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Identification of quantitative trait loci for yield traits and fine-mapping of *qGW4* using the chromosome segment substitution line-Z708 and dissected single-segment substitution lines

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Identifying quantitative trait loci (QTL) for yield traits using single-segment substitution lines (SSSL) is essential for both targeted breeding and functional analysis of key genes. Here, a wide-grain rice chromosome segment substitution line (CSSL), Z708, carrying four substitution segments from Jinhui35 in the genetic background of Xihui18, was used to identify the QTL associated with grain size. Seven QTL for yield-related traits (*qGW4*, *qRLW4*, *qGWT4*, *qGW5*, *qRLW5*, *qGWT5*, and *qGPP5*) were identified on the substitution segments of the fourth and fifth chromosomes of Z708. Subsequently, four SSSLs (S1-S4), which harbored 16 QTL for yield traits, were constructed using molecular marker-assisted selection. These lines (S1-S4) exhibited a significant increase in yield per plant compared to that of Xihui18. Among them, *qGW4*, which controls wide grains, belongs to a single dominant gene action in S1 based on the frequency distribution of grain width and chi-square test analysis. Finally, *qGW4* was fine-mapped to the interval of 80-kb (minimum) and 310-kb (maximum) using both traditional fine mapping and overlapping substitution mapping of the newly constructed secondary SSSLs (S5-S8). Within this interval, four previously unreported candidate genes were predicted.

KEYWORDS

chromosome segment substitution line, yield traits, QTL, qGW4, additive effect

1 Introduction

Rice is a staple food crop that provides nearly half the daily caloric intake for humans worldwide (Bin et al., 2023). Rice production plays an essential role in global food security (Rezvi et al., 2023). According to the Food and Agriculture Organization (FAO) statistics for 2023, China is the largest rice producer globally, accounting for approximately 28% of the total rice production. Increasing rice yields has become a key focus in meeting the demands of food security for the growing population (Shalmani et al., 2023). However, rice yield, as a complex trait, is composed of the number of grains per panicle, number of effective panicles per plant, and 1000-grain weight. The 1000-grain weight is determined by the grain size, including the length, width, length-to-width ratio, and filling rate of the grains. Thus, yield traits are typical quantitative traits controlled by multiple minor-effect genes (Kumari et al., 2023; Zhong et al., 2020; McKenzie et al., 1983). These complex traits have been divided into several discrete Mendelian factors through quantitative trait locus (QTL) mapping using numerous molecular markers (Ali et al., 2010; Maurer et al., 2017; Shabir et al., 2017).

Chromosomal segment substitution lines (CSSLs), each carrying one or a few specific marker-defined donor segments in the genetic background of the adapted cultivar (Surapaneni et al., 2017), can improve the accuracy of QTL mapping (Ando et al., 2008; Ookawa et al., 2016). A CSSL that carries a single substitution segment from a donor, is called a single-segment substitution line (SSSL). Many QTL detected by SSSLs can be directly used in breeding by design. Therefore, SSSL libraries serve as a valuable platform for breeding by design through target chromosome segment substitutions (Zhu et al., 2009; Li et al., 2019; Zhang et al., 2021a).

Currently, several genes related to rice yield traits have been cloned using CSSLs and other primary segregated populations. These genes are involved in various signaling pathways, including phytohormones, G-protein signaling, MAPK signaling, the ubiquitin–proteasome pathway, and transcriptional factors (Li et al., 2021). Genes for grain number include *Grain number 1a (Gn1a)*, *Grain Number per Panicle1 (GNP1)*, *Plant Architecture and Yield 1* (PAY1), Frizzy panicles(*FZP*), *Regulator of Grain Number1 (RGN1)* etc. (Li et al., 2021; Huang et al., 2018; Li et al., 2022). *Gn1a* encodes cytokinin oxidase/dehydrogenase (OsCKX2), which degrades the phytohormone cytokinin to control rice grain number (Ashikari et al., 2005). *GNP1* encodes *GA20ox1*, which participates in GA biosynthesis (Zhao et al., 2015). *PAY1*, encoding a protein containing a peptidase S64 domain, which affects the transport activity of polar auxins and alters the endogenous distribution of indole acetic acid (IAA) (Wu et al., 2016). The COMPASS-like complex, formed by OsWDR5a and OsTrx1, promotes flowering and panicle branching by modulating H3K4me3 levels, further highlighting its critical role in rice yield regulation (Jiang et al., 2018). *FZP* regulates rice secondary branches and grain numbers, a 4-bp tandem repeat deletion about 2.7 kb upstream of *FZP* affect the binding activities of auxin response factors to the *FZP* promoter, decrease *FZP* expression and increase secondary branches and grain yield in cultivated rice. In addition, OsPTB1/2 can mediate *FZP* translational repression by interacting with CUREs in the 3′ UTR of *FZP* mRNA, leading to changes in the NSB and GNP (Huang et al., 2018; Chen et al., 2022). *RGN1* regulates lateral grain formation by controlling *LOG*, a key gene in cytokinin biosynthesis. The favorable allele $RGN1^{C}$ from wild rice promotes longer panicles and higher grain yield (Li et al., 2022). *DEP1* is a gain-of-function mutation causing truncation of a phosphatidylethanolamine-binding protein-like domain protein. In addition, the G- protein βγ subunits of *DEP1* also regulates grain size by interaction with MADS-domain transcription factors in rice. Furthermore, *DEP1-GNA* is a regulating module for rice panicle development, which is important to enhance rice yield (Huang et al., 2009; Liu et al., 2018; Zhang et al., 2025). Genes associated with grain size include *GS3, SMG1, GW2, GW5/qSW5, TGW6, qRBG1*, and *GLW7* etc. *GS3* encodes the G-protein γ subunit and negatively regulates grain length (Fan et al., 2006). *SMG1/MITOGEN-ACTIVATED PROTEIN KINASE KINASE4 (OsMKK4)* controls rice grain size by participating in the mitogen-activated protein kinase (MAPK) signaling pathway (Duan et al., 2014). *GW2* and *GW5/qSW5* control rice grain size by participating in the ubiquitination pathway (Song et al., 2007; Shomura et al., 2008; Weng et al., 2008). Furthermore, the GW2-WG1-OsbZIP47 pathway coordinates grain growth through ubiquitination and transcriptional repression, highlighting its role in controlling cell proliferation (Hao et al.,2021). *GW5* regulates grain width also via the brassinosteroid (BR) signaling pathway (Liu et al.,2017). *TGW6*, which encodes an IAA-glucose hydrolase, regulates grain size via the phytohormone pathway and negatively regulates grain weight (Ishimaru et al., 2013). *TGW2* encodes CELL NUMBER REGULATOR 1 and regulates rice grain width and weight by influencing cell proliferation and expansion in glumes (Ruan et al., 2020). *TGW3* regulates rice grain size by phosphorylation of OsIAA10-OsARF4 mediated auxin signaling (Ma et al., 2023). *OsNLP3* forms the OsNLP3-OsCEP6.1 and OsNLP3-OsNF-YA8 modules, enhance grain weight (Sun et al., 2024). Natural variation in the promoter of *qRBG1/OsBZR5* enhances rice yield via the BR pathway (Zhang et al., 2024). *GLW7* encodes the transcription factor *OsSPL13* in rice, whose higher expression increases grain length and weight by promoting cell elongation (Si et al., 2016). The genes associated with tiller number include *Monoculm1 (MOC1)* and other related genes. *MOC1* encodes a plant-specific GRAS-family nuclear protein that acts as a vital regulator for controlling the formation of tiller buds (Li et al., 2003).

Although many genes for yield trait have been identified, our understanding of the mechanisms governing these traits remains incomplete. Numerous minor-effect QTL for yield traits still need to be identified. Therefore, identifying these minor QTL using SSSLs is crucial. In this study, we present the fine mapping of *qGW4* and the identification of QTL for yield traits using CSSL-708 and the developed SSSLs. We further demonstrate that Z708 carries a 4-segment substitution from Jinhui35 in the Xihui18 background. These findings are essential for exploring previously unknown genes for yield traits and providing ideal breeding germplasms for breeding by design.

2 Materials and methods

2.1 Plant materials

The wide-grained CSSL-Z708 was used as the primary material. Z708 was developed by crossing the progeny (F_4) of the recipient parents Xihui18 and Z403 using marker-assisted selection (MAS). Z403 was found to contain 10 substitution fragments from the donor Jinhui35 in the Xihui18 background, which was developed from Xihui18 as the recipient parent and Jinhui35 as the donor parent by advanced backcrosses in combination with single-sequence repeat (SSR) MAS from the BC_2F_1 to BC_3F_7 generations. Specific construction methods used for the development of Z403 have been described previously (Xu et al., 2023). Xihui18 and Jinhui35 are *indica* rice restorer lines bred by Southwest University, China. The Z708 chromosome substitution segment was identified as described previously (Sun et al., 2022). The specific flow chart of the genetic material construction was showed in the flow chart (Figure 1). The estimated length of the substitution segment was calculated as described previously (Paterson et al., 1991).

Because differences were found in only four chromosomal segments between its recipients Xihui18 and Z708, the material of the QTL mapping population was a secondary F_2 population of 150 individuals constructed using Xihui18/Z708.

2.2 Planting methods

In July 2020, Xihui18 was crossed with Z708 and the hybrid seeds were harvested at the experimental station (Xiema town, Beibei distinct at 106.38° east longitude and 29.76° north latitude) of Southwest University, Chongqing, China. In September of the same year, the hybrids were sown at the Lingshui base (109.86° east longitude and 18.42° north latitude) in Hainan Province, China, and the F_1 seeds were harvested. In March 2021, the parent seeds of Xihui18, Z708, Jinhui35, and the F_2 population were sown in a field at the Rice Research Institute of the Southwestern University of Chongqing, China. In April 2021, 30 seedlings of Xihui18, Jinhui35, and Z708 and 150 individuals from the F_2 population were transplanted to the same field, with 26.4 cm spacing between rows, 16.5 cm between hills, and 10 plants per row. In March and April 2022, 30 seedlings each of Xihui18 and Z708 and four F_2 individuals for the development of SSSLs were cultivated and transplanted in the same field in Chongqing. Further, an F_3 population was sown for the fine-mapping of *qGW4*, and all plants were transplanted into the same field in Chongqing. In March and April 2023, four SSSLs for QTL identification, as well as Xihui18 and Z708 were sown and transplanted (30 individuals for each line) in Chongqing. Additionally, four secondary heterozygous F_3 individuals for the development of SSSLs and one NCL (all lanes of markers were the same as Xihui18) were cultivated for overlapping substitution mapping of *qGW4* (100 plants for each line). Field management was the same as local standard practices.

2.3 Measurement of agronomic traits

At the maturity stage, 10 plants each of Xihui18 and Z708 and 150 F_2 plants were harvested. The following eight yield traits for yield-related traits were measured: plant height (PH), panicle number per plant (PN), panicle length (PL), grains per panicle

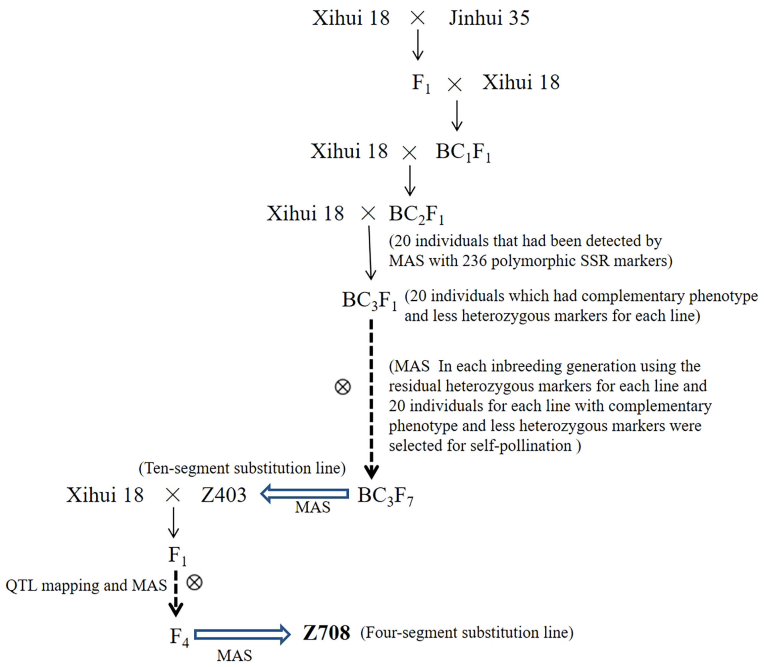

FIGURE 1
Flow chart of Z708 development.

(GPP), 10-grain length (GL), 10-grain width (GW), 1000-grain weight (GWT), and yield per plant (YD). The ratio of length to width (RLW) was calculated by dividing the grain length by grain width as described by Hui (Hui et al., 2020). Finally, simple statistical analyses, including the determining the mean value of each trait and the standard deviation, Student's *t*-test for comparison between eight traits between Xihui18 and Z708, frequency distribution analysis of grain width in the F_3 population, and the chi-square test, were performed using the statistical functions in Microsoft Excel 2016.

2.4 QTL mapping

The DNA of the parental plants and 150 F_2 individuals used for QTL mapping was extracted using the CTAB method described previously (McCouch et al., 1988). PCR amplification, non-denaturing polyacrylamide gel electrophoresis, and rapid silver staining were performed, as described previously (Zhao et al., 2016). The Xihui18-type band was scored as '-1,' the Z708-type band was scored as '1,' the heterozygote was scored as '0,' and a missing band was scored as'.'. The specific description of QTL mapping using the mixed linear model (MLM) method in SAS 9.3 software was the same as that described in a previous study (Zhao et al., 2016). The threshold for determining whether a QTL existed was set at $p < 0.05$.

2.5 Development of SSSLs

Based on the QTL mapping information, four F_2 individuals carrying only one single target substitution segment and 0-1 heterozygous markers for each line were selected and planted as Z1229, Z1231, Z1232, and Z1234 in 2023, with 30 plants per line. Then, the leaves of 20 individuals were taken from each line to construct SSSLs using MAS with the target substitution markers and residual heterozygous markers. Finally, the homozygous SSSLs (S1, S2, S3, and S4) were screened.

2.6 Identification and analysis of the additive effect of QTL for yield traits using four SSSLs

At maturity, 10 plants each of Xihui18 and SSSLs (S1-S4) were harvested. Eight yield traits were measured: grain length, grain width, ratio of length to width, 1000-grain weight, panicle number per plant, panicle length, grain number per panicle, and yield per plant. The specific method has been described by Xu and Sun (Xu et al. 2023; Sun et al. 2022). For each SSSL (S1-S4), QTL were identified using one-way analysis of variance (ANOVA) and least significant difference (LSD) multiple comparisons with Xihui18 for each $SSSL_i$ in IBM SPSS Statistics 25.0. At *p*-value < 0.05, a QTL for a certain trait was considered to exist in the $SSSL_i$. The genetic model in a certain environment was $p_0 = \mu_0 + \varepsilon$ for Xihui18 and $p_i = \mu_0 + a_i + \varepsilon$ for an SSSL carrying a specific QTL (p_0 and p_i represented the phenotypic value of any plant in a plot of xihui18 and the $SSSL_i$, μ_0 represented the mean value of the Xihui18 population, a_i represented the additive effect of the QTL). Thus, a_i was equal to half of the difference between p_i and p_0 (half was estimated as the genetic effect).

2.7 Inheritance analysis, fine-mapping, and overlapping substitution mapping of *qGW4*

An F_3 population comprising 285 individuals developed from an F_2 recombinant plant of *qGW4* was used for *qGW4* inheritance analysis and fine mapping. Among them, 74 recessive individuals (narrow grains) and seven newly designed polymorphism SSR markers together with RM3276, were utilized to analyze the linkage with *qGW4*. Moreover, four individuals with different genotypes were screened to construct secondary SSSLs of *qGW4* in the F_3 generation. Furthermore, the grain widths of all SSSL individuals and one NCL population (10 individuals) (all lanes of markers were the same as Xihui18) were measured to be utilized for the overlapping substitution mapping of *qGW4*. The specific method has been described previously (Yang et al., 2021). When the grain width differed significantly between a secondary SSSL and Xihui18, the QTL controlling grain width was located in the substitution segment of the SSSL. When multiple substitution segments in the SSSLs overlapped with the grain width, the QTL was mapped to the overlapping region.

3 Results

3.1 Detection of substitution segments in CSSL-Z708

Based on the previous breeding of Z708 with 236 polymorphic SSR markers between two parents selected from 429 markers covering the whole rice genome 16 SSR markers in four substitution fragments and 36 outside them from 12 chromosomes were used to detect the accuracy of the substitution segments and the purity of genetic background in ten plants of Z708. The substitution segments of the 10 individuals of Z708 were identical, and no other residual fragments from Jinhui35 were detected. These results confirm the accuracy of the genotype in CSSL-Z708. Z708 contained four substitution segments from Jinhui35 in the Xihui18 background with a total length of 14.08 Mb and an average substitution length of 3.52 Mb, which were distributed on chromosomes 4, 5, 6, and 12 (Figure 2).

3.2 Phenotype analysis of Z708 and Xihui18

As only four substitution segments differed from Xihui18, Z708 was considered a near-isogenic line (NIL) relative to Xihui18. The Z708 plant type (Figure 3A) resembled that of Xihui18, but still exhibited a heavy-spike phenotype. The most attractive characteristics of Z708

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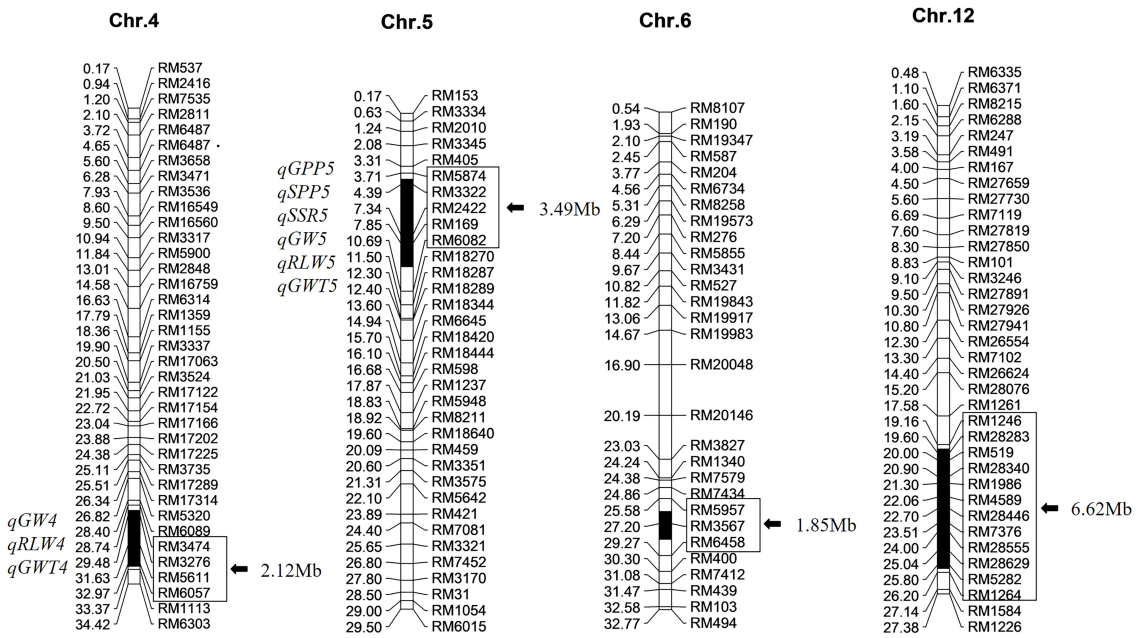

FIGURE 2
Chromosome substitution segments and the harbored QTL of Z708. The reference genome is indica rice cultivar '9311'. The physical distances (Mb) and QTL are marked on the left of each chromosome, the markers and lengths of the substitution segments exhibit on the right. Black sections on each chromosome indicate the substitution segments.

were its large grain size (Figures 3B–G) and less grain number (Figures 3H–L, Table 1). Compared to Xihui18, the grain width of Z708 increased significantly by 25.48% to 3.78 mm (Figures 3B, C, Table 1), while the 1000-grain weight (39.38 g) of Z708 increased by 36.31% (Figure 3G). In contrast, the grain length (10.54 mm) (Figures 3D, E) and the ratio of length to width (Figure 3F, Table 1) in Z708 were significantly lower, by 1.12% and 21.20%, respectively. Additionally, the number of primary branches (13.81), secondary branches (30.82), spikelets per panicle (170.18), and grains per panicle (145.75) of Z708 decreased significantly by 20.27%, 26.50%, 32.96%, and 33.79%, respectively, compared to those in Xihui18 (Figures 3I–L, Table 1).

3.3 QTL for yield traits harbored in the substitution segments of Z708

Using an F_2 segregated population (the specific parameters in Table 1) from Xihui18/Z708 for QTL mapping, nine QTL were detected on chromosomes 4 and 5, including six for grain size and three for grain number related traits (Table 2). The wide grain of Z708 was controlled by *qGW4* and *qGW5*, both with additive effects of 0.08 mm, which explained 17.17% and 15.32% of the phenotypic variation, respectively. The additive effects of *qRLW4* and *qRLW5* from Jinhui35 reduced ratio of length to width of Z708 by 0.09 and 0.07, respectively, explaining the variation of 24.94% and 16.37%, respectively. The additive effects of *qGWT4* and *qGWT5* from Jinhui35 alleles enhanced the 1000-grain weight of Z708 by 1.13 g and 1.15 g, explaining the variation of 16.23% and 16.86%, respectively. The less grain numbers of Z708 was resulted from *qGPP5*, *qSPP5* and *qSSR5* from Jinhui35, whose additive effect reduced 9.54 grains per panicle, 8.30 spikeletes per panicle and 1.25 pencent points of seed setting rate in Z708, explaining 4.04%, 2.68% and 2.11% of the according variation, respectively (Table 2).

3.4 Construction of SSSLs and identification of QTL for yield traits

Four SSSLs (S1, S2, S3, and S4) were developed using marker-assisted selection (MAS) based on the QTL mapping results (Figure 4A). The single-segment substitution line S1 harbored the substitution segment RM3476–RM3276-RM5611–RM6057 of chromosome 4. S2 carried the substitution segment RM5874–RM3322-RM2422-RM169–RM6082 of chromosome 5. S3 contained the substitution segment RM5957–RM3567–RM6458 of chromosome 6. S4 harbored the substitution segment RM1246–RM1986-RM2855-RM5282–RM1264 of chromosome12. The seven QTL identified above were validated using S1 and S2. Further, nine QTL for yield traits were detected by S1, S2, S3, and S4, including *qGPP4*, *qGPP6* and *qGPP12* for grain number per panicle; *qYD4*, *qYD5*, *qYD6* and *qYD12* for yield per plant; and *qPN4* and *qPN6* for panicle number per plant (Figure 4A).

The grain width (3.11and 3.74 mm) of S1 carrying *qGW4* (a = 0.05 mm) and S2 containing *qGW5* (a =0.36 mm) was significantly wider than that of Xihui18 (3.01 mm), whereas S3 and S4 without QTL for *GW* showed no significant differences in grain width (3.02 and 3.02 mm) from Xihui18 (3.01 mm) (Figure 4B). The ratios of the length to width (3.36 and 2.82) of S1 harboring *qRLW4* (a = −0.05) and S2 with *qRLW5* (a = −0.32) were significantly less than

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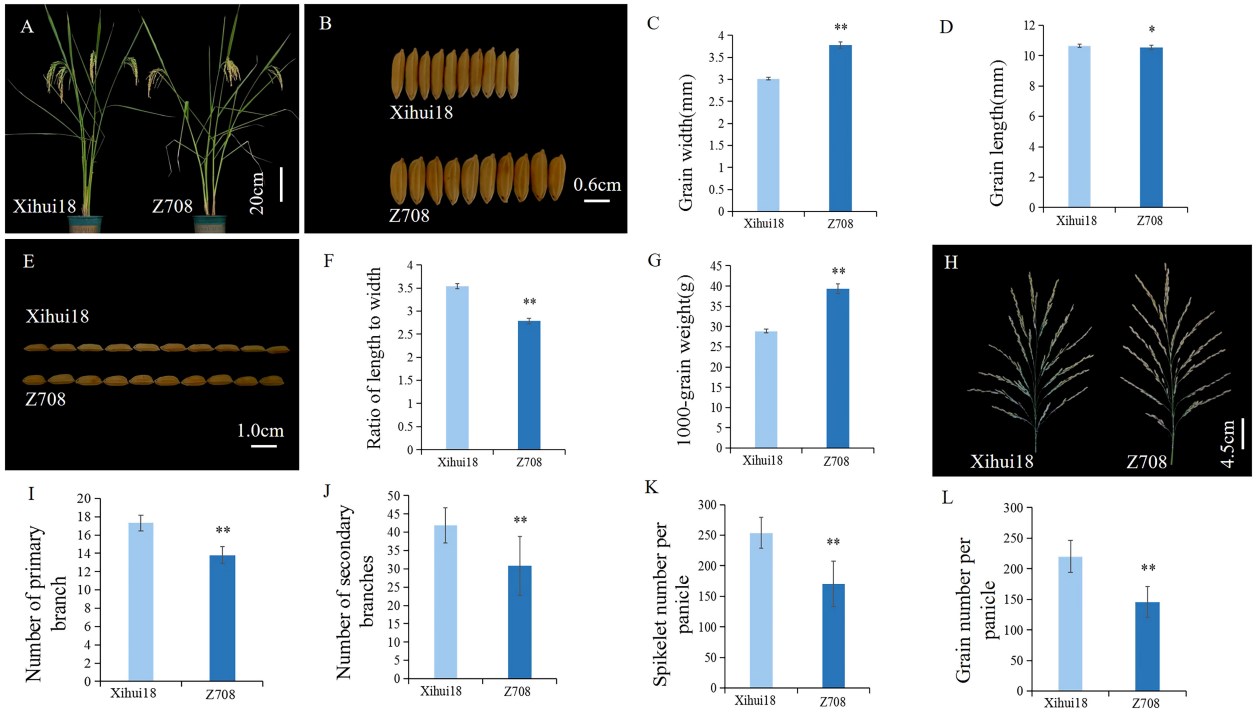

FIGURE 3
Plant-type and grain size analysis of Xihui18 and Z708. **(A)** The phenotype of Xihui18 and Z708 at the milky stage. **(B, C)** Grain width of Xihui18 and Z708. **(D, E)** Grain length of Xihui18 and Z708. **(F)** Ratio of length to width, **(G)** 1000-grain weight. **(H)** spike shape of Xihui18 and Z708. **(I)** Number of primary branch. **(J)** Number of Secondary branches. **(K)** Spikelet number per panicle. **(L)** Grain number per panicle. * and ** represent differences at the 0.05 and 0.01 levels, respectively.

those (3.46, 3.42 and 3.44) of the recipient parent Xihui18, S3, and S4 without QTL for RLW (Figure 4C). The 1000-grain weights (30.02 and 41.42 g) of S1 carrying *qGWT4* (a = 0.42 g) and S2 with *qGWT5* (a = 6.12 g) were significantly higher than those of Xihui18, S3, and S4 (29.18, 28.97, and 29.24 g, respectively) without QTL for GWT (Figure 4D). The grain length of S1-S4 without QTL for this trait was the same as that of Xihui18 (Figure 4E).

The panicle number per plant (5.4 and 5.1) in S1 *qGW4* harboring *qPN4* (a = 0.70) and S3 carrying *qPN6* (a = 0.55) was significantly higher than that of Xihui18 (4.00) and those of S2 and S4 (4.50 and 4.50, respectively) without QTL for PN (Figure 4F). Grain numbers per panicle (204.97 and 215.30) of S3 containing *qGPP6* (a = 9.09) and S4 carrying *qGPP12* (a = 14.26) were significantly greater whereas those of S1 containing *qGPP4* (a =

TABLE 1 Yield traits of Xihui18, Z708 and the F_2 population.

Traits	Mean ± SD (Parents)		F_2 population			
	Xihui18	Z708	Mean±SD	Range	Skew	Kurt
Number of primary branches	17.32±0.85	13.81±0.90**	15.20±1.14	11.17-17.71	-0.39	0.55
Number of secondary branches	41.93±4.80	30.82±8.03**	41.04±8.84	18.00-64.57	-0.02	-0.13
Spikelets per panicle	253.86±25.10	170.18±37.12**	221.29±51.388	40.67-365.00	-0.22	0.99
Grains per panicle	220.16±26.00	145.75±25.36**	193.52±48.2	17.33-328.25	-0.48	1.60
Seed setting rate (%)	86.87±3.72	86.19±4.86^{NS}	86.92±8.63	42.21-95.42	-3.03	11.1
Grain length (mm)	10.66±0.10	10.54±0.13*	10.27±0.19	9.80-10.70	0.02	-0.40
Grain width (mm)	3.01±0.03	3.78±0.07**	3.18±0.22	2.80-4.00	0.99	0.79
Ratio of length-width	3.54±0.06	2.79±0.06**	3.24±0.21	2.61-3.60	-0.77	-0.08
1000-grain weight (g)	28.89±0.50	39.38±1.20*	31.42±3.17	25.10-40.70	0.83	0.28

* and ** indicate a significant difference between the two parents at $P < 0.05$ and $P < 0.01$, respectively.

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TABLE 2 QTL for yield-related traits carried by the substitution fragments of Z708.

Trait	QTL	Chr.	Marker interval	Additive effect	Var. (%)	*P*-value
Grain width (mm)	*qGW4*	4	RM3276-RM5611	0.08	17.17	0.0009
	qGW5	5	RM3322-RM169	0.08	15.72	<0.0001
Ratio of length to width	*qRLW4*	4	RM3276-RM5611	-0.09	24.94	<0.0001
	qRLW5	5	RM3322-RM169	-0.07	16.37	<0.0001
1000-grain weight (g)	*qGWT4*	4	RM3276-RM5611	1.13	16.23	0.0013
	qGWT5	5	RM3322-RM169	1.15	16.86	<0.0001
Grains per panicle	*qGPP5*	5	RM3322-RM169	–9.54	4.04	0.0079
Spikeletes per panicle	*qSPP5*	5	RM3322-RM169	-8.30	2.68	0.0238
Seed setting rate (%)	*qSSR5*	5	RM3322-RM169	-1.25	2.11	0.0376

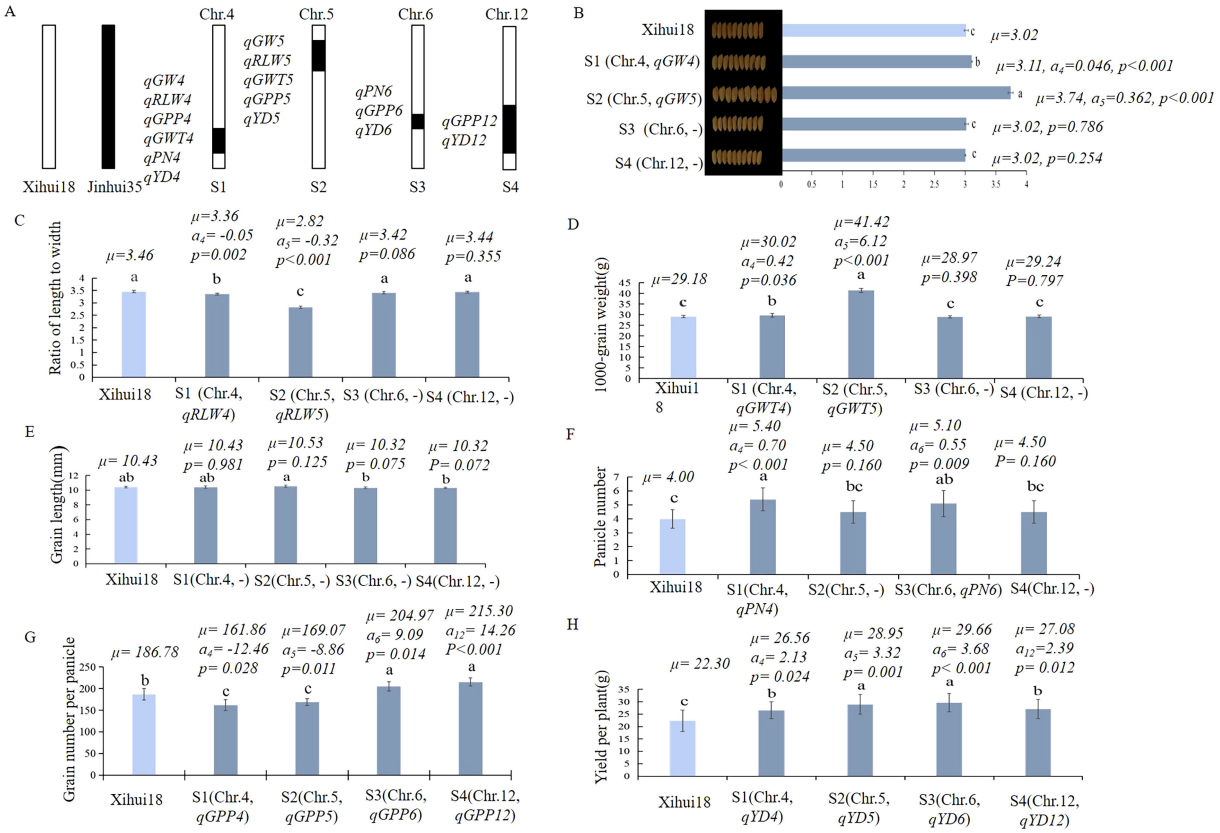

FIGURE 4
Construction of SSSL dissected from Z708 and analysis of additive effects of QTL for yield traits within them. **(A)** Diagram of the substitution segments and QTL in S1-S4. B-L: The QTL parameters in the different SSSLs. **(B)** Grain width; **(C)** Ratio of length to width; **(D)** 1000-grain weight; **(E)** Grain length; **(F)**: Panicle number per plant; **(G)** Grain number per panicle; **(H)** Yield per plant. Different lowercase letters represent significant difference ($p < 0.05$), which determined by Duncan's multiple comparison. μ: the average value of each line; a: additive effect for each QTL controlling the trait. S1 (Chr.4: RM3476 (28.74 Mb)–RM3276 (29.48 Mb)-RM5611 (31.63 Mb)–RM6057 (32.97 Mb)); S2 (Chr.5: RM5874 (3.71 Mb)–RM3322 (4.39 Mb)-RM2422 (7.34 Mb)-RM169 (7.85 Mb)–RM6082 (10.69 Mb)); S3 (Chr.6: RM5957 (25.58 Mb)–RM3567 (27.20 Mb–RM6458 (29.27 Mb)); S4 (Chr.12: RM1246 (19.16 Mb)– RM1986(21.30Mb)-RM2855 (24.0Mb)-RM5282 (25.80Mb)–RM1264 (26.20 Mb)). The middle markers connected with hyphens represent the substitution fragment from the donor, while the markers of the border linked with"–"indicate that recombination might have occurred.

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-12.46) and S2 with *qGPP5* (a = -8.86) (161.86 and 169.07, respectively) were significantly less than that (186.79) of Xihui18 (Figure 4G). The yield per plant of S1 with *qYD4* (a = 2.13 g), S2 carrying *qYD5* (a = 3.27 g), S3 containing *qYD6* (a= 3.68 g), and S4 harboring *qYD12* (a= 2.39 g) (26.56, 28.85, 29.66, and 27.08 g, respectively) was significantly higher than that of Xihui18 (22.30 g) (Figure 4H).

3.5 Genetic analysis of *qGW4* for grain width

The donor parent Jinhui35 exhibited a wide grain type, whereas the recipient parent Xihui18 displayed a narrow grain type. Z708, which carried four substitution fragments from Jinhui35 in a Xihui18 background, exhibited broad grains. In the F_3 population consisting of 285 individuals constructed using a *qGW4* recombinant plant, the frequency of grain width displayed a bimodal distribution, one peak for a narrow grain type (from 2.91 mm to 3.06 mm), with 74 plants, and the other for broad grain type (from 3.06 to 3.45 mm), with 211 individuals (Figure 5). The chi-square test indicated that the broad-grain plants (211) and thin-grain individuals (74) fitted a separation ratio of 3:1 ($\chi^2 = 0.17 < \chi^2_{(0.05,\ 1)} = 3.84$) (Table 3). These results suggested that *qGW4* controlling wide grains from Jinhui35 in S1 displayed a single dominant gene action (Figure 5).

3.6 Fine-mapping of *qGW4* controlling wide grains

Given the *qGW4* linkage with RM3276 in S1 (Figure 6A), *qGW4* was further fine-mapped using 74 recessive plants in the above F_3 population. The grain width of these recessive plants (3.01 mm) was not significantly different from that of Xihui 18 (3.01 mm) (Figure 6B). Within the largest substitution interval of RM17389 and RM17485 of S1, ten SSR markers were designed, seven of which showed polymorphisms between Xihui18 and Jinhui35. Among the seven markers, the bands of three in S1 were the same as those in Jinhui35, indicating that these markers were in the substitution segment, whereas the other four were the same as the recipient Xihui18, suggesting that they belonged to the genetic background of S1. Therefore, by linkage analysis of all markers in the substitution segment using 74 recessive plants, the genetic distances of *qGW4* were 6.20, 2.64, 0.79, and 0.79 cM from RM17468, RM17450, RM7453 and RM3276, respectively. Thus, *qGW4* was fine-mapped between RM17453 and RM3276, with a physical distance of 80 Kb (Figure 6A). We also developed a series of secondary SSSLs (S5–S8) for the overlapping substitution mapping of *qGW4* in the F_4 generation (Figure 6C). The grain width (3.03 mm) of the negative control line (NCL), whose lanes of all markers in the substitution fragment were the same as those of Xihui18, displayed no significant difference from that of Xihui18 (3.01 mm). However, S5 carrying the RM17450-RM17453-RM3276-RM17468 substitution segment, S7 containing the RM3276 segment, and S8 harboring the RM17450-RM17453- RM3276 segment exhibited significantly wider grains (3.10, 3.59, and 3.55 mm, respectively) than those of Xihui18 (3.01 mm). Further, S6 carrying the RM17468 segment, showed no difference in grain width (3.05 mm) from that of Xihui18 (3.01 mm). Therefore, *qGW4* should be in 155 kb of the estimated length, and 310 Kb of the largest substitution length of RM17453–RM3276–RM17468 (Figure 6C). These results were consistent. *qGW4* should be in 80 Kb of the minimum distance between RM17453 and RM3276, 155 Kb of the estimated length, and 301 kb of the largest substitution length of RM17453–RM3276–RM17468 (Figures 6A, C). By predicting the candidate genes within 301 kb of the largest substitution length, four candidate genes of *qGW4* were predicted to encode a MYB protein, DNA-containing protein, PAP fibrinogen family protein, and DUF domain protein (Table 4).

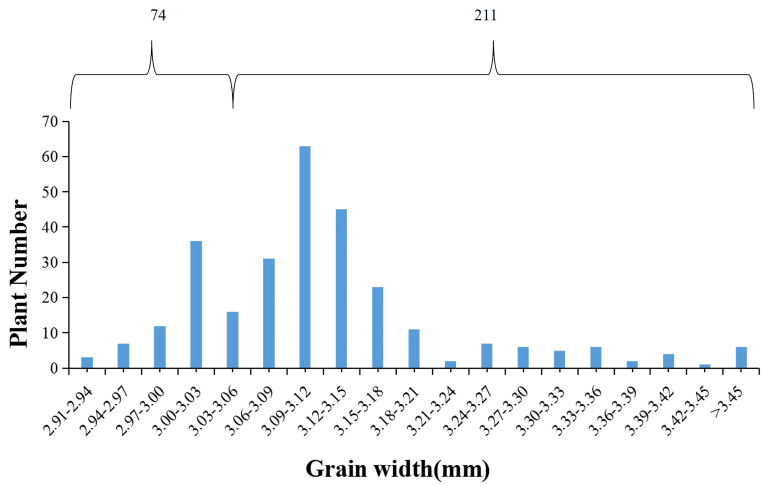

FIGURE 5
Frequency distribution of grain width in F_3 population (285 plants) derived from a recombinant individual of *qGW4*.

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TABLE 3 The chi-square test of *qGW4* in the F_3 population.

Trait	O(observed numbers)	E(theoretical number)	$(\|O-E\|-1/2)^2$	$(\|O-E\|-1/2)^2/E$	χ^2
Wide grain	211	213.75	5.06	0.02	0.09
Narrow grain	74	71.25	5.06	0.07	

4 Discussion

4.1 Eight SSSLs dissected by CSSL-Z708 have promising prospects for enhancing rice yield in breeding application

Rice grain yield is determined by three major "visible" morphological traits: grain weight, grain number per panicle, and panicle number (Li et al., 2021). These yield traits exhibit complex inheritance patterns that are often controlled by several minor QTL. Chromosomal segment substitution lines are ideal materials for the genetic dissection and pyramiding of favorable QTL (Ebitani et al., 2005; Ando et al., 2008; Balakrishnan et al., 2019; Nagata et al., 2023). Here, we characterized a less-grain and wide-grain rice CSSL, Z708, which contained four substitution fragments from Jinhui35 in a Xihui18 background, with an average substitution length of 3.52 Mb.

Although Z708 has some favorable alleles from Jinhui35 for wide and large grains, Its direct use in rice breeding is challenging because of the presence of multiple QTL. To construct more suitable materials for the direct breeding and functional analysis of key genes, eight SSSLs containing various QTL were developed from Z708. As each SSSL has only one difference of a single substitution fragment from its recipient parent, they can identify QTL accurately, which can be verified by the fact that S1-S4 detected more QTL (16) for yield traits than those (7) detected using the F_2 population of Xihui18/Z708. Many studies have confirmed the high efficiency of QTL detection (Ando et al., 2008; Teng et al., 2012; Ookawa et al., 2016; Zhang et al., 2021a; Hui et al., 2020). Notably, S1, S2, S3, and S4 displayed a significant increase in yield per plant compared to that of Xihui18. For instance, S1 which harbors *qPN4* (a = 0.70), *qGW4* (a = 0.05 mm), *qGWT4* (a = 0.42 g), and *qGPP4* (a = -12.46) exhibited a 17.01% increase in yield per plant relative to Xihui18 (22.30 g); S2 carrying *qGW5* (a = 0.36 mm), *qGWT5* (a = 6.12 g), and *qGPP5* (a = -8.86) showed a 29.4% increase in yield; S3 containing *qPN6* (a = 0.50) and *qGPP6* (a = 9.09) achieved a 33.00% increase, while S4 with *qGPP12* (a = 14.26) increased the yield per plant by 21.40% than that of Xihui18 (22.30 g) (Figure 4). Notably, both Xihui18 and Jinhui35 are strong restorer lines carrying restorer genes *Rf1* and *Rf3* on Chr.1, *Rf2* on Chr.2, and *Rf4* on Chr.10 (Akagi et al., 2004; Itabashi et al., 2011; Cai et al., 2013), Thus, some of them could be used as restorer lines to breed new hybrid cultivars by combining with sterile *indica*

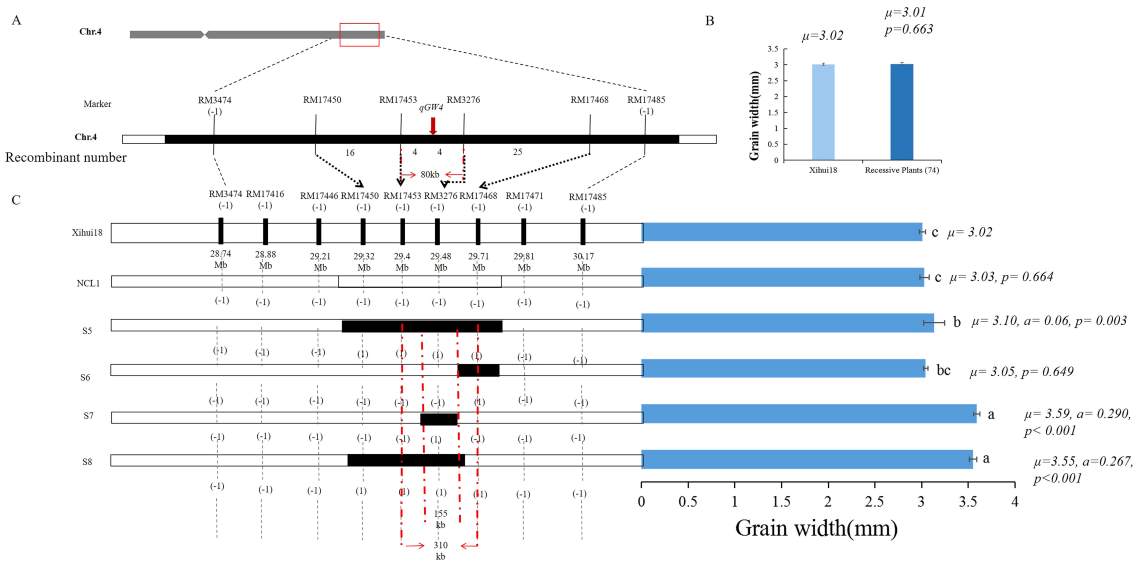

FIGURE 6
Fine mapping and substitution mapping of *qGW4*. **(A)** Fine-mapping of *qGW4* by linkage analysis. **(B)** Statistic analysis of grain width between Xihui18 (10 plants) and the recessive individuals (74 plants) in the F_3 population. **(C)** Overlapping substitution mapping of *qGW4*. The black regions represent the estimated substitution length. NCL, negative control line (the lanes of all marker in corresponding substitution intervals same with Xihui18). Different lowercase letters **a**, **b** and **c** on each top column represent existing difference at 0.05 level, which is determined by Duncan's multiple comparison.

TABLE 4 Candidate genes predicted within the *qGW4* fine-mapping interval.

Possible candidate genes	encoding proteins
Candidate gene 1	LOC_Os04g51800 (MYB protein)
Candidate gene 2	LOC_Os04g51794 (DNA binding protein)
Candidate gene 3	LOC_Os04g51792 (PAP fibrinogen family protein)
Candidate gene 4	LOC_Os04g51786 (DUF domain protein)

lines. Therefore, these SSSLs have promising prospects for improving rice yield in breeding by design.

4.2 Comparison of QTL for yield traits identified in the study with the reported genes

In total, 16 QTL for yield traits were detected in both the F_2 population derived from Xihui18/Z708 and the dissected SSSLs. We compared these QTL with previously reported genes located within the corresponding substitution intervals, *qGW4*, *qRLW4*, *qGWT4*, *qPN4*, *qGPP4*, and *qYD4*, all linked to RM3276. Within the substitution interval, *OsAGO2* regulates the distribution of cytokinins by activating *BG3*, thereby increasing rice grain weight, but has little effect on grain width (Yin et al., 2020). Thus, *OsAGO2* may be a candidate gene for *qGWT4*. Determining whether *OsAGO2* is a candidate gene for *qGWT4* requires genetic complementarity studies in the future. Finally, *qGW4* was fine-mapped to 80 Kb of the minimum interval and 301 Kb of the largest substation interval, and four candidate genes were predicted. *MONOCULM 3* encoding *WUSCHEL*, is a key gene involved in the formation of rice tiller buds (Lu et al., 2015). Thus, *MOC3* may be a candidate gene for *qPN4*. *qGW5*, *qRLW5*, *qGWT5*, *qGPP5*, and *qYD5*, all linked to RM2422. *OsGSK2* was found in the substitution interval. *OsGSK2*, a homolog of Arabidopsis *BIN2*, affects cell proliferation and expansion to negatively control grain size by phosphorylating substrates, including *OFP3* (Tong et al., 2012; Xiao et al., 2020). Thus, *OsGSK2* may be a candidate gene for *qGW5*, *qRLW5*, *qGWT5*. Again, *qGW5* and *qGWT5* were also identified as *qGW5* and *qGWT5-2* by Sun et al. using CSSL-Z431 (Sun et al., 2022). However, they came from different donors (Huhan 3), and whether they are different alleles requires further study. *qPN6*, *qGPP6*, and *qYD6* were linked to RM3567. Within the substitution interval of S3, *MOC1/GNP6* regulates tiller and grain number development (Zhang et al., 2021b). Thus, *MOC1/GNP6* is a candidate gene for *qPN6*, *qGPP6*, and *qYD6*. *qGPP12* and *qYD12* were linked to RM1986. Within the substitution interval of S4, *GNP12* encodes *RGH1A* protein and regulates rice yield by regulating panicle length, grain number per panicle, and grain length (Pan et al., 2022). *OsVIL2* improves biomas and grain production by suppressing *OsCKX2* chromatin (Yang et al., 2019). Thus, *GNP12* and OsVIL2 may be candidate genes for *qGPP12* and *qYD12*. However, whether these genes are candidate genes for the related QTL requires further verification using genetic complementarity experiments.

In conclusion, *qGW4*, *qGPP4*, *qYD4*, *qGPP5*, and *qYD5*, which have not been previously reported, provide a good foundation for the functional analysis of these QTLs. Further, S1-S4 carrying favorable alleles, such as *qPN4*, *qGW4*, *qYD4*, *qGW5*, *qGWT5*, *qYD5*, *qPN6*, *qGPP6*, *qYD6*, *qGPP12*, and *qYD12* could be used as new restorer lines for breeding improved hybrid varieties.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

Author contributions

KZ: Data curation, Formal analysis, Investigation, Writing – review & editing. JY: Investigation, Writing – review & editing. ZY: Investigation, Writing – review & editing. CC: Investigation, Writing – review & editing. JR: Investigation, Writing – review & editing. ZZ: Writing – review & editing. HZ: Writing – review & editing. YL: Writing – review & editing. CZ: Writing – review & editing. FZ: Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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