

Article

Identification and Fine Mapping of Quantitative Trait Loci for Tiller Angle Using Chromosome Segment Substitution Lines in Rice (*Oryza Sativa* L.)

Yujia Leng ^{1,2,†}, Tao Tao ^{1,†}, Shuai Lu ^{1,†}, Ran Liu ¹, Qingqing Yang ¹, Mingqiu Zhang ¹, Lianmin Hong ¹, Qianqian Guo ¹, Xinzhe Ren ¹, Zhidi Yang ¹, Xiuling Cai ^{1,2}, Sukui Jin ^{1,2,3} and Jiping Gao ^{1,2,*} 

¹ Jiangsu Key Laboratory of Crop Genomics and Molecular Breeding/Zhongshan Biological Breeding Laboratory/Key Laboratory of Plant Functional Genomics of the Ministry of Education, Agricultural College of Yangzhou University, Yangzhou 225009, China; yujialeng@yzu.edu.cn (Y.L.); skjin@yzu.edu.cn (S.J.)

² Jiangsu Co-Innovation Center for Modern Production Technology of Grain Crops/Jiangsu Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou 225009, China

³ Joint International Research Laboratory of Agriculture and Agri-Product Safety, the Ministry of Education of China, Yangzhou University, Yangzhou 225009, China

* Correspondence: jpgao@yzu.edu.cn

† These authors contributed equally to this work.

Abstract: The tiller angle, which is an important agronomic trait, determines plant architecture and greatly influences the grain yield of rice. In this study, a population of chromosome segment substitution lines derived from a cross between a *japonica* variety with a compact plant architecture—Koshihikari—and an *indica* variety with a spread-out plant architecture—Nona Bokra—was used to investigate the genetic basis of the tiller angle. Five quantitative trait loci (*qTA1*, *qTA5*, *qTA9-1*, *qTA9-2*, and *qTA11*) for the tiller angle were detected on chromosomes 1, 5, 9, 9, and 11 in two different environments. The phenotypic variation in these QTLs ranged from 3.78% to 8.22%. Two pairs of digenic epistatic QTLs were detected in Lingshui. The epistatic interaction explained 15.19% and 13.60% of the phenotypic variance, respectively. Among the five QTLs, *qTA9-2* was detected in both environments. An F₂ mapping population containing the *qTA9-2* QTL was established. The location of *qTA9-2* was narrowed down to a 187 kb region between InDel markers M9 and M10 on chromosome 9. Thirty open reading frames (ORFs), including *TAC1*, a gene known to regulate the tiller angle, were identified in this region. The gene sequencing results suggested that a base substitution from G to A at position 1557 in the 3'-untranslated region led to a difference in the expression of *qTA9-2* in Koshihikari and Nona Bokra. These findings provide a potential gene resource for the improvement of rice plant architecture.

Keywords: chromosome segment substitution line (CSSL); quantitative trait locus mapping; rice (*Oryza sativa* L.); *qTA9-2*; tiller angle



Citation: Leng, Y.; Tao, T.; Lu, S.; Liu, R.; Yang, Q.; Zhang, M.; Hong, L.; Guo, Q.; Ren, X.; Yang, Z.; et al. Identification and Fine Mapping of Quantitative Trait Loci for Tiller Angle Using Chromosome Segment Substitution Lines in Rice (*Oryza Sativa* L.). *Agriculture* **2024**, *14*, 1002. <https://doi.org/10.3390/agriculture14071002>

Academic Editors: Yong-Feng Shi, Yu-Jun Zhu and Ye-Yang Fan

Received: 15 May 2024

Revised: 20 June 2024

Accepted: 22 June 2024

Published: 26 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops, and it constitutes the greatest proportion of staple food for humans. Improving the yield of rice is a primary objective for breeders, and better plant architecture increases rice yield [1]. As part of the plant architecture, tiller angle affects the yield per unit area by regulating the light energy utilization efficiency of rice populations [2,3]. Consequently, the identification of genes that regulate the rice tiller angle may be an efficient and valuable approach to increasing rice yield.

Previous studies demonstrated that the tiller angle is a quantitative trait with a complex genetic mechanism [4]. To date, several quantitative trait loci (QTLs) associated with the tiller angle have been reported. Li et al. (1999) identified five QTLs for the tiller angle

using an F₂ population from a cross between the *japonica* variety Lemont and the *indica* variety Teqing [5]. Qian et al. (2001) identified three QTLs (*qTA-9a*, *qTA-9b*, and *qTA-12*) on chromosomes 9, 9, and 10, respectively, which explained 22.7%, 11.9%, and 20.9% of phenotypic variation, respectively [6]. Using recombinant inbred lines (RILs) derived from two *indica* varieties—Xieqingzao B and Miyang 46—Shen et al. (2005) detected two QTLs on chromosomes 8 and 9 and three pairs of additive-interaction QTLs associated with the tiller angle [7]. Another study using RILs derived from Asominori and IR24 reported five QTLs—*qTA-2*, *qTA-7a*, *qTA-7b*, *qTA-9*, and *qTA-11*—that controlled the tiller angle in rice, located on chromosomes 2, 7, 7, 9, and 11, respectively [8]. Furthermore, genome-wide association studies (GWAS) identified 18 QTLs for the tiller angle, including two previously reported QTLs and 16 novel QTLs [9]. Dong et al. (2016) identified 30 QTLs for the tiller angle in two environments, which included seven QTLs common to both environments and 23 unique QTLs identified in a single environment [10].

Several genes regulating the tiller angle in rice have also been cloned. *Tiller Angle Control 1* (*TAC1*) was identified as a key regulator for the tiller angle, and it was cloned by using an F₂ population derived from a cross between the *indica* rice variety IR24 (spread-out plant architecture) and an introgressed line, IL55 (compact plant architecture), derived from the *japonica* rice variety Asominori [11]. A base mutation at the 3' splicing site of the fourth intron in *TAC1* led to a compact plant architecture in *japonica* [11]. *TAC1* has been shown to have a conserved function in regulating the tiller angle or branching in *Arabidopsis*, wheat (*Triticum aestivum*), peach (*Prunus persica*), and *Miscanthus sinensis* [12–14]. *PROSTRATE GROWTH 1* (*PROG1*) was found to encode a C2H2-type zinc-finger transcription factor and control the tiller angle and the number of tillers in Asian wild rice (*Oryza rufipogon*) [15,16]. Deficiency in *PROG1* led to a transition from the prostrate growth of Asian wild rice to the erect growth of Asian cultivated rice (*Oryza sativa*) [3]. Similarly, *PROSTRATE GROWTH 7* (*PROG7*) was found to encode a C2H2-type zinc-finger transcription factor and led to a transition from the prostrate growth of African wild rice (*Oryza barthii*) to the erect tiller angle of African cultivated rice (*Oryza glaberrima*) [17]. *RICE PLANT ARCHITECTURE DOMESTICATION* (*RPAD*) was found to harbor a tandem repeat of seven zinc-finger genes that were homologs of *PROG1* and *PROG7* [18]. A 110 kb deletion and a 113 kb deletion at the *RPAD* locus led to a transition from prostrate growth to erect growth in *Oryza sativa* and *Oryza glaberrima*, respectively [18,19]. The tiller angle is also associated with shoot gravitropism [19]. In recent years, several key genes involved in auxin transport and redistribution upon gravistimulation have been identified in rice. These genes play important roles in regulating rice shoot gravitropism and the tiller angle [19]. *LAZY1* (*LA1*) was found to encode a protein in the IGT gene family and to regulate rice shoot gravitropism and the tiller angle by mediating the asymmetric redistribution of auxin upon gravistimulation [20,21]. It was further confirmed that *LA1* is able to interact with Brevis Radix-Like 4 (*BRXL4*) to regulate rice shoot gravitropism and the tiller angle by affecting the nuclear localization of *LA1* [22]. *LA1* was also regulated by the upstream regulator HEAT STRESS TRANSCRIPTION FACTOR 2D (*HSFA2D*) to induce the asymmetric redistribution of auxin, further affecting the asymmetric expression of downstream transcription factors *WOX6* and *WOX11* [23]. *OsHOX1* and *OsHOX28* were found to encode class II homeodomain-leucine zipper proteins and were able to directly bind to the promoter of *HSFA2D* to suppress its expression [24]. *LAZY2* (*LA2*) was found to encode a chloroplastic protein that could interact with the starch biosynthesis enzyme *O. sativa* plastidic phosphoglucomutase (*OspPGM*) to regulate the tiller angle through the starch–statolith-dependent shoot gravitropism regulatory pathway in rice [25]. *Tiller Angle Control 4* (*TAC4*) was found to encode a plant-specific and conserved nuclear localization protein and regulate the tiller angle by affecting polar auxin transport and distribution [26]. *PLANT ARCHITECTURE AND YIELD 1* (*PAY1*) was found to encode a protein that contained a peptidase S64 domain and to regulate plant architecture by affecting polar auxin transport and distribution [27]. In addition, other genes controlling plant architecture or the tiller angle have been identified, including *Ideal Plant Architecture 1* (*IPA1*) [28,29], *Loose Plant Architecture 1* (*LPA1*) [30], *Tiller*

Angle Control 3 (TAC3) [10], and *TILLER INCLINED GROWTH 1 (TIG1)* [31]. Although several genes involved in the tiller angle have been cloned, the complex molecular mechanism of the tiller angle in rice remains largely unclear. Therefore, it is necessary to identify more genes to reveal the molecular mechanism of the tiller angle.

Chromosome segment substitution lines (CSSLs) are genetic populations that represent the complete genome of any genotype in the background of a cultivar as overlapping segments [32]. Ideally, each CSSL has a single and small chromosome fragment from the donor parent and has the maximum recurrent parent genome recovered in the background [32,33]. CSSLs are a powerful genetic stock for fine mapping of the genes of quantitative traits in rice and for marker-assisted breeding [33]. Based on CSSLs, many QTLs or genes for agronomic traits have been identified [34–36]. The *japonica* variety Koshihikari is a premium short-grain rice cultivar, is widely grown in Japan, and exhibits a compact plant architecture [37]. The *indica* variety Nona Bokra, which originates from India, is a well-known salt-tolerant cultivar for rice breeding, and it exhibits a spread-out plant architecture [38]. In this study, a genetic analysis of QTLs determining the tiller angle was performed using CSSLs derived from a cross between Koshihikari and Nona Bokra in two environments. An F₂ mapping population derived from a cross between CSSL113 (spread-out plant architecture) and Nona Bokra was conducted for the fine mapping of a major tiller angle QTL—*qTA9-2*. The aim of this study was to map and clone the genes regulating the tiller angle to determine its genetic basis and improve plant architecture in rice breeding.

2. Materials and Methods

2.1. Plant Materials

CSSLs were constructed by Hao et al. (2009), and they consisted of 154 lines that were established to detect tiller angle QTLs [39]. The CSSLs were developed by using the *indica* cultivar Nona Bokra with a spread-out plant architecture as the donor parent and the *japonica* cultivar Koshihikari with a compact plant architecture as the recipient parent. In brief, the F₁ plants were derived from a cross between Nona Bokra and Koshihikari and then backcrossed with Koshihikari three times to produce 680 BC₃F₁ seeds. Genotyping was performed in the BC₃F₁ lines using 102 polymorphism markers distributed across the 12 chromosomes. According to the genotypes, 71 individuals with a single and relatively long heterozygous chromosome segment were selected. Based on marker-assisted selection (MAS), 154 BC₃F₂ plants were finally selected from 3266 BC₃F₂ individuals for the construction of the CSSLs.

The CSSL113 line, which showed a spread-out plant architecture and carried a homozygous introgression across the entire *qTA9-2* region, was selected for the construction of the F₂ mapping population. The F₂ mapping population was derived from a cross between CSSL113 and Koshihikari. The parental lines, CSSLs, and F₂ mapping population were germinated and sown at the experimental farms of Yangzhou University in Lingshui (110°00' E, 18°31' N) and Yangzhou (119°42' E, 32°39' N) during the 2021 and 2022 rice-growing seasons, respectively. After 30 days, the seedlings were transplanted to the field at a density of 18 cm × 18 cm. The parents and CSSLs were planted in six rows, with each row containing six plants. Approximately 150 kg N ha⁻¹ (as urea), 130 kg K₂O ha⁻¹, and 60 kg P₂O₅ ha⁻¹ were added to each field. Field management, including irrigation and pest control, was performed by following normal agricultural practices.

2.2. Evaluation of the Tiller Angle

The tiller angle is defined as the angle between the main culm and side tillers with the maximum inclination [19]. The tiller angle was measured using a GEELII DIGITAL LEVEL 55454 (GEELII, Remscheid, Germany) according to the manufacturer's instructions. The tiller angle was measured 30 days after heading in 10 plants each for the parental lines and CSSLs.

2.3. DNA Extraction, PCR, and Gene Sequencing

Genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide (CTAB) method with slight modifications [40]. The polymorphic insertion–deletion (InDel) markers used for gene mapping are listed in Table S1. PCR was performed in 20 μ L of reaction mixture using $2 \times$ Taq Master Mix (Vazyme, Nanjing, China), and the PCR products were electrophoresed on a 4% agarose gel at 120 V for 50 min; the gel was visualized using the Tanon 2500B gel image system (Tanon, Shanghai, China). For gene sequencing, a 3048 bp DNA fragment containing the entire genomic sequence of *TAC1* and a 1725 bp downstream sequence of the terminator codon were amplified from Koshihikari and CSSL113. PCR was performed in 50 μ L of reaction mixture of 1 μ L KOD FX (Toyobo, Tokyo, Japan) polymerase, 10 μ L dNTP mix, 25 μ L KOD Buffer, 1.5 μ L 10 mM forward and reverse primers, and 1.5 μ L DNA template, and sterile distilled water was added to bring the total reaction volume to 50 μ L. The primer sequences used for gene sequencing are listed in Table S2. The PCR cycling parameters were 1 cycle of 94 °C for 2 min; 35 cycles of 98 °C for 10 s, 57 °C for 30 s, and 68 °C for 4 min; and 1 cycle of 68 °C for 5 min. The PCR product was sequenced using an ABI 3730xl DNA analyzer (Applied Biosystems, Carlsbad, CA, USA). The alignment of *TAC1* sequences between Koshihikari and CSSL113 was performed using the DNA star Lasergene (Version 7.1, DNASTar Inc., Madison, WI, USA).

2.4. Identification of Quantitative Trait Loci

In total, 126 polymorphism markers, which included 104 simple sequence repeats (SSR) and 22 sequence-tagged site (STS) markers distributed on 12 rice chromosomes, were used to detect the genotypes of the CSSLs [41]. QTL mapping for the tiller angle was performed using QTL IciMapping (Version 4.2) with the inclusive composite interval mapping (ICIM) model [42,43]. The presence of a putative QTL was determined when the likelihood of odds (LOD) score was higher than 2.5. The contributions of each QTL to the phenotypic variance and the additive effect were estimated. The QTLs were named according to the standard described by McCouch et al. [44].

2.5. RNA Isolation and Quantitative Reverse Transcription PCR

Total RNA was extracted from Koshihikari and CSSL113 leaves using the RNAprep Pure Plant kit (Tiangen, Beijing, China). Reverse transcription was performed using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Osaka, Japan) according to the manufacturer's instructions. cDNA was used for quantitative reverse-transcription (qRT) PCR with the TB Green® Premix EX Taq™ kit (Takara, Osaka, Japan) and gene-specific primers (Table S3) in a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). *Ubiquitin* (*UBQ*) was used as a normalizer gene. The relative expression levels of *TAC1* were calculated using the $2^{-\Delta\Delta C_t}$ method [45]. qRT-PCR was completed using three biological replicates for each sample. *t*-tests were employed for statistical analyses.

2.6. Statistical Analysis

One-way ANOVA for the parents and data description for the CSSLs were conducted using Excel 2020. Ten biological replicates of the parents were measured. $p < 0.05$ and $p < 0.01$ were defined as significant and highly significant, respectively.

3. Results

3.1. Phenotypic Variations in the Tiller Angle in the Chromosome Segment Substitution Lines

The *japonica* variety Koshihikari (with a compact plant architecture), the *indica* variety Nona Bokra (with a spread-out plant architecture), and their 154 CSSLs were used to investigate the tiller angle in two environments (Lingshui and Yangzhou). The tiller angles of the parents and their CSSLs are summarized in Figure 1 and Table 1. The mean tiller angles for Koshihikari cultivated in the two environments were 6.1° and 5.2°, respectively, which were significantly lower (over two-fold) than those of Nona Bokra (13.1° and 16.3°,

respectively). In addition, for Koshihikari, the tiller angle was smaller in Yangzhou (5.2°) than in Lingshui (6.1°), whereas the opposite trend was observed for Nona Bokra.

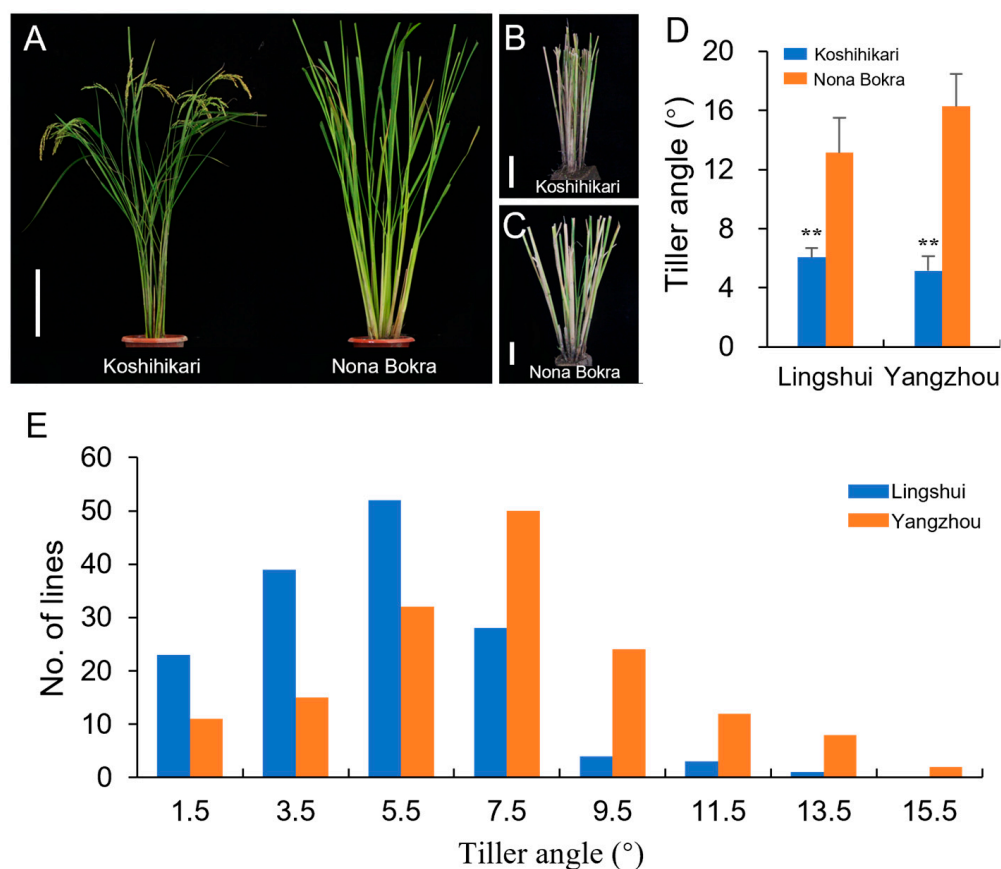


Figure 1. Phenotypes of Koshihikari and Nona Bokra and frequency distributions of the tiller angles in the CSSLs. (A) Phenotypes of Koshihikari and Nona Bokra at the mature stage in Yangzhou. Bar = 20 cm. (B) The base of Koshihikari at the mature stage in Lingshui. Bar = 5 cm. (C) The base of Nona Bokra at the mature stage in Lingshui. Bar = 5 cm. (D) Statistical analysis of the tiller angles of Koshihikari and Nona Bokra. The results are the mean $\pm$ SD of ten biological replicates. ** shows significance at the level of 1%. (E) Frequency distributions of the tiller angles in the CSSLs in the two environments.

Table 1. Description of the tiller angles of the two parents and their CSSLs in two environments.

Location	Parents (Mean $\pm$ SD)			CSSLs				
	Koshihikari	Nona Bokra	<i>p</i> Value	Mean $\pm$ SD	Range	Skewness	Kurtosis	<i>p</i> Value ^a
Lingshui	6.1 $\pm$ 0.64	13.1 $\pm$ 2.37	<0.0001	6.3 $\pm$ 2.68	1.49~15.05	1.09	1.80	0.065
Yangzhou	5.2 $\pm$ 0.98	16.3 $\pm$ 2.2	<0.0001	8.3 $\pm$ 3.05	1.85~18.38	0.41	0.51	0.333

^a The significance of normal distribution of these data by Shapiro–Wilk test.

In the CSSLs, the mean tiller angle in Lingshui (6.3°) was lower than that in Yangzhou (8.3°). Moreover, the range of tiller angles in the CSSLs was larger in Yangzhou (1.85° – 18.38°) than that in Lingshui (1.49° – 15.05°). The skewness and kurtosis of the tiller angle were near zero in the two environments, which indicated that the population was suitable for QTL mapping (Table 1). The Shapiro–Wilk test of CSSL showed that its distribution was close to normal (Table 1). Furthermore, the distribution of the tiller angles was similar between the two environments (Figure 1E). The distribution exhibited a continuous distribution, which indicated that the tiller angle is controlled by a polygene. This result was consistent with those of previous studies [7,8].

3.2. Quantitative Trait Locus Analysis for the Tiller Angle

In total, five QTLs distributed on chromosomes 1, 5, 9, 9, and 11 were identified in the CSSLs in the two environments (Figure 2A, Table 2). One QTL, *qTA9-2*, was detected in both Lingshui and Yangzhou. The LOD values of *qTA9-2* were 3.04 and 7.56 and explained 5.82% and 8.06% of the phenotypic variation, respectively. With this QTL, the allele in Nona Bokra increased the tiller angle by 0.62° and 2.32° (Table 2). *qTA1*, which was located on chromosome 1 between markers RM11051 and RM11268, was detected only in Lingshui. The LOD value of the QTL was 2.64, explaining 4.12% of the phenotypic variation. The *qTA1* allele in Nona Bokra increased the tiller angle by 0.06°. Three QTLs for the tiller angle, *qTA5*, *qTA9-1*, and *qTA11*, were detected only in Yangzhou. The LOD values of the three QTLs were 2.97, 5.52, and 2.71 and explained 3.78%, 7.12%, and 8.22% of the phenotypic variation, respectively (Table 2). The *qTA5* and *qTA11* alleles in Nona Bokra decreased the tiller angle by 1.34° and 0.78, respectively, and the *qTA9-1* allele in Nona Bokra increased the tiller angle by 1.93° (Table 2). Among the five QTLs detected in this study, only one QTL, *qTA9-2*, was detected in both environments, indicating that the tiller angle was greatly affected by the environment.

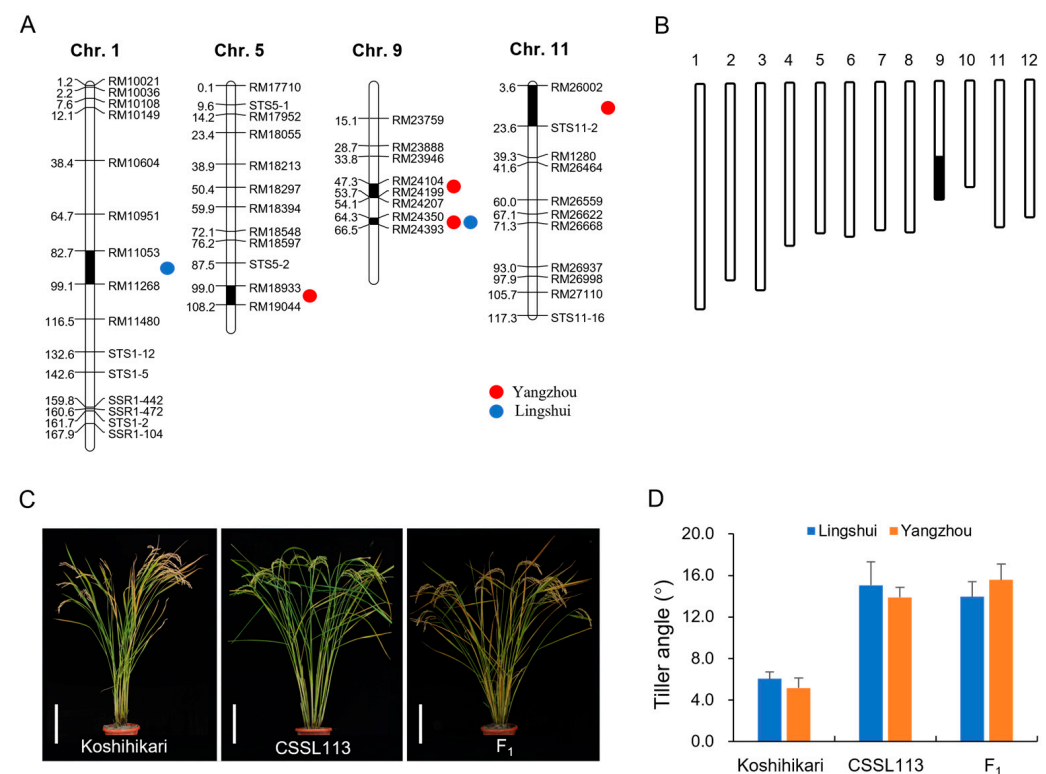


Figure 2. QTL mapping for tiller angle and development of the mapping population. (A) QTL analysis for the tiller angle in CSSLs. Blue and red circles indicate the positions of QTLs on the rice chromosomes in Lingshui (2021) and Yangzhou (2022), respectively. DNA markers are shown on the right side of each chromosome and the genetic distances are shown on the left side of each chromosome. (B) Graphical genotype of CSSL113, a substitution line of chromosome 9. The black bar indicates the genome fragment from Nona Bokra. (C) A comparison of the tillering phenotype for Koshihikari, CSSL113, and F₁ (the cross between Koshihikari and CSSL113). Bar = 20 cm. (D) The tiller angles for Koshihikari, CSSL113, and F₁. The results are the mean $\pm$ SD of ten biological replicates.

Table 2. QTLs for the tiller angle in the CSSLs of Koshihikari and Nona Bokra.

QTL	Chr.	Marker Interval	Position (cM)	LOD		Additive Effect		PVE (%)	
				Lingshui	Yangzhou	Lingshui	Yangzhou	Lingshui	Yangzhou
<i>qTA1</i>	1	RM11053–RM11268	82.7~99.1	2.64	—	0.06	—	4.12	—
<i>qTA5</i>	5	RM18933–RM19044	99.0~108.2	—	2.97	—	−1.34	—	3.78
<i>qTA9-1</i>	9	RM24104–RM24199	47.3~53.7	—	5.52	—	1.93	—	7.12
<i>qTA9-2</i>	9	RM24350–RM24393	64.3~66.5	7.56	3.04	2.32	0.62	8.06	5.82
<i>qTA11</i>	11	RM26002–STS11-2	3.6~23.6	—	2.71	—	−0.78	—	8.22

Several QTL analyses of the tiller angle in rice have been conducted, and four of the five QTLs identified in this study were located in the same regions as those that were previously reported or close to them [5–7,46]. Yan et al. (1999) identified five QTLs for the tiller angle using a DH population derived from a cross between IR64 and Azucena, and one QTL, *ta5*, overlapped with *qTA5* in our study [47]. The interval of *qTA5* was also close to that of another QTL, *tt5.1*, reported by Thomson et al. (2003) [46]. *qTA9-1* was located on chromosome 9 between markers RM24104 and RM24119, and the interval of *qTA9-1* was close to that of another QTL, *ta9*, reported by Li et al. (2006) [48]. One QTL, *qTA9-2*, was identified in the interval from RM24350 to RM24393 (64.3 cM to 66.5 cM), which overlapped with another QTL, *tt9.1*, which was identified in the interval from RZ422 to RM245 (51 cM to 90.2 cM) on chromosome 9 [46]. Similarly, the interval of *qTA9-2* was close to that of another QTL, *ta9*, reported by Yan et al. (1999) [47]. *qTA11* was located on chromosome 11 between markers RM26002 and RM2439; the interval of *qTA11* was close to that of another QTL reported by Kennard et al. (2002) on chromosome 11 [49]. *qTA1* was identified for the tiller angle but has not been previously reported.

A total of two pairs of digenic epistatic QTLs were detected in Lingshui (Table S4). The QTL located in the interval RM24350–RM24393 on chromosome 9 was epistatic with the QTL located in the interval RM13263–RM13549 on chromosome 2 and RM25092–STS10-4 on chromosome 10, respectively. The epistatic interaction explained 15.19% and 13.60% of the phenotypic variance, respectively. We noticed that the phenotypic variance explained (PVE) of the five QTLs identified in this study was small (less than 10), while the PVE of some QTLs in previous studies on the QTL mapping of tiller angle was large. For example, the PVE of *tt9.1* (34.3% and 46.3%), which was identified by Thomson et al. (2003) [46], is significantly higher than that of *qTA9-2* (5.82% and 8.06%). One possible reason is that the small effect of *qTA9-2* was affected by epistatic interaction QTLs.

3.3. Fine Mapping of *qTA9-2* and Analysis of Candidate Genes

In this study, *qTA9-2* was detected in two environments, and this QTL explained a large amount of phenotypic variation (Table 2). Therefore, we selected *qTA9-2* on chromosome 9 for gene cloning. To clone *qTA9-2*, line CSSL113 with the spread-out plant architecture phenotype, which contained the *qTA9-2* fragment from Nona Bokra, was crossed with Koshihikari to construct the F₂ mapping population (Figure 2B). Genetic analysis showed that the F₁ plant exhibited a spread-out plant architecture phenotype, and the segregated ratio of the F₂ progeny was three (spread-out plant architecture phenotype) to one (compact plant architecture) ($\chi^2_{(3:1)} = 0.1004 < \chi^2_{(0.05)} = 3.84$), indicating that the compact plant architecture phenotype was recessive and controlled by a single gene (Figure 2C,D, Table S5). This result was consistent with that obtained for *PROG1* [15,16]. However, as shown by the genes regulating the tiller angle that were cloned with mutants, the spread-out plant architecture phenotype was controlled by a recessive gene, such as *LA1* [20,21], *TAC4* [26], or *LPA1* [30].

A total of 2681 plants showing compact plant architecture were selected for gene mapping in Lingshui. *qTA9-2* was mapped to a 9.6 cM interval between markers M4 and M5 on chromosome 9. Twenty-one heterozygous recombinant plants were harvested and then planted in Yangzhou. A total of 2359 F₃ plants with a compact plant architecture were

selected for fine mapping. Finally, the interval of *qTA9-2* was narrowed down to a 187 kb region between the markers M9 and M10 (Figure 3A). According to the prediction of the Rice Genome Annotation Project (<http://rice.uga.edu/> (accessed on 2 August 2023)), a total of 30 open reading frames (ORFs) were identified in this interval, of which 19 ORFs had annotated protein functions, 10 ORFs encoded expressed protein, and 1 ORF encoded a retrotransposon protein (Table S6). It is worth noting that a known gene controlling the tiller angle, *TAC1*, was identified in these ORFs [11]. *TAC1* encodes a Gramineae-specific protein associated with the regulation of tiller angle. According to Yu et al. (2007), a mutation from AGGA to GGGA was found in the 3' splicing site of the 1.5 kb intron in the 3' untranslated region (UTR) within the *TAC1* locus, which led to a compact plant architecture [11]. Therefore, we sequenced the *TAC1* locus, and the results showed that a four-base deletion (AGTT), a one-base deletion (T), and ten base substitutions were found between CSSL113 and Koshihikari. In the gene-coding region, only one A-to-G substitution was found in the third exon, but it did not change the amino acid sequence. In the 3'-UTR region, one base deletion and seven base substitutions were found between CSSL113 and Koshihikari. A base substitution from G to A at position 1557 in the 3'-UTR exhibited the same mutation type as that reported by Yu et al. (2007) [11] (Figure 3B). We further analyzed the expression of *TAC1* in CSSL113 and Koshihikari using qRT-PCR. The results showed that the expression of *TAC1* in CSSL113 was significantly lower than that in Koshihikari (Supplementary Materials Figure S1), which further confirmed that *qTA9-2* might be *TAC1*.

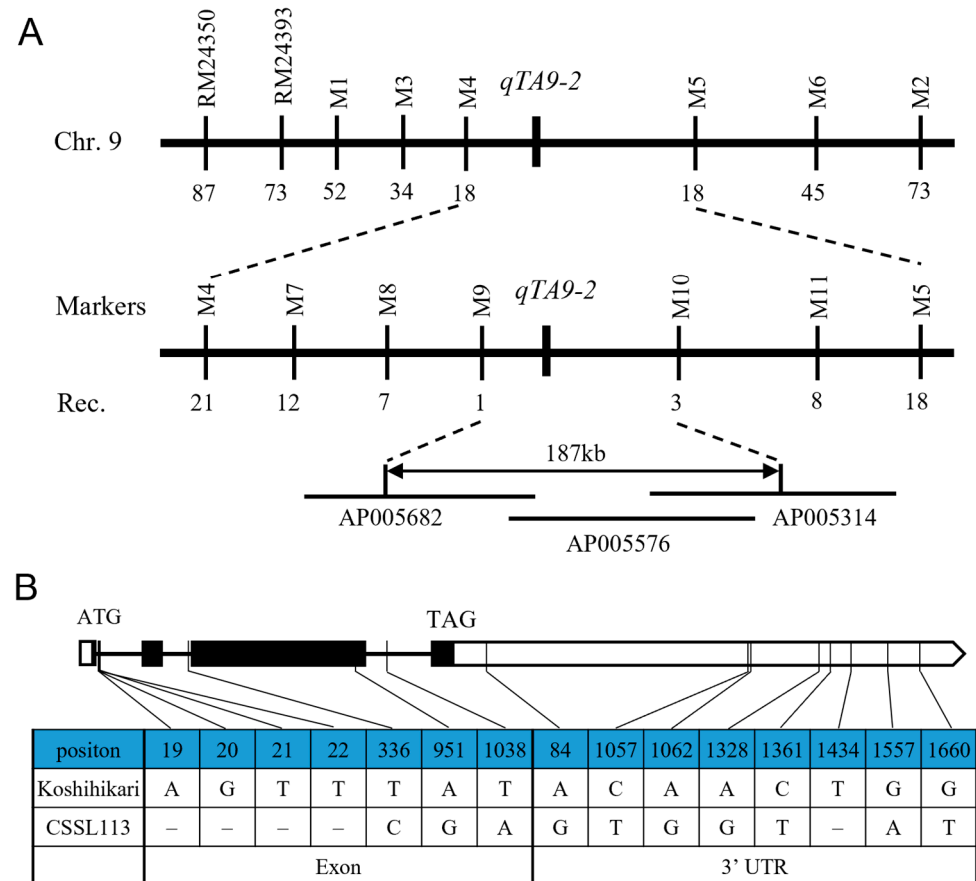


Figure 3. Fine mapping of *qTA9-2*. **(A)** Genetic mapping of *qTA9-2*; the locus was mapped to a 187 kb region on chromosome 9. Chr.: chromosome. Rec.: recombination. **(B)** Schematic diagram of *qTA9-2* and the position of the difference in sequences between Koshihikari and CSSL113. Black rectangles represent exons. White rectangles represent the 5'-UTR. White arrows represent the 3'-UTR.

3.4. Potential Utilization of CSSLs to Improve Rice Architecture

The plant architecture of rice was important for grain yield and was determined by the tiller number and angle, plant height, and panicle morphology [28]. The tiller angle has a crucial influence on planting density, photosynthetic efficiency, lodging, disease resistance, etc. A moderate tiller angle is a key factor for an ideal plant architecture (IPA) and the achievement of high-yield breeding in rice [50]. The tiller angle, which can be extremely spread out or compact, has little practical breeding value. In contrast, the utilization of the tiller angle genes cloned from the varieties has great value for the improvement of plant architecture. In our study, the tiller angle showed wide variation throughout the CSSLs and exhibited transgressive segregants in two environments relative to the parents (Figure 1E). The line contained the entire *qTA9-2* fragment from Nona Bokra and was able to increase the appropriate tillering angle of rice. This provides breeders with breeding materials of interest for the improvement of plant architecture.

A base substitution from G to A at position 1557 in the 3'-UTR of *qTA9-2* changed the plant architecture of rice from loose to compact (Figures 2C and 3B). Sequence analysis showed that significant differentiation between *indica* and *japonica* was found at this locus [51]. The *indica* variety with a spread-out plant architecture at this locus was 'G', whereas the *japonica* variety with a compact plant architecture at this locus was 'A'. Therefore, SNP variations at position 1557 in the 3'-UTR can be used as a functional molecular marker in rice breeding.

Previous studies have shown that the regulation of the rice tiller angle is complex [52]. Although several genes that control the rice tiller angle have been cloned, the molecular mechanism that regulates the tiller angle is not clear. Therefore, further understanding of the regulatory mechanism for *qTA9-2* will provide more information for shaping the ideal plant architecture and breeding elite varieties of rice.

4. Conclusions

In this study, five QTLs for the tiller angle were detected using a population of CSSLs derived from Koshihikari (the recipient parent) and Nona Bokra (the donor parent), which were cultivated in Lingshui and Yangzhou. Among the five QTLs, three were detected only in Yangzhou, and one was detected only in Lingshui. Only one QTL, *qTA9-2*, was detected in both environments. One QTL (*qTA1*) for the tiller angle that was not previously reported was identified. In addition, two pairs of digenic epistatic QTLs were detected in Lingshui, which were both overlapping with *qTA9-2*. In order to clone *qTA9-2*, CSSL113, which showed the phenotype of a spread-out plant architecture and carried a major QTL (*qTA9-2*), was crossed with Koshihikari to establish an F₂ mapping population. The locus was mapped to a 187 kb region between the markers M9 and M10 on chromosome 9. A known tiller angle gene, *TAC1*, was found in this region. The G-to-A base substitution at position 1557 in the 3'-UTR led to a difference in the expression of *qTA9-2* in Koshihikari and Nona Bokra. We demonstrated that *qTA9-2* is essential for the regulation of the tiller angle. The results of this study are expected to provide useful information for the further improvement of the tiller angle in rice.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture14071002/s1>, Figure S1: The expression of *qTA9-2* between Koshihikari and CSSL113. Table S1: The primers used for gene mapping in this study. Table S2: The primers used for gene sequencing in this study. Table S3: The primers used for qRT-PCR in this study. Table S4: Epistasis effect for tiller angle in CSSLs population of Koshihikari and Nona Bokra. Table S5: Genetic analysis of *qTA9-2*. Table S6: Putative function annotation for 30 ORFs.

Author Contributions: Y.L., S.J. and J.G. conceived the project and designed the study. Y.L., T.T., S.L., R.L., Q.Y., M.Z., L.H., Q.G., X.R., Z.Y. and X.C. performed the experiments. Y.L., T.T., S.L., R.L., Q.Y. and M.Z. analyzed and interpreted the data. Y.L., T.T., S.L., S.J. and J.G. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the National Key Research and Development Program of China (2022YFD1200103), the Jiangsu Province Government (BE2022336), the Project of Zhongshan Biological Breeding Laboratory (BM2022008-02), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (22KJA210003), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding author upon request.

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

References

- Wang, Y.; Li, J. Branching in rice. *Curr. Opin. Plant Biol.* **2011**, *14*, 94–99. [\[CrossRef\]](#)
- Wang, Y.; Li, J. Molecular basis of plant architecture. *Annu. Rev. Plant Biol.* **2008**, *59*, 253–279. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, Y.; Li, J. Rice, rising. *Nat. Genet.* **2008**, *40*, 1273–1275. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xu, Y.B.; Susan, R.M.; Shen, Z.T. Transgressive segregation of tiller angle in rice caused by complementary gene action. *Crop Sci.* **1998**, *38*, 12–19. [\[CrossRef\]](#)
- Li, Z.K.; Paterson, A.H.; Pinson, S.R.M.; Stansel, J.W. RFLP facilitated analysis of tiller and leaf angles in rice (*Oryza sativa* L.). *Euphytica* **1999**, *109*, 79–84. [\[CrossRef\]](#)
- Qian, Q.; He, P.; Teng, S.; Zeng, D.L.; Zhu, L.H. QTLs analysis of tiller angle in rice (*Oryza sativa* L.). *Acta Genet. Sin.* **2001**, *28*, 29–32.
- Shen, S.Q.; Zhuang, J.Y.; Bao, J.S.; Zheng, K.L.; Xia, Y.W.; Shu, Q.Y. Analysis of QTLs with additive, epistasis and G×E interaction effects of the tillering angle trait in rice. *J. Agric. Biotechnol.* **2005**, *13*, 16–20.
- Yu, C.Y.; Liu, Y.Q.; Jiang, L.; Wang, C.M.; Zhai, H.Q.; Wan, J.M. QTLs mapping and genetic analysis of tiller angle in rice (*Oryza sativa* L.). *Acta Genet. Sin.* **2005**, *32*, 948–954.
- Bai, S.; Hong, J.; Su, S.; Li, Z.K.; Wang, W.S.; Shi, J.X.; Liang, W.; Zhang, D. Genetic basis underlying tiller angle in rice (*Oryza sativa* L.) by genome-wide association study. *Plant Cell Rep.* **2022**, *41*, 1707–1720. [\[CrossRef\]](#)
- Dong, H.; Zhao, H.; Xie, W.; Han, Z.; Li, G.; Yao, W.; Bai, X.F.; Hu, Y.; Guo, Z.L.; Lu, K.; et al. A novel tiller angle gene, *TAC3*, together with *TAC1* and *D2* largely determine the natural variation of tiller angle in rice cultivars. *PLoS Genet.* **2016**, *12*, e1006412. [\[CrossRef\]](#)
- Yu, B.; Lin, Z.; Li, H.; Li, X.; Li, J.; Wang, Y.; Zhang, X.; Zhu, Z.F.; Zhai, W.X.; Wang, X.K.; et al. *TAC1*, a major quantitative trait locus controlling tiller angle in rice. *Plant J.* **2007**, *52*, 891–898. [\[CrossRef\]](#) [\[PubMed\]](#)
- Dardick, C.; Callahan, A.; Horn, R.; Ruiz, K.B.; Zhebentyayeva, T.; Hollender, C.; Whitaker, M.; Abbott, A.; Scorza, R. PpTAC1 promotes the horizontal growth of branches in peach trees and is a member of a functionally conserved gene family found in diverse plants species. *Plant J.* **2013**, *75*, 618–630. [\[CrossRef\]](#) [\[PubMed\]](#)
- Waite, J.M.; Dardick, C. *TILLER ANGLE CONTROL 1* modulates plant architecture in response to photosynthetic signals. *J. Exp. Bot.* **2018**, *69*, 4935–4944. [\[CrossRef\]](#) [\[PubMed\]](#)
- Zhao, H.; Huai, Z.X.; Xiao, Y.J.; Wang, X.H.; Yu, J.Y.; Ding, G.D.; Peng, J.H. Natural variation and genetic analysis of the tiller angle gene *MstTAC1* in *Miscanthus sinensis*. *Planta* **2014**, *240*, 161–175. [\[CrossRef\]](#) [\[PubMed\]](#)
- Jin, J.; Huang, W.; Gao, J.P.; Yang, J.; Shi, M.; Zhu, M.Z.; Luo, D.; Lin, H.X. Genetic control of rice plant architecture under domestication. *Nat. Genet.* **2008**, *40*, 1365–1369. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tan, L.B.; Li, X.R.; Liu, F.X.; Sun, X.Y.; Li, C.G.; Zhu, Z.F.; Fu, Y.C.; Cai, H.W.; Wang, X.K.; Xie, D.X.; et al. Control of a key transition from prostrate to erect growth in rice domestication. *Nat. Genet.* **2008**, *40*, 1360–1364. [\[CrossRef\]](#) [\[PubMed\]](#)
- Hu, M.; Lv, S.; Wu, W.; Fu, Y.; Liu, F.; Wang, B.; Li, W.; Gu, P.; Cai, H.; Sun, C.; et al. The domestication of plant architecture in African rice. *Plant J.* **2018**, *94*, 661–669. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wu, Y.; Zhao, S.; Li, X.; Zhang, B.; Jiang, L.; Tang, Y.; Zhao, J.; Ma, X.; Cai, H.; Sun, C.; et al. Deletions linked to *PROG1* gene participate in plant architecture domestication in Asian and African rice. *Nat. Commun.* **2018**, *9*, 4157. [\[CrossRef\]](#)
- Wang, W.; Gao, H.; Liang, Y.; Li, J.; Wang, Y. Molecular basis underlying rice tiller angle: Current progress and future perspectives. *Mol. Plant* **2022**, *15*, 125–137. [\[CrossRef\]](#)
- Li, P.; Wang, Y.; Qian, Q.; Fu, Z.; Wang, M.; Zeng, D.; Li, B.; Wang, X.J.; Li, J.Y. *LAZY1* controls rice shoot gravitropism through regulating polar auxin transport. *Cell Res.* **2007**, *17*, 402–410. [\[CrossRef\]](#)
- Yoshihara, T.; Iino, M. Identification of the gravitropism-related rice gene *LAZY1* and elucidation of *LAZY1*-dependent and -independent gravity signaling pathways. *Plant Cell Physiol.* **2007**, *48*, 678–688. [\[CrossRef\]](#) [\[PubMed\]](#)
- Li, Z.; Liang, Y.; Yuan, Y.; Wang, L.; Meng, X.; Xiong, G.; Zhou, J.; Cai, Y.; Han, N.; Hua, L.; et al. OsBRXL4 regulates shoot gravitropism and rice tiller angle through affecting *LAZY1* nuclear localization. *Mol. Plant* **2019**, *12*, 1143–1156. [\[CrossRef\]](#) [\[PubMed\]](#)

23. Zhang, N.; Yu, H.; Yu, H.; Cai, Y.; Huang, L.; Xu, C.; Xiong, G.; Meng, X.; Wang, J.; Chen, H.; et al. A core regulatory pathway controlling rice tiller angle mediated by the LAZY1-dependent asymmetric distribution of auxin. *Plant Cell* **2018**, *30*, 1461–1475. [[CrossRef](#)] [[PubMed](#)]
24. Hu, Y.; Li, S.L.; Fan, X.; Song, S.; Zhou, X.; Weng, X.; Xiao, J.; Li, X.; Xiong, L.; You, A.; et al. OsHOX1 and OsHOX28 redundantly shape rice tiller angle by reducing HSFA2D expression and auxin content. *Plant Physiol.* **2020**, *184*, 1424–1437. [[CrossRef](#)] [[PubMed](#)]
25. Huang, L.Z.; Wang, W.G.; Zhang, N.; Cai, Y.Y.; Liang, Y.; Meng, X.B.; Yuan, Y.D.; Li, J.Y.; Wu, D.X.; Wang, Y.H. LAZY2 controls rice tiller angle through regulating starch biosynthesis in gravity-sensing cells. *New Phytol.* **2021**, *231*, 1073–1087. [[CrossRef](#)]
26. Li, H.; Sun, H.Y.; Jiang, J.H.; Sun, X.Y.; Tan, L.B.; Sun, C.Q. TAC4 controls tiller angle by regulating the endogenous auxin content and distribution in rice. *Plant Biotechnol. J.* **2021**, *19*, 64–73. [[CrossRef](#)]
27. Zhao, L.; Tan, L.B.; Zhu, Z.F.; Xiao, L.T.; Xie, D.X.; Sun, C.Q. PAY1 improves plant architecture and enhances grain yield in rice. *Plant J.* **2015**, *83*, 528–536. [[CrossRef](#)]
28. Jiao, Y.; Wang, Y.; Xue, D.; Wang, J.; Yan, M.; Liu, G.; Dong, G.; Zeng, D.; Lu, Z.; Zhu, X.; et al. Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nat. Genet.* **2010**, *42*, 541–544. [[CrossRef](#)] [[PubMed](#)]
29. Miura, K.; Ikeda, M.; Matsubara, A.; Song, X.J.; Ito, M.; Asano, K.; Matsuoka, M.; Kitano, H.; Ashikari, M. OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nat. Genet.* **2010**, *42*, 545–549. [[CrossRef](#)]
30. Wu, X.R.; Tang, D.; Li, M.; Wang, K.J.; Cheng, Z.K. Loose plant architecture1, an indeterminate domain protein involved in shoot gravitropism, regulates plant architecture in rice. *Plant Physiol.* **2013**, *161*, 317–329. [[CrossRef](#)]
31. Zhang, W.F.; Tan, L.B.; Sun, H.Y.; Zhao, X.H.; Liu, F.X.; Cai, H.W.; Fu, Y.C.; Sun, X.Y.; Gu, P.; Zhu, Z.F.; et al. Natural variations at TIG1 encoding a TCP transcription factor contribute to plant architecture domestication in rice. *Mol. Plant* **2019**, *12*, 1075–1089. [[CrossRef](#)] [[PubMed](#)]
32. Balakrishnan, D.; Surapaneni, M.; Mesapogu, S.; Neelamraju, S. Development and use of chromosome segment substitution lines as a genetic resource for crop improvement. *Theor. Appl. Genet.* **2019**, *132*, 1–25. [[CrossRef](#)] [[PubMed](#)]
33. Fan, J.; Hua, H.; Luo, Z.; Zhang, Q.; Chen, M.; Gong, J.; Wei, X.; Huang, Z.; Huang, X.; Wang, Q. Whole-genome sequencing of 117 chromosome segment substitution lines for genetic analyses of complex traits in rice. *Rice* **2022**, *15*, 5. [[CrossRef](#)] [[PubMed](#)]
34. Wang, H.; Zhang, J.Y.; Naz, F.; Li, J.; Sun, S.F.; He, G.H.; Zhang, T.; Ling, Y.H.; Zhao, F.M. Identification of rice QTLs for important agronomic traits with long-kernel CSSL-Z741 and three SSSLs. *Rice Sci.* **2020**, *27*, 414–422.
35. Liang, P.Y.; Wang, H.; Zhang, Q.L.; Zhou, K.; Li, M.M.; Li, R.X.; Xiang, S.Q.; Zhang, T.; Ling, Y.H.; Yang, Z.L.; et al. Identification and pyramiding of QTLs for rice grain size based on short-wide grain CSSL-Z563 and fine-mapping of *qGL3-2*. *Rice* **2021**, *14*, 35. [[CrossRef](#)] [[PubMed](#)]
36. Bian, J.M.; Jiang, L.; Liu, L.L.; Wei, X.J.; Xiao, Y.H.; Zhang, L.J.; Zhao, Z.G.; Zhai, H.Q.; Wan, J.M. Construction of a new set of rice chromosome segment substitution lines and identification of grain weight and related traits QTLs. *Breed. Sci.* **2010**, *60*, 305–313. [[CrossRef](#)]
37. Kobayashi, A.; Hori, K.; Yamamoto, T.; Yano, M. Koshihikari: A premium short-grain rice cultivar—Its expansion and breeding in Japan. *Rice* **2018**, *11*, 15. [[CrossRef](#)] [[PubMed](#)]
38. Jin, S.K.; Xu, L.N.; Yang, Q.Q.; Zhang, M.Q.; Wang, S.L.; Wang, R.A.; Tao, T.; Hong, L.M.; Guo, Q.Q.; Jia, S.W.; et al. High-resolution quantitative trait locus mapping for rice grain quality traits using genotyping by sequencing. *Front. Plant Sci.* **2023**, *13*, 1050882. [[CrossRef](#)] [[PubMed](#)]
39. Hao, W.; Zhu, M.Z.; Gao, J.P.; Sun, S.Y.; Lin, H.X. Identification of quantitative trait loci for rice quality in a population of chromosome segment substitution lines. *J. Integr. Plant Biol.* **2009**, *51*, 500–512. [[CrossRef](#)]
40. Murray, M.G.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* **1980**, *8*, 4321–4326. [[CrossRef](#)]
41. Leng, Y.; Hong, L.; Tao, T.; Guo, Q.; Yang, Q.; Zhang, M.; Ren, X.; Jin, S.; Cai, X.; Gao, J. Mapping of QTLs for brown rice traits based on chromosome segment substitution line in rice (*Oryza sativa* L.). *Agriculture* **2023**, *13*, 928. [[CrossRef](#)]
42. Li, H.H.; Ye, G.Y.; Wang, J.K. A modified algorithm for the improvement of composite interval mapping. *Genetics* **2007**, *175*, 361–374. [[CrossRef](#)] [[PubMed](#)]
43. Li, M.; Li, H.H.; Zhang, L.Y.; Wang, J.K. QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. *Crop J.* **2015**, *3*, 269–283.
44. McCouch, S.R.; Cho, Y.G.; Yano, M.; Paul, E.; Blinstrub, M.; Morishima, H.; Kinosita, T. Report on QTL nomenclature. *Rice Genet. Newsl.* **1997**, *14*, 11–13.
45. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
46. Thomson, M.J.; Tai, T.H.; McClung, A.M.; Lai, X.H.; Hinga, M.E.; Lobos, K.B.; Xu, Y.; Martinez, C.P.; McCouch, S.R. Mapping quantitative trait loci for yield, yield components and morphological traits in an advanced backcross population between *Oryza rufipogon* and the *Oryza sativa* cultivar Jefferson. *Theor. Appl. Genet.* **2003**, *107*, 479–493. [[CrossRef](#)]
47. Yan, J.; Zhu, J.; He, C.; Benmoussa, M.; Wu, P. Molecular marker-assisted dissection of genotype $\times$ environment interaction for plant type traits in rice (*Oryza sativa* L.). *Crop Sci.* **1999**, *39*, 538–544. [[CrossRef](#)]
48. Li, H.; Zhou, A.; Sang, T. Genetic analysis of rice domestication syndrome with the wild annual species, *Oryza nivara*. *New Phytol.* **2006**, *170*, 185–193. [[CrossRef](#)] [[PubMed](#)]

49. Kennard, C.; Phillips, L.; Porter, A. Genetic dissection of seed shattering, agronomic, and color traits in American wildrice (*Zizania palustris* var. *interior* L.) with a comparative map. *Theor. Appl. Genet.* **2002**, *105*, 1075–1086. [[CrossRef](#)]
50. Gao, J.; Liang, H.; Huang, J.; Qing, D.; Wu, H.; Zhou, W.; Chen, W.; Pan, Y.; Dai, G.; Gao, L.; et al. Development of the PARMS marker of the TAC1 gene and its utilization in rice plant architecture breeding. *Euphytica* **2021**, *217*, 49. [[CrossRef](#)]
51. Jiang, J.; Tan, L.; Zhu, Z.; Fu, Y.; Liu, F.; Cai, H.; Sun, C. Molecular evolution of the *TAC1* gene from rice (*Oryza sativa* L.). *J. Genet. Genom.* **2012**, *39*, 551–560. [[CrossRef](#)] [[PubMed](#)]
52. He, Y.; Li, L.; Jiang, D. Understanding the regulatory mechanisms of rice tiller angle, then and now. *Plant Mol. Biol. Rep.* **2021**, *39*, 640–647. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.