cm; c-f, 2 mm.

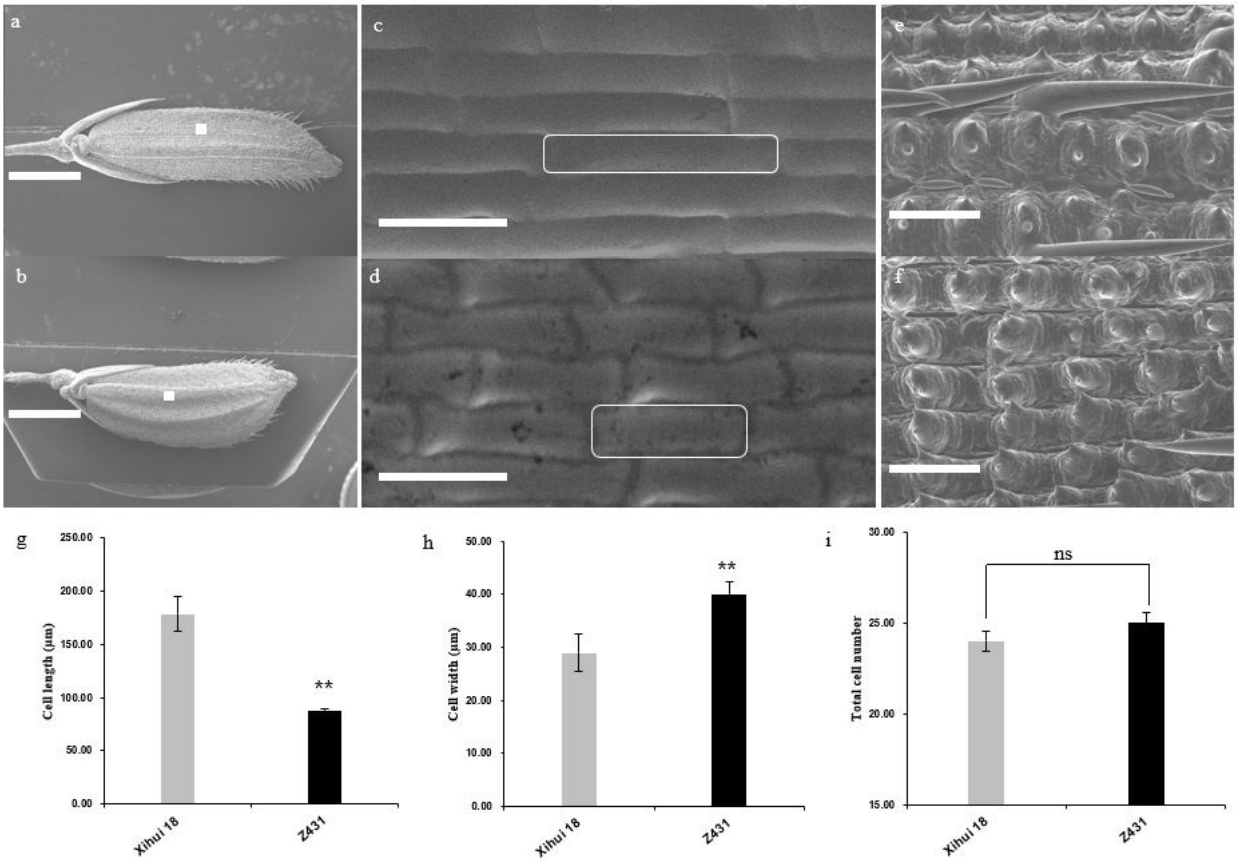

Figure 3

Scanning electron microscopy observation and analysis of glume in Z431 Scanning electron micrograph of glume, inner epidermis, and outer epidermic of glume in Xihui18 (a, c, e) and Z431 (b, d, f). g-h, Cell length and cell width in the inner epidermis of Xihui18 and Z431. i, Total cell number in the outer epidermis of the lemma along the longitudinal axis of Xihui18 and Z431. Bars in a and b, 1mm; c-f, 100μm. Data are given as the mean and SE (n = 10). Asterisks (**) indicate a significant difference between the Xihui18 and Z431 at $p<0.01$, ns indicate no significant difference between the Xihui18 and Z431.

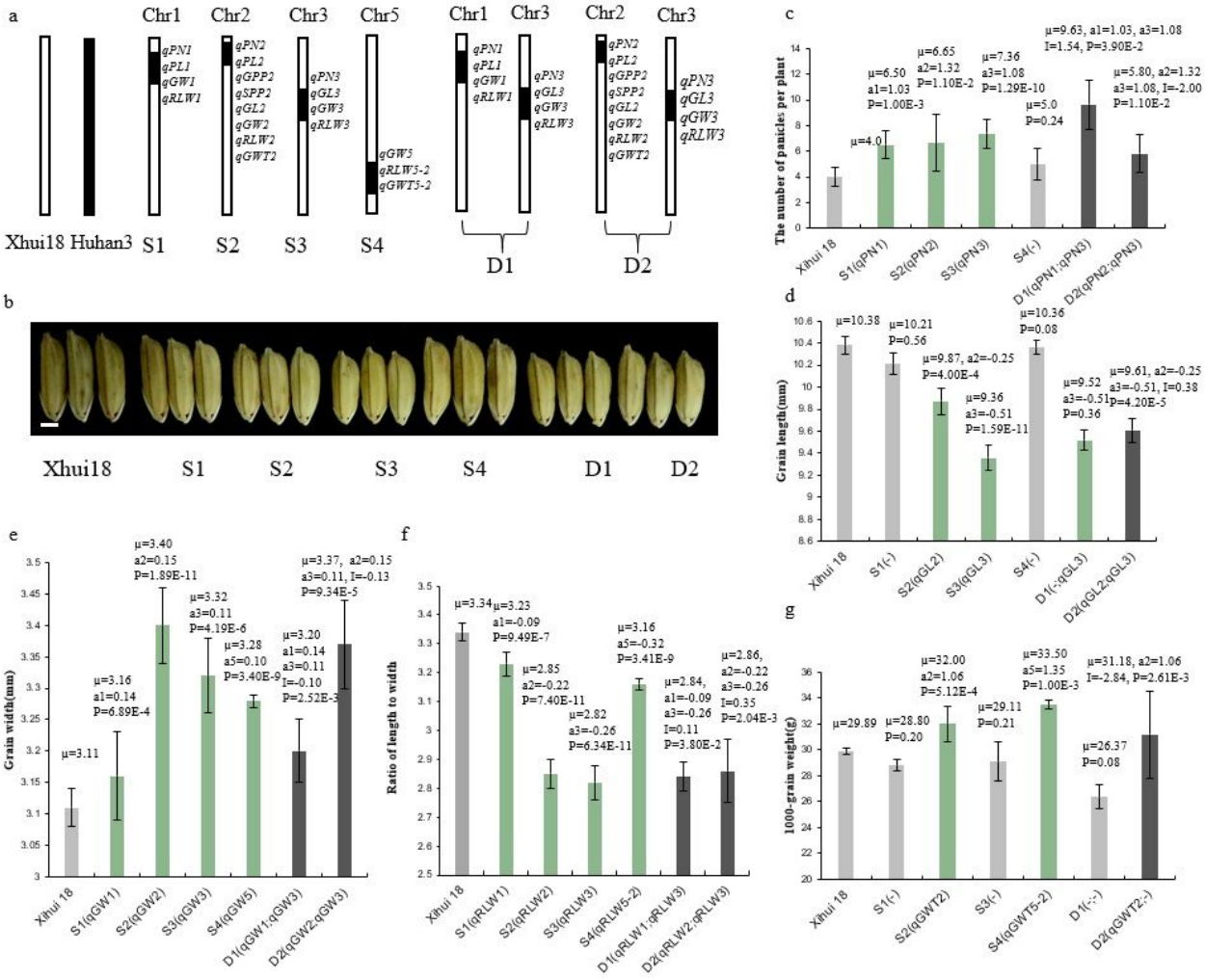

Figure 4

Verification and pyramid QTLs by SSSLs and DSSLs a, Schematic graph of substitution segment lines and QTLs located on them of SSSLs, DSSSLs. Xihui18 was the recipient parent and Huhan3 was the donor parent of the SSSLs and DSSLs. b, Grains of Xihui18, S1-S4, D1-D2. Scale bar, 2 mm. c-g, Analysis of additive and epistatic effects of QTLs for the number of panicles per plant (c), grain length (d), grain width (e), ratio of length to width (f), 1000-grain weight(g), respectively. μ represent the mean value of corresponding trait, ai represent additive effect of QTL, I represent epistatic effect of QTLs, P-value in S1-S4 indicate probability of t-test between Xihui18 and SSSL, when P<0.05 indicate existing a QTL, P-value in D1-D2 indicate probability of t-test between (DSSL+Xihui18) and (SSSLi+SSSLj), when P<0.05 indicate existing epistatic interact between QTLs or substitution segment without QTL. S1: Chr.1, RM283–RM8111-RM259–RM5496; S2: Chr.2, short arm–RM3440-RM2770–RM236; S3: Chr.3, RM5864–RM6266–RM5626; S4: Chr.5, RM2422–RM169–RM6082. D1: Chr.1, Chr.3, RM283–RM8111-RM259–RM5496, RM5864–RM6266–RM5626; D2: Chr.2, Chr.3, short arm–RM3440-RM2770–RM236, RM5864–RM6266–RM5626. The single lines in the middle of markers indicate substitution segment, while the double lines on the border of markers indicate segments recombination might appear.

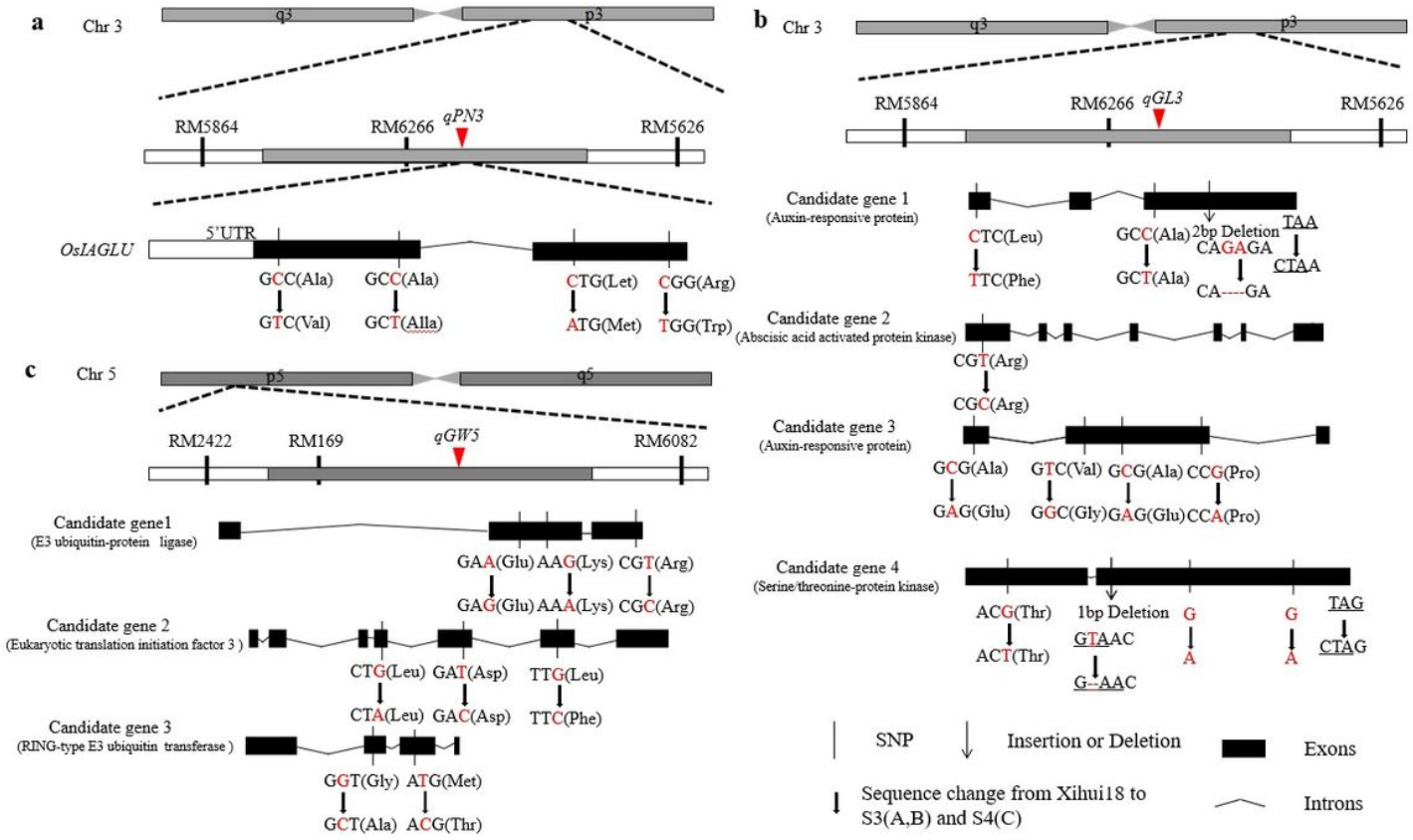

Figure 5

Sequence analysis of candidate genes a, The DNA sequence of OsIAGLU in S3 compared with Xihui18. b-c, Annotation information and comparative analysis of candidate genes in the qGL3 and qGW5 location interval, respectively.