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Genetic Dissection of Tiller Number *qTN4* in Rice

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Abstract: Tiller number (TN) is an important trait that contributes to yield in rice (*Oryza sativa* L.). A set of 76 chromosome segment substitution lines (CSSLs) derived from the super-hybrid cross between Zhonghui 9308 (ZH9308) and Xieqingzao B (XQZB) was used to map quantitative trait loci (QTL) controlling tiller number (TN). A total of four QTLs were detected in Fuyang, Zhejiang Province (30.15° N, 120° E). Two QTLs were detected in Lingshui, Hainan Province (18.5° N, 110° E) in our previous study. To further map the QTL on chromosome 4, namely *qTN4*, the line CSSL29 with a lower tiller number was selected to cross with ZH9308 to develop the secondary F_2 population. In the $F_{2:3}$ population, the *qTN4* was validated and subsequently narrowed down to a 4.08 Mb region. What is more, combined phenotype with genotype, *qTN4* was dissected into two QTLs, *qTN4.1* and *qTN4.2*, in the $F_{4:5}$ population. The *qTN4.1* and *qTN4.2* explained 34.31% and 32.05% of the phenotypic variance, with an additive effect of 1.47 and 1.38, respectively. Finally, the *qTN4.1* and *qTN4.2* were fine-mapped into a 193.55 Kb and 175.12 Kb intervals on chromosome 4, respectively. Based on genotype and phenotype, four near-isogenic lines (NILs) were selected in the mapping populations. Compared with NIL^{CSSL29}, tiller number (TN), grain setting rate, grain length (GL), the ratio of grain length to width (LWR) and grain yield per plant of NIL^{ZH9308}, NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308} were increased, and the heading date of these three lines were earlier than that of NIL^{CSSL29}. Interestingly, among the candidate genes of *qTN4.1* and *qTN4.2*, except for *LOC_Os04g23550*, none of the other genes has been cloned, indicating the existence of a novel gene-controlling tiller number. These results lay a foundation for the analysis of QTL controlling tiller number in ZH9308 and provide a theoretical basis for the application of ZH9308 in super-hybrid breeding.

Keywords: genetic dissection; quantitative trait loci; tiller number; fine mapping

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Citation: Chen, H.; Fan, Y.; Li, D.; Sun, Z.; Ruan, Z.; Yang, H.; Kang, Y.; Wu, W.; Liu, Q.; Zhan, X.; et al. Genetic Dissection of Tiller Number *qTN4* in Rice. *Agriculture* **2023**, *13*, 411. https://doi.org/10.3390/agriculture13020411

Academic Editor: Nour Ahmadi

Received: 17 December 2022
Revised: 27 January 2023
Accepted: 6 February 2023
Published: 9 February 2023

1. Introduction

Rice (*Oryza sativa* L.) is an important cereal crop that is widely grown around the world [1]. Nowadays, there are more and more people, but less and less land for agriculture [2,3]. With more than half of our population selecting rice as the staple food, the yield of rice is crucial to our food security [4]. The yield in rice is determined mainly by three yield components, including panicle number, the number of grains per panicle and the weight of 1000 grains (TGW) [5,6]. The rice tiller is the branch that occurs at the unelongated basal internode [7]. The process of tillering in rice is mainly divided into two stages: the first stage is the formation of an axillary bud at each leaf axil, and the second stage is the subsequent growth of axillary buds [8]. The tiller number determines the number of productive panicles, affecting the yield of rice to a certain extent [9,10]. Thus, it is necessary to understand the genetic and molecular basis of the regulation of tiller numbers.

The molecular mechanisms regulating the tiller number of rice have been analyzed in the past several decades [11]. According to the Gramene Database (https://archive.gramene.org/ (accessed on 10 October 2022)), 258 QTLs related to tiller have been detected in rice so far, 213 QTLs controlling the tiller number of rice [12], 35 QTLs for regulating tiller angle, and 10 QTLs for tiller bud dormancy. The distribution of QTLs for tiller numbers was found on all 12 chromosomes of rice, but the discovered QTLs on chromosomes 9 and 10 were less than that on other chromosomes. There are 20 QTLs for tiller number on chromosome 4, according to the Gramene Database. Although the large number of QTLs detected are associated with tiller numbers, few of them have been cloned [9]. Recently, several QTLs, including *OsIAA17q5* and $HTD1^{HZ}$ for tiller number, have been cloned, or some cloned QTLs for other traits have been found to affect tiller number, such as *An-2* and *Hd3a* [13–18]. The novel gene *OsIAA17q5*, which is a major QTL for tiller number and belongs to the member of the auxin-responsive gene family, was identified on chromosome 5 [13]. The gene $HTD1^{HZ}$, an allele of *HTD1*, was cloned by QTL mapping for the tiller number. $HTD1^{HZ}$ can maintain strigolactone (SL) at a certain level so that it can partially relieve the inhibition of tiller bud growth, but still maintain normal panicle development [14]. *An-2* related to awn length was first discovered in 2015 [15], and subsequent studies found that this gene encodes a cytokinin synthase and reduces plant yield by reducing the grains and tiller number [15,16]. *Hd3a* is a photoperiod-sensitive gene in rice, which was originally mapped on chromosome 6 in rice as a QTL associated with the heading date [17]. It was found that *Hd3a* promotes tiller number by activating lateral bud growth in subsequent studies [18]. Meanwhile, great progress has been made for key genes regulating tiller number in rice using mutants. Since the key gene *MOC1* regulating tiller number was cloned [7], many genes affecting tiller number have been discovered through mutants [11], such as *MOC3* [19,20], *FON1* [8,21], *DLT10* [22], and so on.

It is difficult to detect and clone QTLs regulating tiller number because detection for quantitative traits is affected by some key environmental factors. Proper sowing density is beneficial to form the tiller number for winter rye [23] and single season, density, and interactions between season and density, have different effects on the tiller number in rice [24]. Tiller numbers are related to photothermal quotient (PTQ, i.e., radiation/temperature) and a higher PTQ-positive regulating tiller number [25,26]. Some QTLs associated with tiller number are detected only at certain developmental stages and are extremely unstable. A total of 23 QTLs for tiller numbers were identified using recombinant inbred lines, most of which were detected in only one specific period, suggesting that the number of QTLs affecting the tiller number was different at different measuring stages [27]. QTL was detected at two stages after sowing under sufficient water conditions and low-moisture stress conditions using the double haploid (DH) population, confirming that six and five QTLs were identified under low and sufficient water conditions, respectively, among which only *qNOT8-1* was detected at both measuring stages under sufficient water conditions, and the remaining were detected at only one measuring stage under one condition [28]. In a DH population, *qTNN2-1* and *qTNN4-1* were detected under normal nitrogen conditions, while, only one QTL, namely *qTNL2-1*, was identified under low-nitrogen cultivation conditions in the same population [29]. What is more, *qTNN2-1* and *qTNL2-1* were mapped on different positions at chromosome 2. Researchers compared the results from populations derived from a cross between Zhenshan 97 and Minghui 63, and the populations included an $F_{2:3}$ population [30], a VF_2 population [31], a recombinant inbred line (RIL) population [32] and an immortalized F_2 population [33,34], implying that tiller number is a developmental process and is more susceptible to environmental conditions and management practices under field conditions [5]. Therefore, it is significant to map or clone QTLs related to tiller number in rice, which is beneficial in the cultivation of excellent rice varieties.

Although many scholars have researched the QTL of tiller number in rice, the regulation mechanism of tiller number remains unclear. Therefore, much work needs to be done on tiller numbers in rice. Moreover, the construction of chromosome fragment substitution lines can help to map or clone more favorable and naturally mutated germplasm genes.

In a previous study, Zhonghui 9308 (ZH9308) and Xieqingzao B (XQZB) were used to construct the chromosome segment substitution lines (CSSLs) populations [35]. ZH9308 is an excellent restorer line and has been widely used in hybrid rice breeding, while the XQZB maintainer line is one of the parents of Xieqingzao A (XQZA), a male-sterile line. As we know, Xieyou 9308, one of the representative varieties of super-hybrid rice with high yields, derives from the super-hybrid cross between ZH9308 and XQZA. Although QTL associated with grain size was detected using a set of 76 CSSLs, there were phenotypic variations, especially tiller number, in this population, indicating that there was a QTL that regulated tiller number. Here, we identified a novel QTL, named *qTN4*, on chromosome 4 for the tiller number in rice by using these CSSLs. This study aimed to map and analyze the *qTN4* for tiller number in the ZH9308 variety, and to provide a theoretical basis for the application of ZH9308 in breeding in the future.

2. Materials and Methods

2.1. Plant Materials

Using the indica-japonica subspecific line ZH9308 and indica cultivar XQZB as parents, the CSSL population was constructed [31]. The CSSL29 line, one of 76 CSSLs, had significantly fewer tillers and harbored an XQZB segment in the target region on chromosome 4 under the background of ZH9308. The CSSL29 line was crossed with ZH9308 to generate secondary F_2 populations for subsequent mapping of the *qTN4*. There were 67 lines in the $F_{2:3}$ population, each line with 16 individuals (Figure S1). The $F_{3:4}$ population was obtained from the selfing of 22 heterozygous recombinants selected based on the phenotype and genotype in the $F_{2:3}$ progenies for QTL further mapping. The $F_{2:3}$ population and the $F_{3:4}$ population were planted in Fuyang in 2020 and in Lingshui in 2021, respectively. Similarly, 1600 individuals in the $F_{4:5}$ and $F_{5:6}$ with 4096 individuals were generated to narrow down the interval of the *qTN4*. These two populations were planted in Fuyang in 2021 and in Lingshui in 2022, respectively. In addition, combining the 120 markers mentioned in previous studies [31] with 51 new polymorphic markers (Table S1), two near-isogenic lines (NILs) were selected, named NIL^{ZH9308} and NIL^{CSSL29}. These two NILs were planted in Lingshui and Fuyang, and derived from the same residual heterozygote which was heterozygous in the interval of *qTN4* and homozygous in the rest region (Figure 1). The *qTN4* was dissected into two QTLs, *qTN4.1* and *qTN4.2*, in the $F_{4:5}$ population according to the genotype and phenotype of this population. In the $F_{5:6}$ population, we used the previous markers and 28 new polymorphic markers to select two new NILs (Table S1), NIL-$qTN4.1^{ZH9308}$ and NIL-$qTN4.2^{ZH9308}$, whose genotypes were consistent with ZH9308 in the interval of *qTN4.1* and *qTN4.2*, respectively (Figure 1).

The NIL^{CSSL29} was sown in two stages in Fuyang in 2022 with an interval of 10 days. On one stage, NIL^{CSSL29} together with NIL^{ZH9308} were sown on 10 May 2022, and on the other, NIL^{CSSL29}, NIL-$qTN4.1^{ZH9308}$ and NIL-$qTN4.2^{ZH9308}$ were sown at the same location on 20 May 2022. All the plant materials were grown in the experiment fields of the China National Rice Research Institute (CNRRI) in Lingshui, Hainan Province (18.5° N, 110° E) and Fuyang, Zhejiang Province (30.15° N, 120° E) in winter and spring, respectively. About 25 days after sowing, all the seedings were transplanted to the experiment field. Field management followed local agricultural practices.

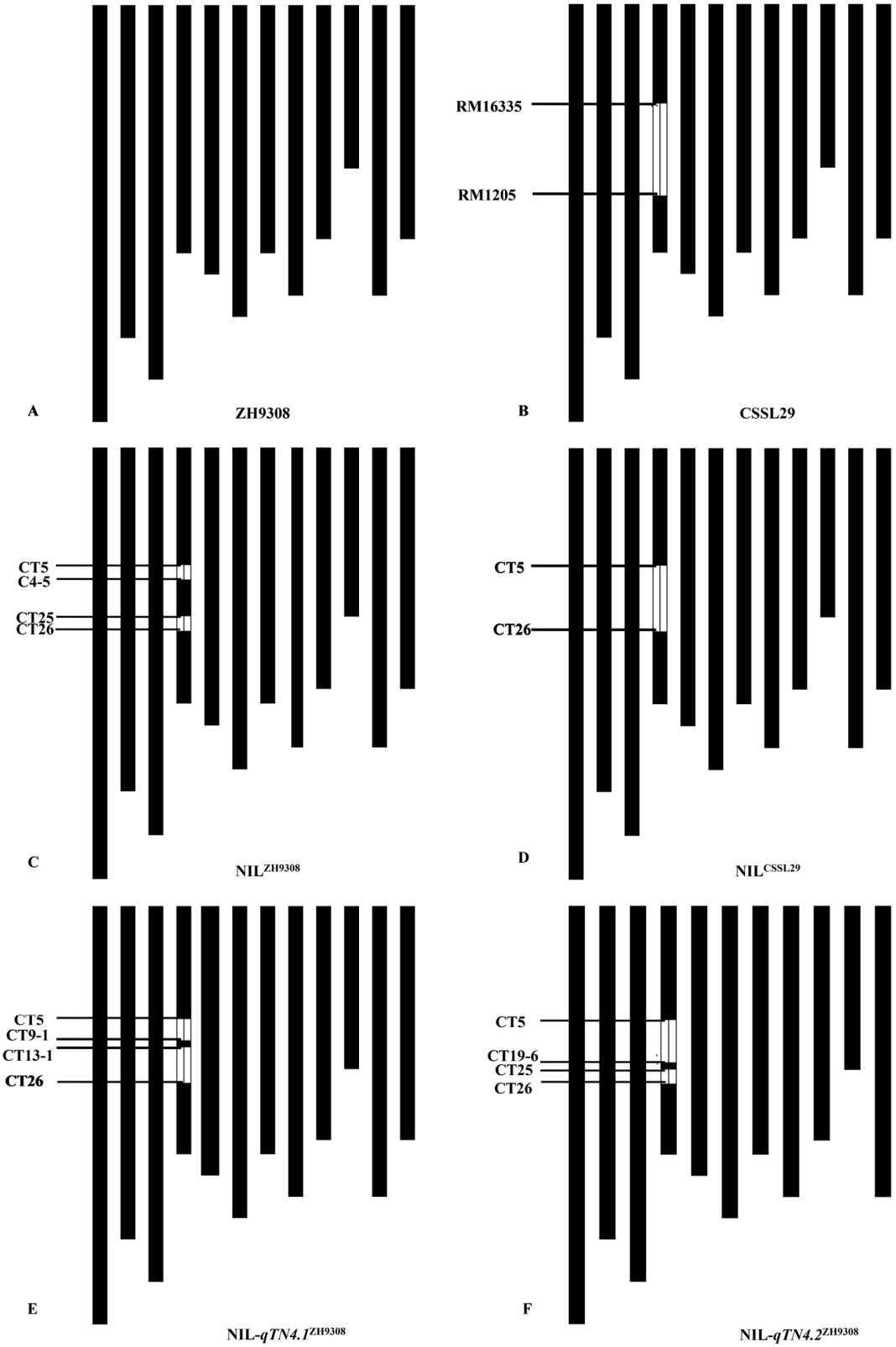


Figure 1. The genotype of ZH9308, CSSL29 and four NILs. (**A**) The genotype of ZH9308. (**B**) The genotype of CSSL29. (**C**) The genotype of NIL^{ZH9308}. (**D**) The genotype of NIL^{CSSL29}. (**E**) The genotype of NIL-*qTN4.1*^{ZH9308}. (**F**) The genotype of NIL-*qTN4.2*^{ZH9308}. The black regions indicate that the regions are homozygous for the ZH9308 genome. The white ones indicate that the regions are homozygous for the XQZB genome. NILs, near-isogenic lines.

2.2. Phenotype Measurement

The tiller number was surveyed at the tillering stage when the tiller number reached the maximum by manual counting. Plant height was measured from the soil surface to the tip of the highest spike, excluding the awn, at the time of maturity. In this study, we investigated the dynamic change in the tiller number of the NIL^{ZH9308}, NIL^{CSSL29}, ZH9308 and CSSL29. The survey was conducted nine times in a seven-day cycle starting from the 29th day after germination. Sun-dried threshed seeds were used to measure the phenotype of the yield trait using the automatic seed examination system (Wanshen Detection Technology Co., Ltd., Hangzhou, China) [36].

2.3. Molecular Markers and Genotyping

Total molecular markers, including SSR (Simple Sequence Repeat) and InDel (insertion-deletion), are shown in Supplemental Table S1. The InDel markers were designed using SnapGene software based on the sequence difference between the ZH9308 and XQZB genomic sequence.

DNA was extracted referring to the CTAB methods with minor modifications [37]. The protocol is as follows: take an appropriate amount of fresh plant leaves in a 2.0 mL centrifuge tube, grind them into powder with liquid nitrogen, next add 500 µL CTAB into the centrifuge tube, incubate at 65 °C for 30 min, and mix upside down every 15 min. Then, add 500 µL a mixture of trichloromethane: isoamyl alcohol (24:1). Mix upside down, centrifuge at 12,000 rpm for 10 min, transfer 200 µL supernatant into 1.5 ml centrifuge tube with pipetting gun, then add 400 µL pre-cooled anhydrous ethanol, incubate at −20 °C for 1 h, centrifuge at 12,000 rpm for 10 min, discard supernatant, dry overnight in a ventilator, add appropriate amount of sterile water to dissolve DNA the next day. Store at −20 °C for later use.

In this study, PCR products obtained by a PCR-amplified procedure were separated to identify the genotype of plants by using polyacrylamide gel electrophoresis (PAGE). The 2 × Rapid Taq Master Mix (Vazyme) was used for PCR experiment, and we added the reaction system according to the manufacturer's instructions of this reagent. Reaction procedure was 95 °C denaturation for 3 min, 42 cycles at 95 °C for 15 s, 60 °C for 15 s, 72 °C for extension with a rate of 15 s/kb, thoroughly extended for 5 min at 72 °C.

PAGE is a technique commonly used to separate proteins and oligonucleotides [38]. The main steps of this experiment are as follows: preparation of glass plate, sealing glass plate with 1% agarose, preparation of PAGE gel, addition of electric buffer, sampling, electrophoresis, silver staining, coloration and recording of results.

2.4. Data Analysis

Student's *t*-test was used to detect the phenotype differences between the parents, and different NILs. Duncan's test was used to compare the phenotype of yield traits of the parents and NILs. QTL identification for tiller number was performed using QTL IciMapping software ver. 4.2 (http://www.isbreeding.net (accessed on 22 July 2020)) with a threshold of LOD 2.5, and validation and analysis for QTL were performed using WinQTLCart software ver. 2.5 (https://brcwebportal.cos.ncsu.edu/qtlcart/WQTLCart.htm (accessed on 10 August 2021)) by composite interval mapping (CIM) method with a threshold of LOD 2.5.

3. Results

3.1. Identification of QTLs, Especially qTN4 in the CSSLs Population

In the previous study, CSSLs population containing 76 lines were developed using the indica rice varieties ZH9308 and XQZB, which were used for detecting the QTL related to grain size [31]. Meanwhile, there were significant differences between ZH9308 and XQZB in the traits of tiller number (TN), heading date (HD) and plant height (PH) (Table S2). Two QTLs on chromosome 4 and 5 related to tiller number were detected in the 76 CSSLs in Lingshui, and four QTLs for tiller number were detected on chromosome 7, 8 and 12 in

the same population in Fuyang (Table S3, Figure 2) in our previous study. The four QTLs detected in Fuyang and the two QTLs detected in Lingshui explained 7.28–37.63% and 10.62–19.45% of the phenotypic variation, with an LOD value of 7.03–21.97 and 2.98–5.11, respectively. Simultaneously, the *qTN4* on chromosome 4 was detected in Lingshui near marker RM5633, which could explain 10.62% of the phenotypic variation. The two QTLs detected on chromosome 7 were close to *Ghd7* associated with the heading date. There was a significant difference in tiller number between the two NILs of *Ghd7* [39]. The QTL on chromosome 8 detected in this study was close to *DTH8* controlling the heading date [40]. The *DTH8* can upregulate the expression of *MOC1*, which controls the occurrence of tillers in rice, thereby increasing the tiller number in rice [41]. The QTL on chromosome 12 was close to *Pita*, which is associated with blast resistance in rice [42,43]. The additive effect of the QTL on chromosome 5 was 1.82, indicating that the allele on XQZB increased the tiller number, and the allele on ZH9308 negatively regulated the tiller number. However, the additive effect of QTL on chromosome 4, *qTN4*, was −1.06, showing that the allele on ZH9308 positively regulated the tiller number, while the allele on XQZB negatively regulated the tiller number. The purpose of this study was to understand the regulatory mechanism of tiller number in ZH9308 and lay a foundation for the future application of ZH9308 in breeding. Taken together, the QTL on chromosome 4 was selected for further study.

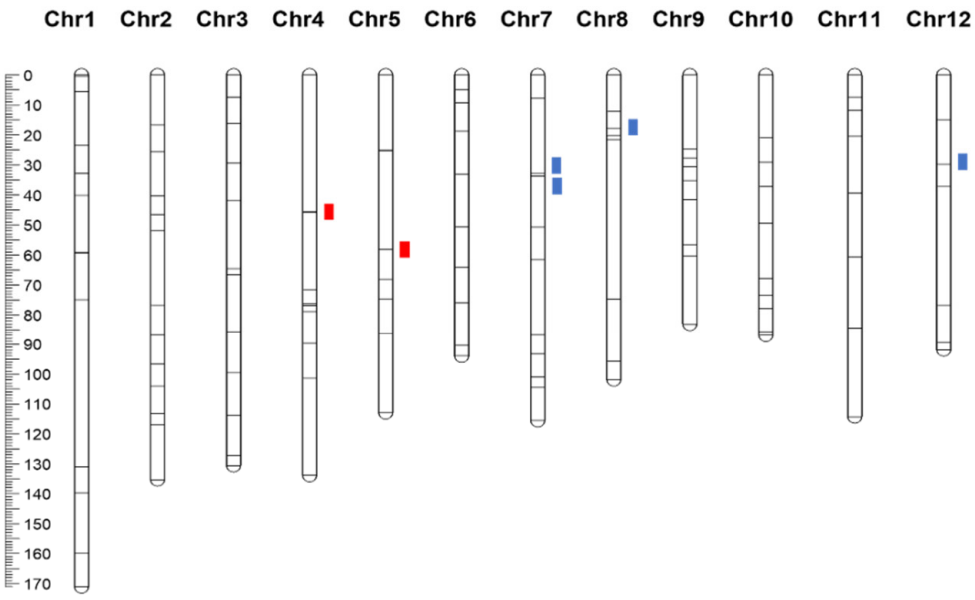
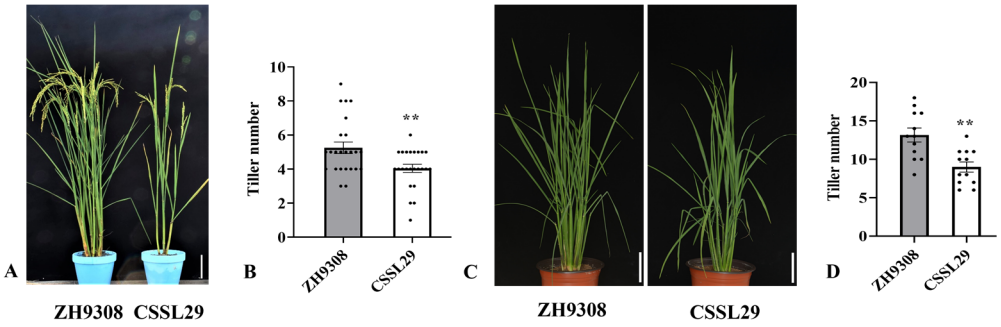


Figure 2. Genetic positions of detected QTLs for tiller number in CSSLs in Fuyang and Lingshui. The blue boxes indicate the detected QTLs in Fuyang, and the red ones show the QTLs detected in Lingshui. The scale on the left indicates map distance in centimorgans (cM). QTL, quantitative trait loci; CSSLs, chromosome segment substitution lines.

3.2. Validation and Mapping of the qTN4

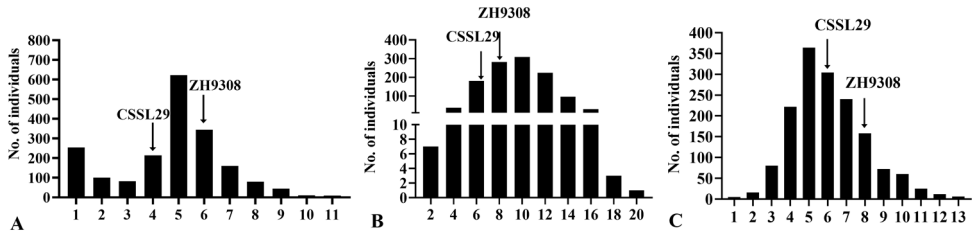
To identify the *qTN4*, we crossed CSSL29, one of the CSSL populations, with ZH9308 to generate secondary F_2 populations. Compared with ZH9308, the tiller number of CSSL29 decreased significantly in both Fuyang and Lingshui (Figure 3). Moreover, the frequency distribution of tiller number showed continuously distributed in the secondary F_2 population (Figure 4). These results suggested that there was a QTL controlling tiller number.



E

Traits	2022LS		2022FY	
	CSSL29	ZH9308	CSSL29	ZH9308
HD (d)	112.8±0.5	101.8±0.9**	83.2±0.4	91.4±0.4**
PH (cm)	72.5±1.2	86.3±0.7**	96.7±4.0	125.8±1.3**
TN	6.0±0.5	8.0±0.6*	9.3±0.9	13.2±0.9**

Figure 3. Phenotype of ZH9308 and CSSL29: (**A**,**B**) the phenotype of ZH9308 and CSSL29 at grain-filling stage in Lingshui in 2021; (**C**,**D**) phenotype of ZH9308 and CSSL29 at tillering stage in Fuyang in 2022; and (**E**) phenotype of ZH9308 and CSSL29 in Lingshui and Fuyang in 2022. HD, heading date; PH, plant height; TN, tiller number. The CSSL29 was regarded as reference. The scale bar in (**A**) and (**C**) is 10 cm. Data in (**B**) and (**D**) are presented as means ± SEM. n = 24 in (**B**); n = 12 in (**D**). * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.



D

Year	Locations	Parents		The secondary population			
		CSSL29	ZH9308	Range	Mean±SEM	Kurtosis	Skewness
2021	Lingshui	4.0±0.2	5.3±0.3**	1.0-11.0	4.7±0.001	−0.08	−0.2
2021	Fuyang	7.3±0.3	8.2±0.6	2.0-20.0	9.1±0.002	−0.03	0.2
2022	Lingshui	6.0±0.5	8.0±0.6*	1.0-13.0	6.1±0.001	0.5	0.6

Figure 4. Frequency distribution of tiller number in the secondary population: (**A**–**C**) frequency distribution of tiller number in the secondary population in Lingshui in 2021, Fuyang in 2021, and Lingshui in 2022, respectively; and (**D**) the phenotypic performance of the parents and the secondary population. The CSSL29 was regarded as reference. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

The previous report revealed that XQZB segment substitution in CSSL29 between the marker RM16335 and RM1205, position ranged from 1,712,026 to 19,635,166 bp [36], and *qTN4* was located at this interval. Then, we used marker-assisted selection (MAS) to purify the genetic background to narrow down the interval of *qTN4* in the secondary F_2 and $F_{2:3}$ populations. Polymorphic markers were used to map the *qTN4* into a 5.38 Mb between marker CT5 and CT26 in the secondary $F_{2:3}$ population.

3.3. Dissection of qTN4 into Two Quantitative Trait Loci

The $F_{3:4}$ population, including 4324 individuals, was generated by 22 heterozygous recombinants obtained in the secondary $F_{2:3}$ population. The *qTN4* was mapped to the 4.08 Mb interval between marker C4-5 and CT25 (from 10,633,439 to 14,710,197 bp) in the $F_{3:4}$ population. Moreover, two new NILs with smaller substitution fragments were selected, named NIL^{ZH9308} and NIL^{CSSL29}, which showed the same genotype as ZH9308 and CSSL29

between markers CT5 and CT26, respectively. There was a significant difference in tiller numbers between NIL^{ZH9308} and NIL^{CSSL29}, and NIL^{ZH9308} had more tiller numbers than NIL^{CSSL29} (Figure 1).

Using polymorphic markers, we selected heterozygous recombinants in the target region in $F_{2:3}$ and $F_{3:4}$ to construct the $F_{3:4}$ and $F_{4:5}$ populations, respectively. Based on genotypic and phenotypic consistency, we dissected the *qTN4* into two independent QTLs in the $F_{4:5}$ population, designated as *qTN4.1* and *qTN4.2*. Combining phenotype with genotype in the $F_{5:6}$ population, we analyzed these two QTLs using WinQTLCart software with the CIM method. The results showed that the *qTN4.1* explained 34.31% of the phenotypic variance, with an additive effect of 1.47 within the region flanking markers C4-5 and CT13-1. Meanwhile, the *qTN4.2* was located between markers CT19-3 and CT25, explaining 32.05% of the phenotypic variance, with an additive effect of 1.38 (Table S4).

In the $F_{5:6}$ population, we selected two new NIL lines as NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308}. The genotype of NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308} which contained the *qTN4.1* and *qTN4.*, respectively, were consistent with the ZH9308 in the target interval. The tiller number of the NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308} was significantly higher than that of the NIL^{CSSL29} (Figure 5).

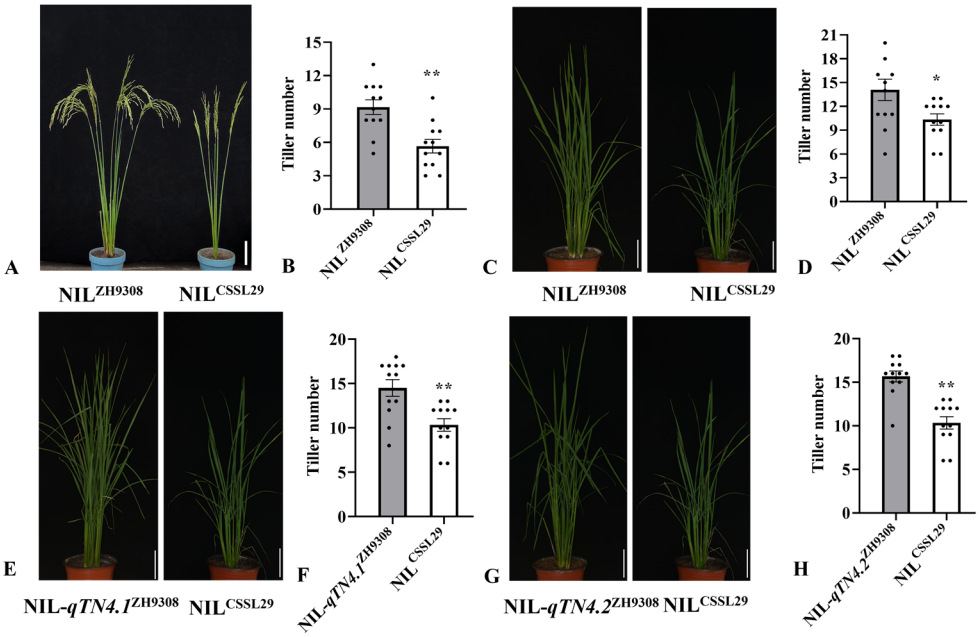


Figure 5. The phenotype of four NILs: (**A**,**B**) the phenotype of NIL^{ZH9308} and NIL^{CSSL29} at grain filling stage in Lingshui in 2021; (**C**,**D**) the phenotype of NIL^{ZH9308} and NIL^{CSSL29} at tillering stage in Fuyang in 2022; and (**E**–**H**) the phenotype of two QTLs' NILs at tillering stage in Fuyang in 2022. The scale bar is 10 cm. Data are presented as means ± SEM. n = 24 in (**B**); n = 12 in (**D**,**F**,**H**). * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

3.4. Fine Mapping of the qTN4.1 and qTN4.2

The $F_{5:6}$ population containing 4096 individuals was generated to further map *qTN4.1* and *qTN4.2*. Compared with PA29 (NIL^{CSSL29}), except for line PA146, PA104 and PA110, tiller number of the lines containing the *qTN4.1* and *qTN4.2* segment increased significantly. Combining the genotype with phenotype of these lines, especially two homozygous recombinants, the PA133 and PA27, we mapped the *qTN4.1* to the 193.55 Kb interval (from 11,504,150 to 11,645,392 bp) between CT9-1 and H6-2-1-3 based on positions of recombinant breakpoints and allelic composition (Figure 6). Meanwhile, the *qTN4.2* was mapped to the 175.12 Kb interval (from 13,433,359 to 13,610,832 bp) between CT19-6 and CT24-1 using the same method.

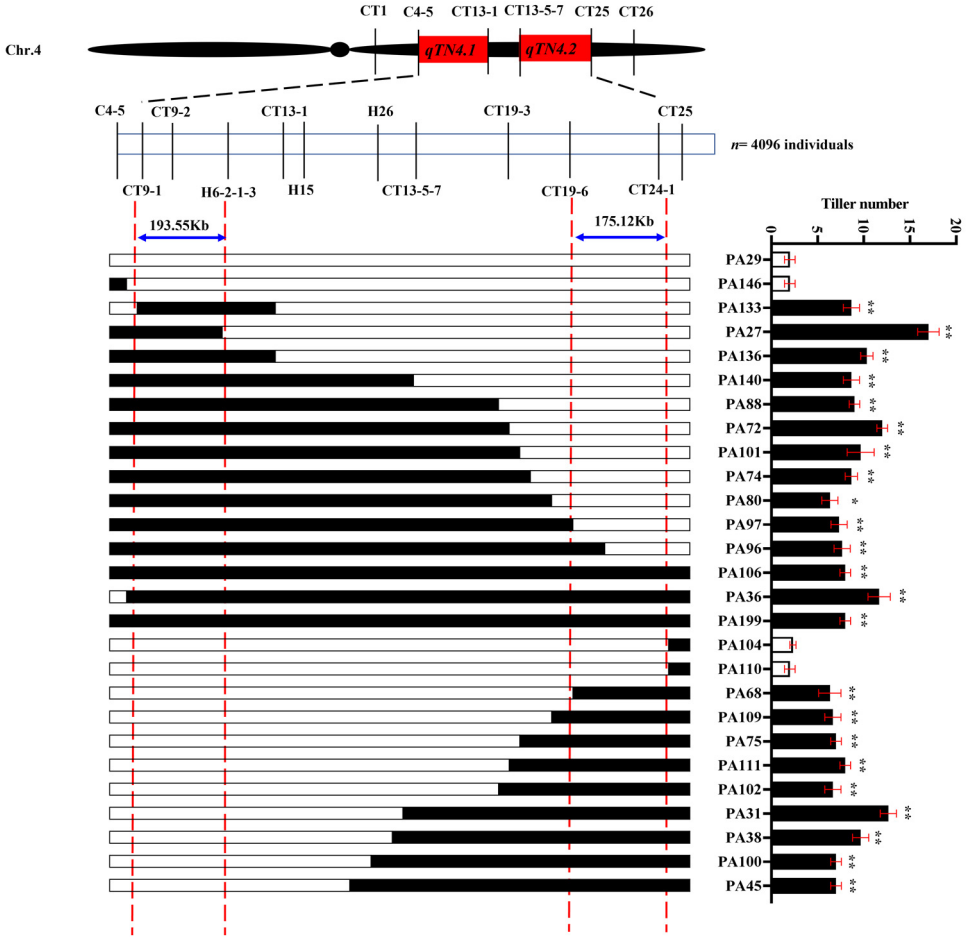


Figure 6. Genetic mapping, dissection, and physical maps of *qTN4.1* and *qTN4.2*. The black regions indicate that the regions are homozygous for the ZH9308 genome. The white regions indicate that the regions are homozygous for the XQZB genome. * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

3.5. Candidate Genes of qTN4.1 and qTN4.2

The above positioning results showed that *qTN4.1* was delimited into 193.55 Kb interval between markers CT9-1 and H6-2-1-3. By searching relevant databases, we found that different genome databases had different candidate genes, among which the Rice Genome Annotation Project Database (http://rice.uga.edu/ (accessed on 10 May 2022)) had the most candidate genes. The *qTN4.1* region contained only eight candidate genes based on the Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/ (accessed on 10 May 2022)) (Table S5). Eight candidate genes were also found in the Gramene Database (http://www.gramene.org (accessed on 10 May 2022)) (Table S5). Nevertheless, according to the Rice Genome Annotation Project (http://rice.uga.edu/ (accessed on 10 May 2022)), there were 16 predicted ORFs within the range of *qTN4.1*, including eight transposons or retrotransposon protein and expressed protein with special domain (Table S5).

The *qTN4.2* was finally mapped to the 175.12 Kb target region between CT19-6 and CT24-1. Referring to different genomic databases, the number of candidate genes varies greatly, containing 15 candidate genes in Gramene Database (Table S6), 10 candidate genes in the Rice Annotation Project Database (Table S6), and 25 candidate genes in the Rice Genome Annotation Project, including three retrotransposon proteins, eight transposon proteins, three lectin protein kinase family proteins, two D-mannose binding lectin family proteins, three hypothetical proteins, three expressed proteins, a basic helix-loop-helix family protein, a xylosyltransferase and a methionyl-tRNA synthetase, respectively (Table S6).

4. Discussion

4.1. Phenotypic Variation among Important Agronomical Traits

Almost important agronomical traits, such as HD, grain setting rate and grain size are related to yield. According to the dynamic development of tiller number (Figure 7A), the tiller number of NIL^{ZH9308} and ZH9308 was significantly higher than that of the NIL^{CSSL29} and CSSL29 from 36th to 78th days after germination. In addition, the tiller number of the four lines reached the maximum at about the 54th day after germination and then began to decrease, which was due to the death of some unproductive tillers in the later stage. There was no significant difference in the productive panicle number of NIL^{ZH9308}, NIL^{CSSL29} and ZH9308 (Figure 7), which might be caused by the death of unproductive tillers. However, there were differences in other agronomic traits among NILs. The CSSL29 delayed heading compared with ZH9308 in Lingshui, and ZH9308 delayed heading compared with CSSL29 in Fuyang, which may be caused by different light lengths. Lingshui has natural short-day conditions, while Fuyang has natural long-day conditions. The GL, the LWR, TGW and grain setting rate of NIL^{ZH9308} were higher than that of NIL^{CSSL29} (Figure 7). The grain-setting rate and grain yield per plant of all materials were extremely poor, because the materials planted in Fuyang in 2022 encountered high temperatures during heading. According to the Zhejiang Meteorological Bureau (http://zj.cma.gov.cn/ (accessed on 1 December 2022)), the temperature of Zhejiang Province was higher than that in the same period of the year, and the temperature was higher in the range of 0.4~4.2 °C. High temperatures will lead to short growth duration, low head rice rate and poor grain filling, which will result in lower rice yields [44], which was consistent with the low grain-setting rate in this study. What is more, the phenotype of NIL-*qTN4.1*^{ZH9308}, NIL-*qTN4.2*^{ZH9308} and NIL^{CSSL29} showed that the alleles of *qTN4.1* and *qTN4.2* from ZH9308 positively regulated tiller number, grain length, grain width and grain setting rate compared with that from CSSL29 (Figure S2). What is more, NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308} would head earlier than NIL^{CSSL29}. Although the genotype of NIL^{CSSL29} had not changed, its phenotypic data changed slightly under different planting times. NIL^{CSSL29} together with NIL^{ZH9308} was planted 10 days earlier than that of NIL-*qTN4.1*^{ZH9308} and NIL-*qTN4.2*^{ZH9308}. The different planting dates resulted in different light lengths, temperatures, and other environmental factors in each stage, which resulted in the differences in yield traits. All results showed that the target region of *qTN4* contained a gene-regulating tiller number and/or yield-related traits.

4.2. Fine-Mapping for qTN4.1 and qTN4.2 and Analysis of Candidate Genes

Although researchers have made some progress in rice tiller number studies, few QTLs have been reported on chromosome 4. Some QTLs, including *tn4-1*, *qTN-4-1* and *qNOT4-1*, were reported in previous studies directly detecting the tiller number in rice on chromosome 4 [10,27,28,45], but none of these QTLs were located in the fine-mapping interval of *qTN4*. Using the recombinant inbred (RI) population developed by the cross between CO39 and Moroberekan varieties, the researchers identified a QTL-affecting tiller number when they mapped the QTL associated with root penetration ability, and this QTL was mapped within an interval from 8,610,617 to 20,087,362 bp containing the *qTN4* on chromosome 4 [45]. The *tn4-1* was located between markers RG190 and RG908 (from 8,610,617 to 13,635,012 bp) in a double haploid population from Azucena/TR64 by conditional QTL mapping methods [10]. The *qTN-4-1* was mapped on chromosome 4 within the interval from 12,642,088 to 12,642,399 bp by using a conditional composite interval mapping approach [27]. The *qNOT4-1* was mapped within the interval RG91-RG449 (from 13,634,515 to 13,635,012 bp) on chromosome 4, by using a DH developed from a cross between IR64 and Azucena varieties, was detected at 85 days after sowing under low-moisture stress, and the *qNOT4-1* explained 12.4% of phenotypic variance [28]. Taken together, *qTN4* is a new genetic locus on chromosome 4 that regulates the tiller number in rice. Consequently, the map-based cloning was used for the fine-mapping of *qTN4*.

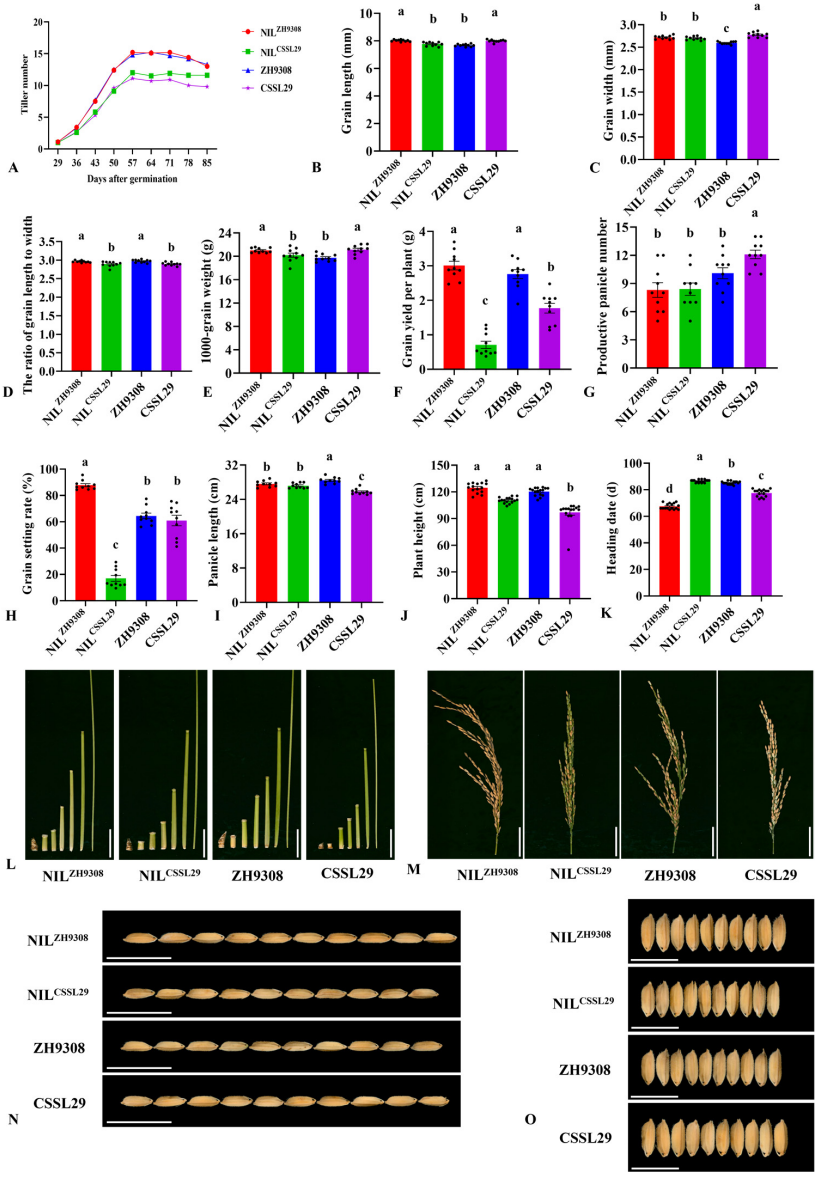


Figure 7. Agronomic trait comparison between NIL^{ZH9308}, NIL^{CSSL29}, ZH9308 and CSSL29 in 2022, Fuyang: (**A**) the dynamic tiller number of the NIL^{ZH9308}, NIL^{CSSL29}, ZH9308 and CSSL29; and (**B**–**O**) agronomic trait comparison between NIL^{ZH9308}, NIL^{CSSL29}, ZH9308 and CSSL29. Data shown represent means ± SEM. n = 30 in (**A**); n = 10 in (**B**–**I**); n = 16 in (**J**–**K**). Different letters indicate significant differences between lines according to Duncan'n test ($p < 0.05$). Scale bar is 10 cm in (**L**–**M**), 1 cm in (**N**–**O**).

Interestingly, the *qTN4* was not detected in Fuyang in 2016, but was detected in Fuyang in 2021 and 2022. We suspected that some of the reasons were as follows: First, this QTL was affected by the environment, such as light, temperature, fertility, etc.; Second, the evaluation of the tiller number was at the maturity stage when some unproductive tillers died in Fuyang in 2016, while the evaluation of the tiller number was at the tillering stage in Fuyang in 2021 (Table S4). The degree of difference in the tiller number of near-isogenic lines at different stages was different, especially at the peak tillering stage, indicating that the results of QTL detection were different at different developmental stages (Figure 6A, Table S4). By constructing different mapping populations and molecular markers for genotyping, the *qTN4* was finally decomposed into two QTLs, namely *qTN4.1* and *qTN4.2*. The *qTN4.1* was delimited into 193.55 Kb interval between markers CT9-1 and H6-2-1-3 containing sixteen candidate genes. The *qTN4.2* was fine-mapped into the 175.12 Kb

interval between markers CT19-6 and CT24-1 with twenty-five candidate genes. Among the candidate genes of *qTN4.1* and *qTN4.2*, except for *LOC_Os04g23550*, none of the other genes has been cloned. Previous studies have found that the gene *LOC_Os04g23550* (*OsbHLH6*) negatively regulates blast resistance and dynamically regulates salicylic acid and jasmonic acid signaling by shuttling between the nucleus and cytoplasm to control disease resistance in rice [46]. Subsequently, sequence analysis will be conducted for the candidate genes within the two QTLs and detect their expression to validate the causal gene for the tiller number. These results will contribute to the cloning of novel genes for tiller numbers.

4.3. The Prospect and Utilization of qTN4.1 and qTN4.2 in Rice Breeding

Super-rice varieties with excellent traits, such as high resistance, high yield and quality are popular among breeders and farmers. In this study, the NIL-$qTN4.1^{ZH9308}$ and NIL-$qTN4.2^{ZH9308}$ performed better than NIL^{CSSL29} in some yield-related traits, such as grain yield per plant, grain length, grain width and seed setting rate, which can provide better germplasm resources for breeders seeking to increase yields. After subsequent validation of candidate genes, gene function can be further elaborated. For example, based on the differences in tiller number between NILs, the expression levels of tiller-related genes can be examined to establish the interrelationship between *qTN4* and other tiller-regulated genes, and enrich the tiller-regulation network. In addition, as these two QTLs, *qTN4.1* and *qTN4.2*, are derived from natural variation, haplotype analysis is particularly important. To further understand the *qTN4.1* and *qTN4.2* function in regulating tiller number, haplotype analysis should be conducted among numerous germplasm resources to screen beneficial allele for the improvement of plant architecture and grain shape. Moreover, functional markers for *qTN4.1* and *qTN4.2* can be developed to identify and select alleles of these two QTLs in cultivated rice. Molecular marker-assisted selection can be used to introduce favorable genes into cultivated rice to create better rice lines and combinations. Therefore, the polymorphic markers developed in this study can be used for subsequent breeding applications. Molecular polymerization was used to improve the yield of rice varieties. The *qTN4.1* and *qTN4.2* can be polymerized into rice varieties with high quality and high resistance to improve their yield by molecular polymerization.

5. Conclusions

Several QTLs were detected in our previous study, among which the *qTN4* was narrowed down to a 4.08 Mb region on chromosome 4 and dissected into two QTLs, *qTN4.1* and *qTN4.2*, using the secondary F_2 population. The *qTN4.1* and *qTN4.2* explained 34.31% and 32.05% of the phenotypic variance, respectively. Finally, the *qTN4.1* was fine-mapped into a 193.55 Kb interval containing sixteen candidate genes and *qTN4.2* was limited to a 175.12 Kb region containing twenty-five candidate genes according to the MSU Database. These results lay a foundation for the analysis of QTL controlling the tiller number in ZH9308 and offer new resources for yield improvement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13020411/s1, Figure S1: Mapping population development. CSSL, Chromosome segment substitution line.; Figure S2: Agronomic trait comparison between NILs of *qTN4.1* and *qTN4.2* in Fuyang in 2022; Table S1: List of primers.; Table S2: The phenotypic performance of the parental lines, Zhonghui 9308 (ZH9308) and Xieqingzao B (XQZB) in Lingshui (LS, 2022), Fuyang (FY, 2022); Table S3: QTLs detected in CSSLs for tiller number; Table S4: QTLs detected in the secondary population for tiller number; Table S5: List of candidate genes of *qTN4.1* in different Database; Table S6: List of candidate genes of *qTN4.2* in different Database.

Author Contributions: Conceptualization, S.C. and Y.Z.; Formal analysis, H.C. and Y.F.; Investigation, H.C., Y.F., D.L., Z.S. and Z.R.; Methodology, S.C. and Y.Z.; Project administration, W.W., Q.L., X.Z., L.C., M.Z., S.C. and Y.Z.; Resources, S.C. and Y.Z.; Supervision, W.W., Q.L., X.Z., L.C., M.Z., S.C. and Y.Z.; Writing—original draft, H.C.; Writing—review & editing, H.C., Y.F., D.L., Z.S., Z.R., H.Y., Y.K., W.W., Q.L., X.Z., L.C., M.Z., S.C. and Y.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Fundamental Research Funds of Central Public Welfare Research Institutions (CPSIBRF-CNRRI-202102), the Independent Project of State Key Laboratory of Rice Biology and Chinese National Center (2020Z2KT10201),the Chinese Academy of Agricultural Sciences Innovation Project (CAAS-ASTIP-2013-CNRRI), and National Major Agricultural Science and Technology Projects (2022ZD0400202).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The datasets used or analyzed in the present study are available upon demand from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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