BMC Plant Biology

RESEARCH

Open Access

QTL-seq identifies *NAL1* and *OsOFP19* as additive regulators of tiller number in rice (*Oryza sativa* L.)

Thanyakorn Rongsawat[1†], Nattiwong Pankasem[2,3†], Tripop Thianthavon[1], Piyamongkol Mangkalasane[1], Wanchana Aesomnuk[1], Reajina Dumhai[1], Siriphat Ruengphayak[1], Jonaliza L. Siangliw[2], Meechai Siangliw[2], Mutiara K. Pitaloka[4], Rui Xia[5], Theerayut Toojinda[1], Vinitchan Ruanjaichon[2], Julie E. Gray[6], Samart Wanchana[2] and Siwaret Arikit[1,7*]

Abstract

Background Tiller number is a critical component of rice yield, as it directly influences overall productivity. While upland rice varieties are well adapted to lowland environments and prove resilient to fluctuating water availability, their typically low tillering capacity limits their performance in lowland ecosystems where conditions are more conducive to achieving higher yields.

Results To facilitate the marker-assisted selection (MAS) breeding of upland rice cultivars suitable for lowland conditions, we performed QTL-seq analysis using populations derived from a cross between a high-tillering lowland indica parent (PTT1) and a low-tillering upland tropical japonica line (NDCMP49). Two major QTLs associated with tiller number were identified on chromosomes 4 and 5 and designated as *qTN4* and *qTN5*, respectively. Candidate gene analysis revealed *NAL1* and *OsOFP19* as putative genes underlying these loci. Functional validation of *NAL1* using CRISPR-Cas9 knockout mutants confirmed its role as a negative regulator of tillering, as two independent alleles of *nal1* mutant plants exhibited significantly increased tiller numbers compared with the wild type. Marker–trait association analysis further supported the additive effect of *qTN4 (NAL1)* and *qTN5 (OsOFP19)*, indicating their potential for pyramiding in breeding programs. Functional KASP markers of *NAL1* and *OsOFP19* were developed and successfully validated in segregating populations, demonstrating their applicability for marker-assisted selection.

Conclusions Collectively, these findings advance our understanding of the genetic regulation of tillering in rice and provide molecular tools for improving plant architecture and yield in upland rice varieties cultivated under lowland conditions.

Keywords Tiller number, Upland rice, QTL-seq, *NAL1*, *OsOFP19*, Lowland adaptation

†Thanyakorn Rongsawat and Nattiwong Pankasem contributed equally to this work.

*Correspondence:
Siwaret Arikit
siwaret.a@ku.th

Full list of author information is available at the end of the article

Background

Global food security is increasingly threatened by the dual challenges of climate change and rapid population growth [1]. As a staple food for more than half of the world's population, rice (*Oryza sativa* L.) plays a central role in sustaining global food systems [2]. To meet future food demands under increasingly variable environmental conditions, increasing the productivity and adaptability of rice is a key objective of crop improvement programs. Currently, the majority of the world's rice is grown in lowland ecosystems, which rely on the constant water supply to maintain flooded or waterlogged soils that are essential for high yields [3]. In contrast, rice is also grown in upland ecosystems, where aerobic soil conditions and limited water availability have driven the evolution of various adaptive traits [4].

In Southeast Asia, the rice germplasm exhibits considerable genetic diversity, with *indica* being the predominant subspecies, while *aus*, temperate *japonica* and tropical *japonica* varieties are also grown in certain agro-ecological environments [5]. In Thailand, for example, the rice gene pool is dominated by *indica*, while tropical *japonica* varieties are mainly grown in the arid mountainous regions [6]. Tropical *japonica* is thought to have diverged from temperate *japonica* by adapting to the highlands and mountainous regions [7], acquiring traits such as tall stature, deep root system and lower tillering ability. These adaptations are beneficial under drought-prone conditions but often result in lower yield potential compared to varieties adapted to lowland conditions [8]. However, when cultivated under favorable lowland conditions, upland rice often does not produce high yields due to its inherently low tillering ability, which directly limits the number of panicles and total grain production. Improving the tillering ability of upland rice is a key strategy to improve its adaptation and performance in lowland systems.

The number of tillers is an important yield component as it directly determines the number of productive panicles per plant [9]. In water-abundant environments, increased tillering can contribute to higher biomass, better resource utilization and greater yield stability [10, 11]. Despite the physiological importance of tillering, its genetic regulation is complex and involves a network of developmental and hormonal pathways. Auxin and strigolactones act as negative regulators of tiller bud outgrowth, while cytokinin and brassinosteroids promote tiller formation [12, 13]. Tiller development is initiated by the formation of axillary meristems (AMs), which then give rise to tiller buds. Several genes have been shown to regulate these processes, including *MONOCULM1* (*MOC1*), *MONOCULM3TILLER ABSENT1/SRT1*, *LAX PANICLE1* (*LAX1*) and *LAX2* [14–20]. *NAL1* (*Narrow leaf 1*) encodes a plant-specific protein implicated in regulating leaf morphology, photosynthetic capacity, and yield-related traits in rice. Previous work by Jiang et al. linked *NAL1* to tiller number using a mutant derived from random mutagenesis in the Nipponbare background [21]. Further studies have shown that microRNAs and dwarfing genes are involved in the modification of tillering phenotype [13, 22, 23]. Although numerous genes involved in tillering development have been identified, relatively few have been translated into useful molecular markers for breeding purposes. In addition, little attention has been paid to tillering traits in upland rice, although they are of great value for increasing yield in lowland cultivation. Given the particular architecture and adaptation of the upland germplasm, there is a clear need to investigate the genetic basis of tillering in this group to enable targeted trait improvement.

In the present study, we used QTL-seq, a next-generation sequencing-based bulked segregant analysis (BSA) method [24], to identify genomic regions associated with tiller number in rice. F_2 and $F_{2:3}$ populations derived from a cross between an indica lowland parent with high tiller production (PTT1) and a tropical japonica upland line with low tiller production (NDCMP49) were used for QTL-seq analysis. Key QTLs were mapped and candidate genes were identified. Functional markers were developed and validated to facilitate marker-assisted selection (MAS). This work provides new insights into the genetic regulation of tillering in upland rice and facilitates development of molecular markers for breeding programs aimed at increasing yield potential in lowland environments.

Materials and methods

Plant materials and phenotype evaluation

An F_2 population derived from a cross of Niaw Dam Chaw Mai Pai 49 (NDCMP49) x Pathum Thani 1 (PTT1) was used for a QTL-seq analysis to identify QTLs associated with tillering ability under lowland (waterlogged) conditions. NDCMP49, an upland tropical japonica cultivar, was used as a low tiller number (low-TN) parent with an approximate TN of 9 tillers per plant, and PTT1, an elite Thai fragrant lowland *indica* rice cultivar with an approximate TN of 20 tillers per plant, was used as a high tiller number (high-TN) parent. Three-week-old seedlings from a total of 492 F_2 individuals from this cross were grown together with their parents in the field at a distance of 25 cm x 25 cm between the plants. A total of 2.3 g of chemical fertilizer N-P-K (21-7-14) was applied per plant in three splits at 15, 30 and 50 days after transplanting. The standing water level was maintained at 5 cm above the soil throughout the season. The field experiments were conducted at the rice field of the Rice Science Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand.

Bulk construction, DNA isolation and whole-genome re-sequencing

The tillering ability of each F_2 line and the parental lines was evaluated by counting the tiller number at the maximum tillering stage. A total of 30 F_2 individuals with the highest tiller number and the other 30 individuals with the lowest tiller number were selected and assigned into high-TN and low-TN groups, respectively. The leaf samples of all 60 lines were collected individually and stored at -80 °C until they were used for DNA isolation. To select the F_2 lines with robust phenotype from the preselection, we reconfirmed the phenotype in $F_{2:3}$ families corresponding to the selected F_2 lines. Fifteen plants of each $F_{2:3}$ family were grown in the field under the same growing conditions as previously described and the tiller number from each plant was counted at the maximum tillering stage. Ten $F_{2:3}$ families with the highest average tiller numbers and another ten $F_{2:3}$ families with the lowest average tiller number were selected and grouped into high-TN and low-TN bulks. The DNA of the individual F_2 lines corresponding to the 20 selected $F_{2:3}$ families was extracted from the stored leaf samples using Dneasy® Plant Mini Kit (QIAGEN, Hilden, Germany). The DNA of the individual F_2 plants as well as that of the both parents was sequenced the whole genome using an MGI-Seq platform at the China National Gene Bank (CNGB) Shenzhen, China.

Sequencing data analysis and identification of genomic regions associated with tillering ability

Raw sequencing data of each sample were quality-filtered and trimmed using Trimmomatic [25] to remove adapter sequences and low-quality reads, defined as those containing more than 15% bases with a Phred score below 30 or more than 5% ambiguous bases (N). Reads were retained only if ≥ 85% of bases had a Phred score ≥ 30. The resulting high-quality reads were subjected to QTL-seq analysis using the QTL-seq pipeline v2.2.2 (https://github.com/YuSugihara/QTL-seq). The analysis was performed as follows: First, a PTT1-based pseudo-reference genome was generated by aligning clean PTT1 reads to the publicly available Nipponbare reference genome (IRGSP1.0) using BWA-MEM with default parameters [26], followed by conversion, sorting, and indexing with SAMtools [27]. Variants were called with BCFtools mpileup [28] and filtered with thresholds of minimum read depth ≥ 10 and maximum read depth ≤ 250. The Nipponbare genome was then modified by substituting PTT1-specific variants to create the PTT1 pseudo-reference. Equal numbers of clean reads from each F_2 individual were pooled into high-tiller-number (high-TN) and low-tiller-number (low-TN) bulks. Pooled reads were aligned to the PTT1 pseudo-reference genome, and SNPs and indels were called for each bulk. Only variants detected in both bulks were retained for SNP-index calculation, defined as the proportion of alternative allele reads to total read depth. The Δ(SNP-index) was calculated by subtracting the high-TN SNP-index from the low-TN SNP-index [24]. SNP-index and Δ(SNP-index) values were averaged using a sliding window of 2 Mb with a 10 kb step size, and plotted along the 12 rice chromosomes. Statistical significance was determined using the pipeline's simulation-based approach, which performs 10,000 Monte Carlo simulations based on bulk sizes and population type to generate 95% ($p < 0.05$) and 99% ($p < 0.01$) confidence intervals for each window. Genomic regions where the observed Δ(SNP-index) exceeded these thresholds were considered significantly associated with the trait, with peaks above the 99% interval regarded as the most likely QTL candidates. SNPs within QTL regions were annotated using the Variant Effect Predictor (VEP: https://plants.ensembl.org/Oryza_sativa/Tools/VEP), and homologs of previously characterized genes carrying high- to moderate-impact SNPs were identified as potential candidate genes associated with tiller number.

Marker development and QTL validation

The design of the markers was determined by the preliminary marker-trait association of each sequenced F_2 line ($n = 20$). SNPs within selected genomic regions were tested for association with phenotype using ANOVA for a single marker as described in [29]. Briefly, F_2 lines were grouped by SNP alleles and then one-way ANOVA was tested for significant differences between group means. The F-test was used to determine the trait-associated SNP. SNPs showing both high to moderate variant impact and high association with tiller number were targeted for the development of allele-specific PCR genotyping assays using the Kompetitive Allele-Specific PCR Genotyping (KASP) system (LGC Genomics) or TaqMan™ Real-Time PCR Assays (Thermo Fisher Scientific). The KASP genotyping test was performed according to [30], and the TaqMan assay was performed according to [31]. A further marker-trait analysis of a larger F_2 population was performed using the genotypes and phenotypic data obtained from 500 progeny. Two-way analysis of variance (ANOVA) was performed using *jamovi* version 2.6 (https://www.jamovi.org) to evaluate the main and interaction effects between the two QTL genotypes on the tiller number.

Generation of *nal1* mutant lines and growing condition

Two *nal1* mutant lines (T1 generation), *nal1*-1 (BG110220C10) and *nal1*-2 (BG110336H11) generated via the CRISPR-Cas9 system in the japonica rice variety ZhongHua 11 (ZH11) were obtained from Biogle Gene Tech Co, Ltd (Jiangsu, China). The sgRNA sequence for *nal1*-1 was 5′-GGAATCCCACTGCTGTCCCG-3′, and

that for *nal1*-2 was 5′-GACGATAAGGCGCAGCTCTC CGG-3′. Details of edited sites are available at http://biogle.cn/geo/index/geo/val/BG110220C10 for *nal1*-1 and http://biogle.cn/geo/index/geo/val/BG110336H11 for *nal1*-2. Both the *nal1* mutants and ZH11 wild-type plants were grown in pots under waterlogged and dry conditions, simulating lowland and upland environments, respectively, in the greenhouse at South China Agricultural University, China.

Results

Tiller number assessment in parental lines and F_2 population

The development of tillers in the parental lines NDCMP49 and PTT1 was observed over time. In the early growth phase (i.e. before 50 days after sowing, DAS), no significant difference in the number of tillers was observed between the two parental varieties. However, from 60 DAS, PTT1 began to show increased tiller production, with 6–7 tillers per plant observed, compared to 3–4 tillers in NDCMP49. This trend continued throughout plant development, with maximum tiller production reached at 80 DAS. At this stage, PTT1 formed about 20 tillers, while NDCMP49 formed about 7 tillers (Fig. 1A–B). The number of tillers was analyzed at 80 DAS in 492 F_2 individuals from the NDCMP49 × PTT1 cross. The trait showed a continuous, approximately normal distribution, ranging from 2 to 41 tillers, with a mean of 18.78 (Fig. 1C). Transgressive segregation was evident as 36.78% of F_2 individuals exceeded the tiller count of the PTT1 parent (TN = 20), while 3.45% produced fewer tillers than NDCMP49 (TN = 7). To construct high and low-tiller number (TN) bulks for QTL-seq analysis, 30 F_2 individuals with the highest and 30 with the lowest tiller numbers were preliminarily selected. These individuals were further developed to generate $F_{2:3}$ families in which tillering ability was reassessed. From this set, ten families with the highest and ten with the lowest number of tillers were selected. The corresponding F_2 individuals were then used to form high-TN and low-TN bulks, which were subjected to whole-genome resequencing and QTL-seq analysis (Fig. 1D).

Whole-genome re-sequencing and analysis of parents and high/low-tiller F_2 bulks

Whole-genome re-sequencing was performed on 20 selected F_2 lines (consisting of the high-TN and low-TN bulks) and the two parental lines using the MGI-seq platform. The total number of high-quality, cleaned reads of NDCMP49 and PTT1 was 65.55 million and

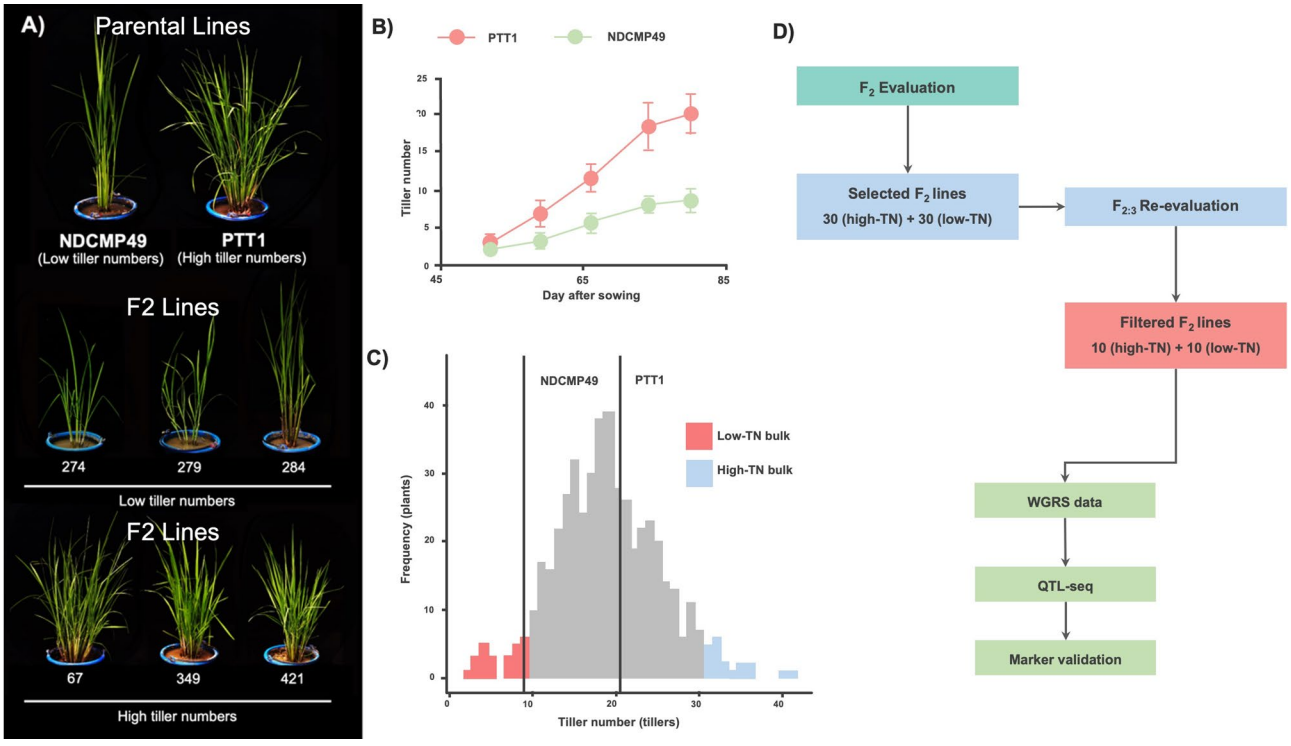

Fig. 1 Development of tiller numbers in PTT1, NDCMP49 and F_2 populations and QTL analysis workflow. **A** Appearance at the maximum tillering stage (80 DAS) of NDCMP49, PTT1 and representative F_2 lines selected for QTL-seq analysis. **B** Development of tiller number in PTT1 and NDCMP49 from 50 to 80 DAS, **C** Distribution of traits based on the number of tillers of the F_2 population at the maximum tillering stage. The vertical lines indicate the average number of tillers of the two parents. The plants chosen for the high-TN and low-TN bulks are indicated in green and red, respectively. **D** Schematic diagram of the experiment workflow

Table 1 Summary of BGI-seq data of parental lines and High-TN and Low-TN bulks

Sample	Cleaned read (million)	Cleaned base (Gb)	%Alignment	Average depth coverage (x)
NDCMP49	65.55	9.83	92.72	23.41
PTT1	78.54	11.69	93.79	27.53
Low-TN bulk	264.9	40	92.95	100
High-TN bulk	264.9	40	92.61	100

Table 2 Chromosome-wise distribution of common SNPs and indels (read depth > 8) identified in high- and low-tiller number bulks

Chr.	Length	SNPs (Depth > 8)	Indels (Depth > 8)
1	43,270,923	61,521	20,308
2	35,937,250	32,559	10,169
3	36,413,819	51,911	16,096
4	35,502,694	31,763	9418
5	29,958,434	37,464	11,014
6	31,248,787	42,264	12,861
7	29,697,621	34,128	10,184
8	28,443,022	29,889	9167
9	23,012,720	41,349	12,256
10	23,207,287	20,735	6115
11	29,021,106	34,434	9441
12	27,531,856	18,464	5537
Total	373,245,519	436,481	132,566

78.54 million, respectively, corresponding to approximately 23.41-fold and 27.53-fold genome coverage (based on an estimated rice genome size of ~400 Mb). Sequencing of individual high-TN and low-TN F_2 lines yielded an average of 126.91 million (between 62.56 and 166.42 million) and 141.57 million (between 86.05 and 190.62 million) paired-end reads, respectively. To create the high-TN bulk, 26.49 million clean reads (corresponding to ~10-fold coverage) were randomly selected from each of the 10 high-TN samples. A similar approach was used to construct the low-TN bulk. The clean reads from the selected individuals were pooled to generate high-TN and low-TN bulks for subsequent analysis (Table 1). Alignment of the PTT1 and NDCMP49 reads to the Nipponbare reference genome identified 1,921,313 and 585,671 SNPs, respectively. Variant calling from the pooled reads revealed 959,597 SNPs in the high-TN bulk and 925,060 SNPs in the low-TN bulk (Table S1). A total of 436,481 common SNPs supported by more than eight reads were selected for QTL-seq analysis (Table 2).

QTL-seq analysis and identification of genomic regions associated with the tiller number in rice

To identify genomic regions associated with tiller number in rice, SNP-index values for each bulk and the Δ(SNP-index) of 436,481 high-confidence SNPs were calculated and plotted across all 12 rice chromosomes (Fig. 2). Significance thresholds were determined based on a null distribution generated assuming no QTL. Two candidate genomic regions were identified where the average Δ(SNP-index) exceeded the 99% confidence interval, indicating a statistically significant association with the trait (Table 3; Fig. 2). On chromosome 4, the QTL labeled *qTN4* was mapped in the interval between 30.7 and 34.3 Mb, with a peak at 33.2 Mb. On chromosome 5, *qTN5* was detected in the region between 9.5 and 15.7 Mb, with a peak at 12.6 Mb (Fig. 2).

Candidate gene annotation and validation

Candidate genes within the *qTN4* and *qTN5* regions were annotated using the RAP-DB database. Genes that had both annotated biological functions and functional SNPs were prioritized, resulting in the identification of 175 genes in the *qTN4* region and 30 genes in the *qTN5* region (Table S2). Based on their known involvement in the regulation of lateral leaf growth and panicle development, *NARROW LEAF1* (*NAL1*; Os04g0615000) was selected as a candidate gene for *qTN4* and and OVATE protein gene, *OsOFP19* (Os05g0324600), as a candidate for *qTN5* (Fig. 3). Three non-synonymous SNPs were identified within *NAL1*. A G-to-A substitution at position 4:31,212,801 in exon 3 resulted in an amino acid change from arginine to histidine (R-to-H). Two additional SNPs were detected in exon 5: a C-to-T substitution at 4:31,214,019, resulting in a change from alanine to valine (A-to-V), and a G-to-A substitution at 4:31,214,045, resulting in a change from valine to isoleucine (V-to-I) (Fig. 4A). In *OsOFP19*, a non-synonymous G-to-A substitution was identified at position 5:15,069,810 in exon 1, resulting in an amino acid change from methionine to valine (M-to-V) (Fig. 4B).

We also analyzed the genotypes of the two candidate genes in the 20 individual F_2 lines used for the QTL-seq analysis. For *NAL1*, F_2 lines carrying the homozygous genotypes of NDCMP49 (A/A at position 4:31,212,801; T/T at 4:31,214,019; and A/A at 4:31,214,045) had a significantly lower number of tillers (TN = 11.60 ± 0.72, $p < 0.01$), while lines with the homozygous PTT1 genotype (G/G, C/C and G/G at the corresponding positions) had a higher number of tillers (TN = 24.15 ± 2.45) (Fig. 5). For *OsOFP19*, the F_2 lines with the homozygous NDCMP49 genotype (A/A at position 5:15,069,810) also had a significantly lower number of tillers (TN = 11.84 ± 1.01), while the lines with the homozygous PTT1 genotype (G/G) had a significantly higher value (TN = 22.90 ± 0.92, $p < 0.01$). Of note, the F_2 lines heterozygous for *NAL1* had a comparable number of tillers to the NDCMP49 homozygotes (TN = 11.67 ± 1.01, no significant difference), suggesting a recessive effect. In contrast, the heterozygous lines for *OsOFP19* had a wide

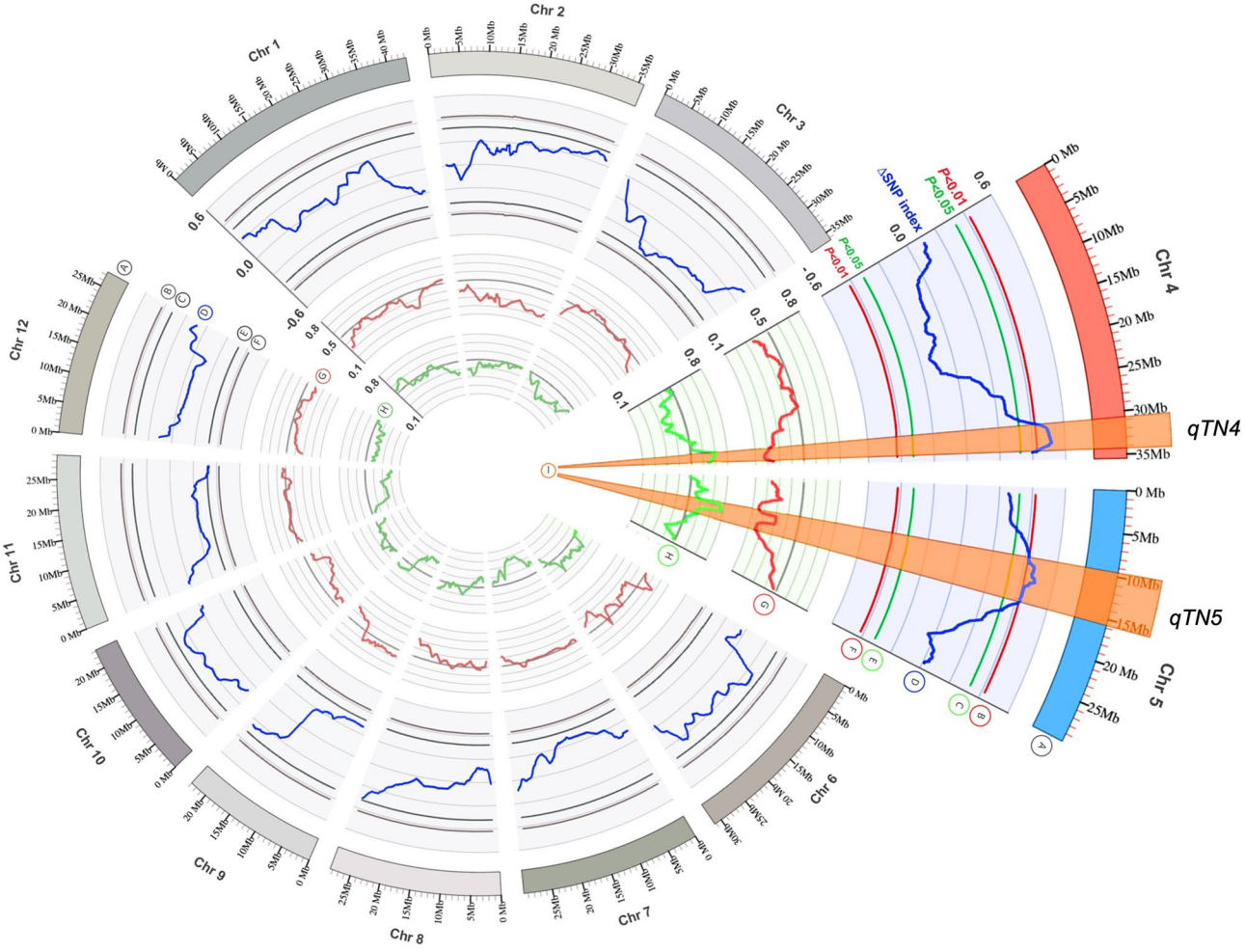

Fig. 2 SNP-index and Δ(SNP-index) diagrams from the QTL-seq analysis. **A** Pseudomolecules of the Nipponbare reference genome (IRGSP 1.0), **B** Upper probability values at 99% confidence ($P < 0.01$), **C** Upper probability values at 95% confidence ($P < 0.05$), **D** The sliding window plots of Δ(SNP-index), with a window size of 2-Mb and 10-kb increments, **E** Lower probability values at 95% confidence ($P < 0.05$), **F** Lower probability values at 99% confidence ($P < 0.01$), **G** Sliding window plots of the average SNP index values in low-bulk, with a 2-Mb window size and 10-kb increments, **H** The sliding window plots of the average SNP-index values in high-bulk, with a 2-Mb window size and 10-kb increments, **I** Candidate genomic regions containing QTLs for tiller number

Table 3 Summary of illumina sequencing data of parental lines and F_2 bulks

QTL	Chr	QTL Region (Mb)	Peak SNP (Mb)	High-TN bulk SNP index	Low-TN bulk SNP index	Confidence interval (95%)	Confident interval (99%)	Delta (SNP index) of peak SNP
qTN4	4	30.7–34.3	33.2	0.17–0.22	0.65–0.70	0.33	0.42–0.43	0.52
qTN5	5	9.5–15.7	12.6	0.16–0.31	0.58–0.79	0.32	0.42	0.49

range of tiller numbers (TN = 10.12–27.94), including both high and low values, suggesting possible incomplete dominance or variable expressivity.

Marker-trait association analysis for *NAL1* and *OsOFP19*

To evaluate the association between SNP genotypes in the candidate genes *NAL1* and *OsOFP19* and tiller number, 500 F_2 individuals from a cross between NDCMP49 and PTT1 were genotyped using a KASP marker (*NAL1*_4_31214045) and a TaqMan probe marker (*OFP19*_5_15069810), developed from G/A SNPs located in exon 3 of *NAL1* (4:31,214,045) and exon 1 of *OsOFP19* (5:15,069,810), respectively (Table S3). Single marker analysis revealed that *NAL1*_4_31214045 had a highly significant association with tiller number ($p < 2.2 \times 10^{-16}$) and explained 22.07% of the phenotypic variance (PVE), while *OFP19*_5_15069810 showed a weaker but still

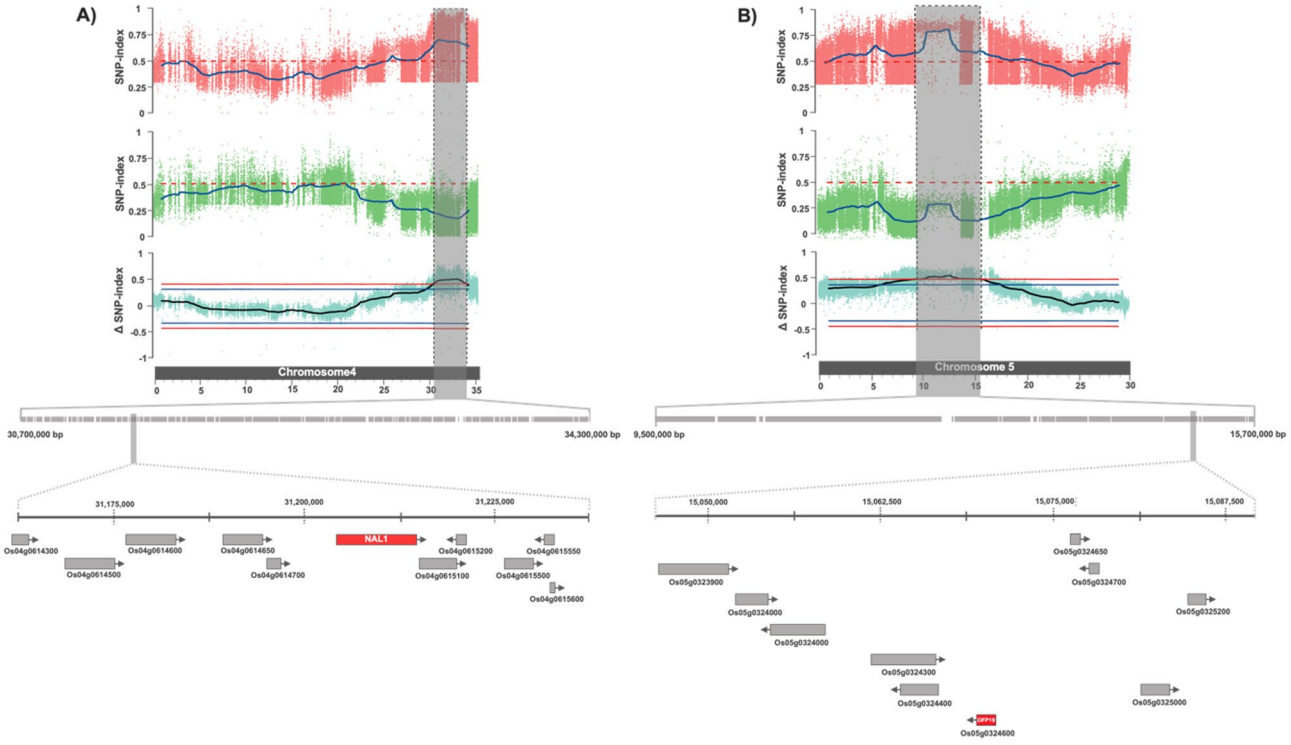

Fig. 3 SNP-index plots and candidate genes on chromosomes 4 and 5. **A** and **B** show the SNP-index plots for low-TN bulk (top, red dots), high-TN bulk (middle, green dots) and Δ(SNP-index) values (bottom, cyan dots) along chromosomes 4 and 5, respectively. Gray shaded areas indicate genomic regions identified as QTLs. Annotated genes within these regions are shown as shaded rectangles, with arrowheads indicating the direction of the open reading frame (ORF). The candidate genes *NAL1* and *OsOFP19* are highlighted by red shaded rectangles

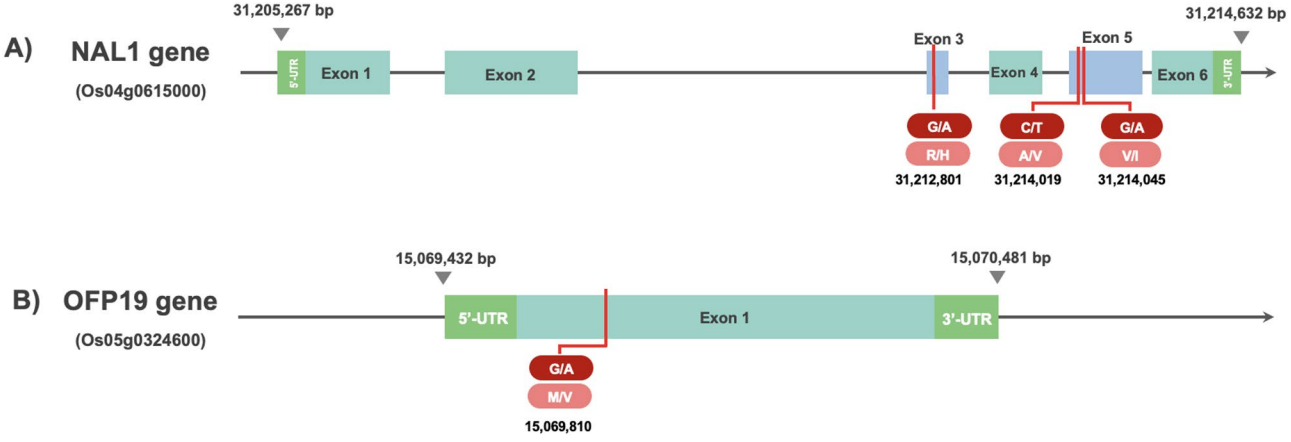

Fig. 4 Gene structures of *NAL1* and *OsOFP19* and polymorphisms in the parental lines and selected F_2 individuals. **A** The structure of the *NAL1* gene shows the exon–intron organization and the positions of three non-synonymous SNPs that distinguish PTT1 and NDCMP49. The amino acid substitutions resulting from each SNP are indicated. **B** Structure of the *OsOFP19* gene showing the position of a non-synonymous SNP identified between the two parents along with the corresponding amino acid change

significant association ($p = 1.99 \times 10^{-5}$), with a PVE of 3.65% (Table 5; Fig. 6A, B).

To further evaluate the combined effect of the two markers, a multiple marker regression analysis was performed. The model showed that both markers contributed significantly to the variation in tiller number, with *NAL1*_4_31214045 showing a strong positive effect (estimate = 4.201, $p < 2 \times 10^{-16}$) and *OFP19*_5_15069810 showing a modest but significant negative effect (estimate = −1.3884, $p = 0.000336$). The overall model explained 24.13% of the phenotypic variance (Table 5; Fig. 6C). The results confirm that the PTT1 allele in *NAL1* contributes significantly to the increase in tiller number, while the PTT1 allele in *OsOFP19* exerts an additive effect,

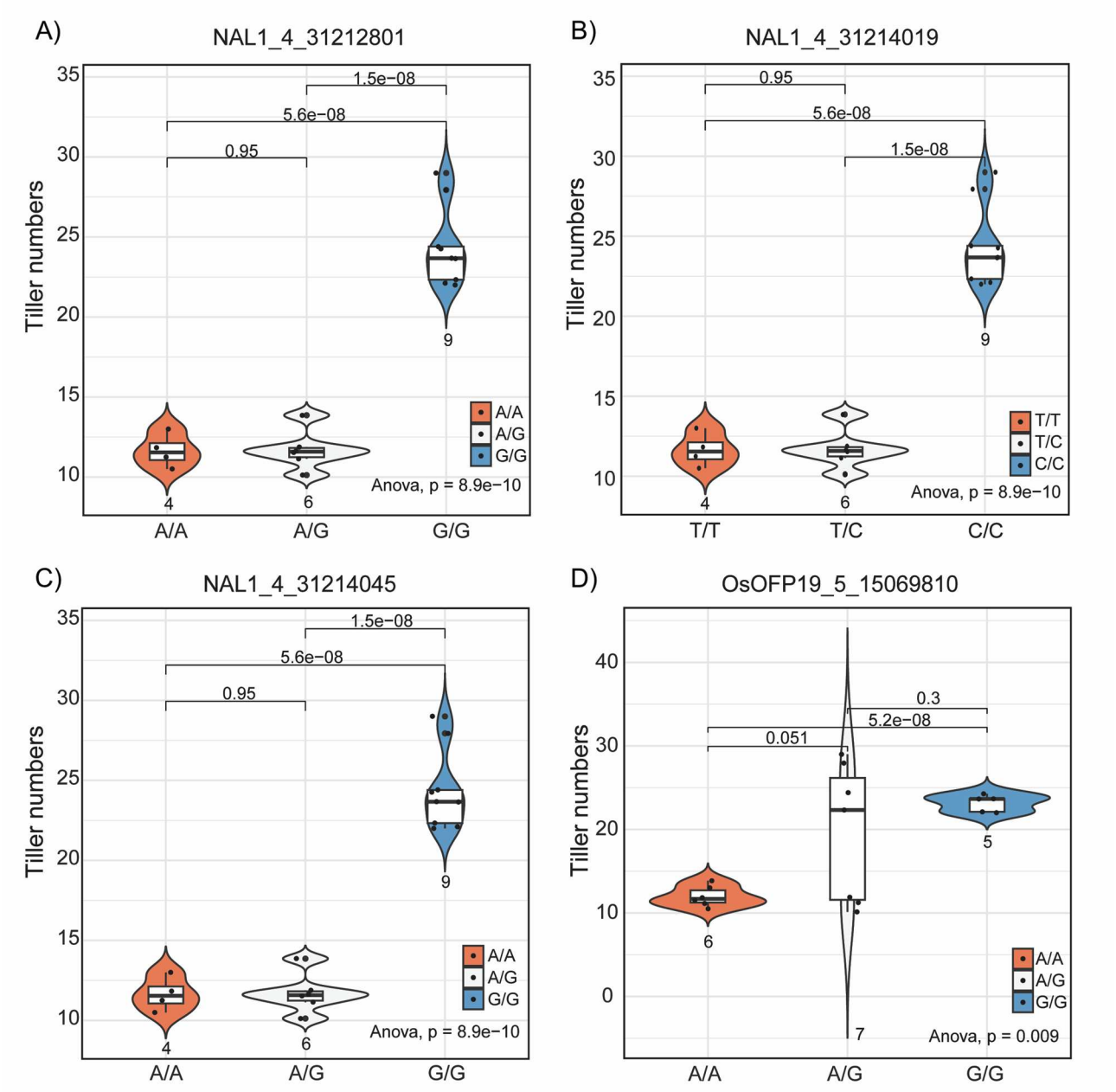

Fig. 5 Association between sequenced SNP genotypes of *NAL1* and *OsOFP19* and tiller number in selected F_2 individuals used for QTL-seq analysis. **A**–**C** Tiller number distribution among *NAL1* genotypes based on SNPs at positions 4:31,212,801, 4:31,214,019 and 4:31,214,045. **D** Tiller number distribution among *OsOFP19* genotypes based the SNP at position 5:15,069,810. Violin plots show the distribution of tiller number across genotype classes, overlaid with boxplots and individual data points (jittered). Genotype classes for the SNPs at positions 4:31,212,801, 4:31,214,019, and 5:15,069,810 are labeled as G/G = homozygous PTT1 allele, A/A = homozygous NDCMP49 allele, and A/G = heterozygous, while genotype classes for 4:31,214,019 are labeled as C/C = homozygous PTT1 allele, T/T = homozygous NDCMP49 allele, and T/C = heterozygous

albeit smaller. To test for potential epistatic interactions between the two QTLs, a two-way analysis of variance (ANOVA) was conducted. The analysis showed significant main effects of both *NAL1* and *OsOFP19* on tiller number, but no significant interaction between the loci ($F_{(4,\ 427)} = 0.57$, $p = 0.68$; Table S4), indicating that their effects are additive rather than epistatic. These results suggest that *NAL1* plays an important role in regulating tiller number in this population and that the effect of *OsOFP19* may be context-dependent or involve interaction with other loci.

Table 5 Analysis of single and multiple markers in the F_2 population

Marker	Tested Sample	Chr.	Position of SNPs	*p*-Value	PVE (%)
Single marker					
NAL1_4_31214045	439	4	31,214,045	< 2.2E-16	22.07
OFP19_5_15069810	498	5	15,069,810	1.99E-05	3.65
Multiple markers					
Tested sample	436				
	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**	**PVE (%)**
(Intercept)	12.9235	1.1781	10.97	< 2e-16 ***	24.13
NAL1_4_31214045	4.201	0.3853	10.903	< 2e-16 ***	
OFP19_5_15069810	-1.3884	0.384	-3.615	0.000336 ***	

Validation of *NAL1* function in regulating tillering ability

CRISPR-Cas9-edited lines targeting *NAL1* were studied to determine the gene's role in regulating tiller number. The *nal1*-1 line contained a 19-bp deletion, and *nal1*-2 carried a 10-bp deletion within the *NAL1* coding sequence (exon 1). Both mutations are predicted to cause frameshift disruptions. At the maximum tillering stage, the edited lines produced a greater number of tillers than the wild type Zhonghua 11 (ZH11; a lowland *Japonica* variety). The average number of tillers was 6.75 in *nal1*-1, 5.75 in *nal1*-2 and 2.75 in ZH11 (Fig. 7). Statistical analysis confirmed significant differences in tiller number among the three genotypes ($p < 0.05$, Duncan's test). The increased number of tillers in the mutant lines was evident in the early vegetation stage and remained consistent through 49 days after sowing (Fig. 7B). The increased number of tillers and narrow leaf width were consistently observed in the mutant lines grown under dry condition, simulating upland condition (Figure S1). These phenotypic results observed in the edited lines are consistent with QTL-seq and marker-trait association results implicating *NAL1* in the control of tillering in rice. In addition to increased tillering, both mutants exhibited a narrow leaf phenotype, with average leaf widths of 0.38 cm (*nal1*-1) and 0.39 cm (*nal1*-2), compared with 0.92 cm in the wild-type background (Fig. 7A). These findings confirm that loss-of-function mutations in NAL1 promote increased tillering and reduced leaf width, supporting its pleiotropic role in rice plant architecture.

Discussion

To improve the performance of upland rice in favorable lowland ecosystems, it is essential to decipher the genetic basis of important agronomic traits such as tillering, which directly influence yield potential under high-input conditions [32]. Numerous quantitative trait loci (QTLs) associated with tiller number have been previously identified using traditional and molecular mapping approaches [33–35]. In recent years, next-generation sequencing (NGS)-based bulked segregant analysis (BSA), i.e. QTL-seq, has emerged as an effective tool to rapidly identify genomic regions associated with phenotypic variation, especially in populations descended from parents with contrasting phenotypes [36].

In this study, we used QTL-seq to analyze an F_2 population derived from a cross between the high tillering lowland indica cultivar PTT1 and the low tillering upland tropical japonica line NDCMP49. QTL-seq analysis identified two genomic regions on chromosomes 4 and 5 that were significantly associated with variation in tiller number. Within these regions, two candidate genes, *NAL1* (Os04g0615000) and *OsOFP19* (Os05g0324600), were identified as likely contributing to the observed phenotypic differences. Both genes have previously been associated with plant architecture traits. *NAL1* has been extensively studied for its role in regulating leaf width via auxin-mediated acid growth mechanisms and cell expansion [37]. The pleiotropic role of *NAL1* has also been associated with vascular bundle formation, vein patterning and cell division [38], as well as physiological traits such as chlorophyll content and photosynthetic efficiency [39]. Favorable alleles of *NAL1* from japonica germplasm have been introgressed into indica backgrounds to improve yield traits, including spikelet number, root architecture, and vascular development [40, 41]. Although *NAL1* has traditionally been associated with narrow leaf morphology, a growing body of evidence, including our results, suggests that it also plays a broader role in shoot architecture and yield-related traits [42]. Our results, derived from two independent *nal1* alleles (*nal1*-1 and *nal1*-2) generated via CRISPR/Cas9 in the ZH11 wild-type background, provide strong genetic evidence consistent with the findings of Jiang et al. [21], who reported similar phenotypes in a mutant generated by random mutation in the Nipponbare background. Consistently, our evaluation under dry (upland) conditions also showed that *nal1* mutants produced more tillers than wild-type plants, indicating that the negative regulatory role of *NAL1* in tiller production is maintained across contrasting water regimes. Together, these studies confirm that loss of *NAL1* function leads to increased tiller number, supporting its role as a negative regulator of tiller production in rice.

Within the *qTN5* interval, we identified 30 annotated genes, among which *OsOFP19* emerged as the most compelling candidate based on our QTL mapping results and prior genetic reports. While additional fine mapping and functional assays are needed to confirm causality, our discovery of previously unreported, naturally occurring missense mutations in *OsOFP19* provides novel evidence for its functional importance. The consistent association

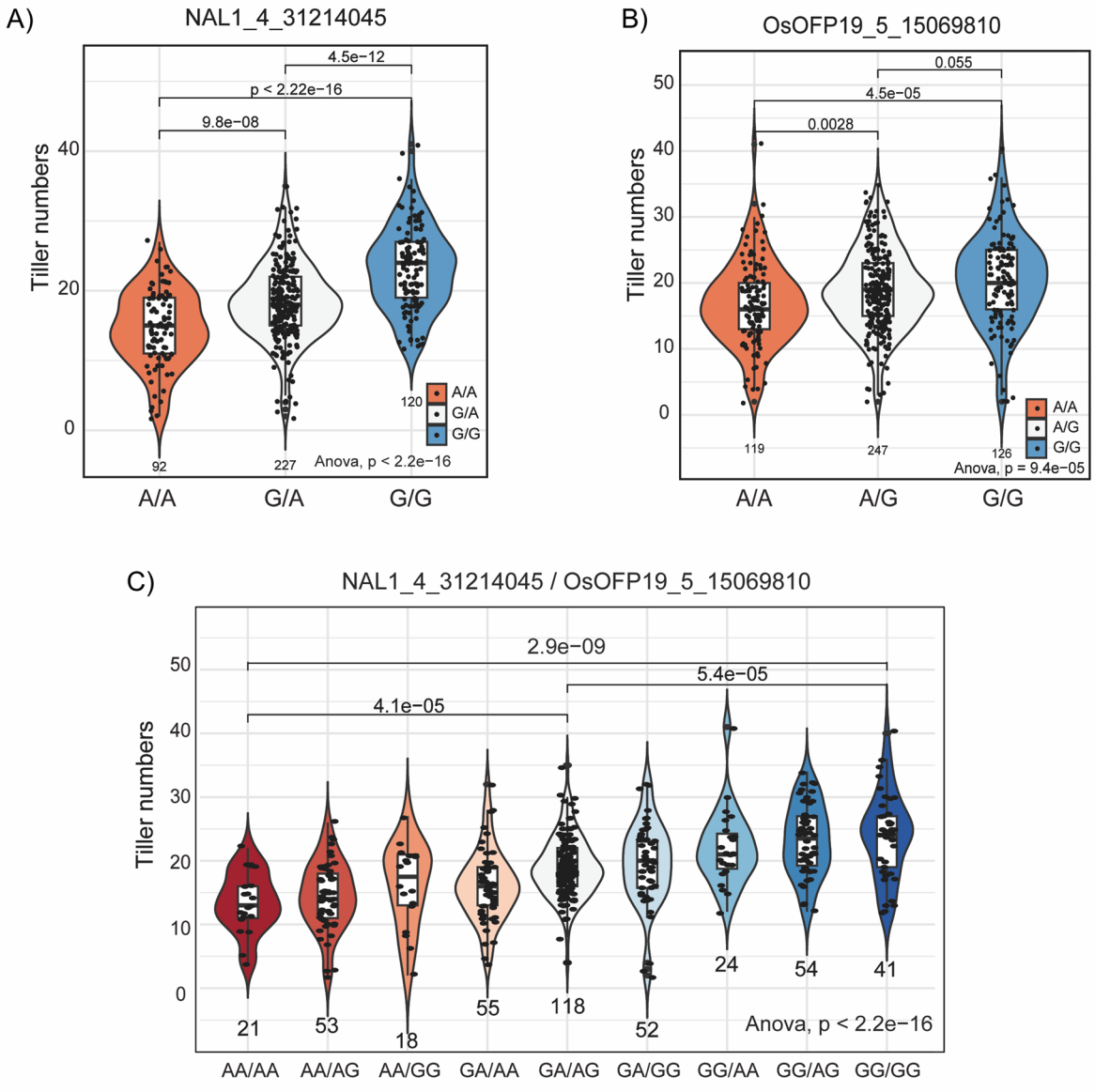

Fig. 6 Marker–trait association analysis of *NAL1* and *OsOFP19* SNP genotypes with tiller number in the F_2 population derived from the NDCMP49 × PTT1 cross. **A** Single-marker analysis of the KASP marker *NAL1*_4_31214045, based on a G/A SNP in exon 3 of *NAL1*. **B** Single-marker analysis of *OFP19*_5_15069810, based on a G/A SNP in exon 1 of *OsOFP19*. **C** Two-marker regression analysis indicates an additive effect between the PTT1 alleles at *NAL1* and *OsOFP19*. In **A** and **B**, genotypes are shown as G/G: homozygous for the PTT1 allele, A/A: homozygous for the NDCMP49 allele, and G/A: heterozygous. In **C**, combined genotypes across the two loci are represented in the format [*NAL1* genotype]/[*OsOFP19* genotype]. For example, GG/GG: homozygous PTT1 at both loci, GG/AA: homozygous PTT1 at *NAL1* and homozygous NDCMP49 at *OsPFP19*, AA/GG: homozygous NDCMP49 at *NAL1* and homozygous PTT1 at *OsOFP19*, AA/AA: homozygous NDCMP49 at both loci, and AG/AG: heterozygous at both loci

of *OsOFP19* with tiller number in the segregating population derived from two genetically distant parental lines highlights its robustness across genetic backgrounds, positioning it as a priority target for future functional studies and rice breeding programs. *OsOFP19* was functionally characterized as part of a protein complex with OSH1 (*Oryza sativa* homeobox 1) and DLT (DWARF AND LOW-TILLERING) that integrates the brassinosteroid (BR) signaling and developmental pathways to modulate plant architecture [43]. Considering that tiller formation is associated with axillary meristem initiation and bud outgrowth, the involvement of *OsOFP19* in hormonal regulation emphasizes its functional importance.

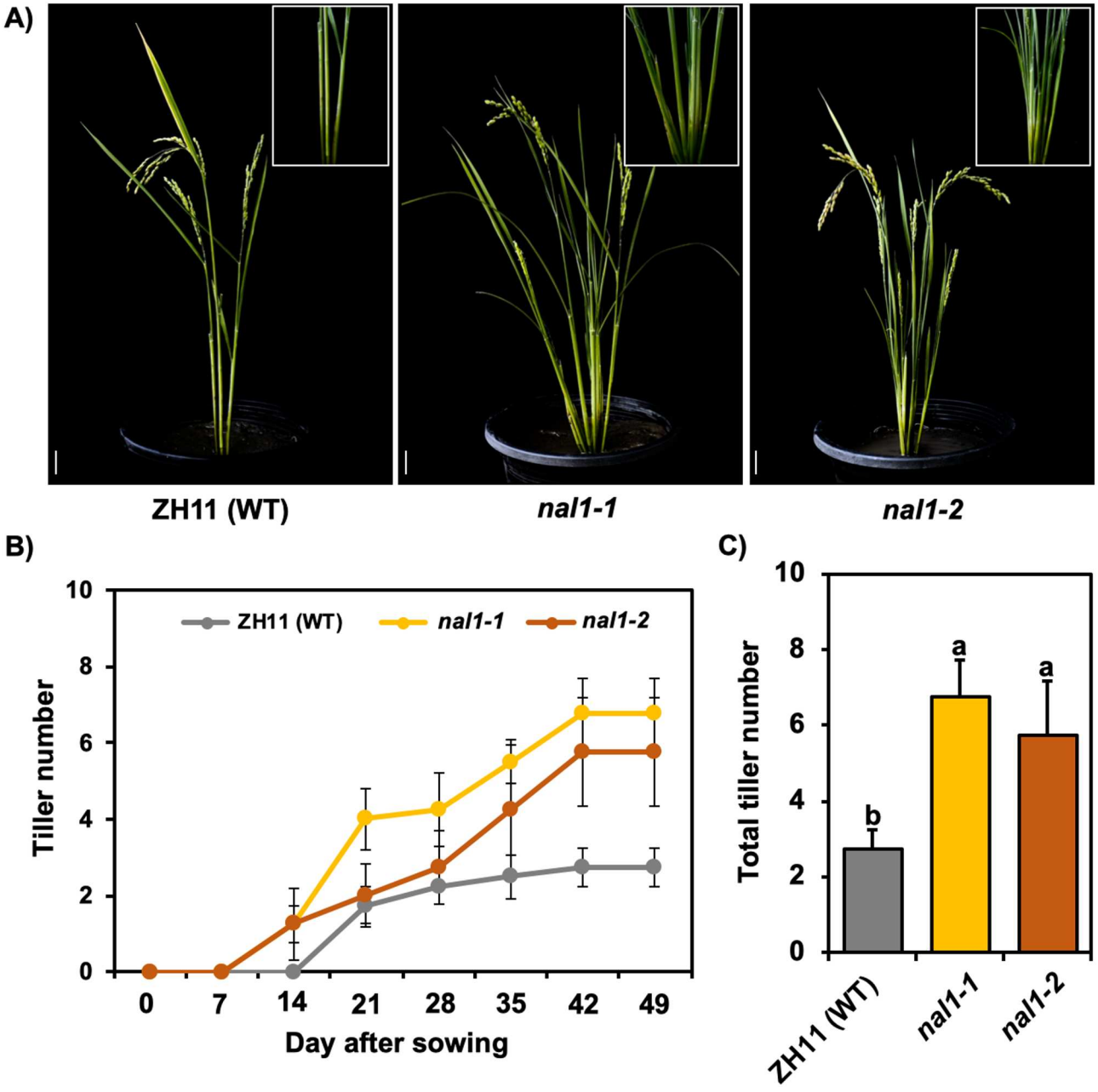

Fig. 7 Whole plant phenotype of ZH11 (WT) and *NAL1* lines at the grain-filling stage under anaerobic condition (**A**). Tiller number of ZH11 (WT), *nal1-1* and *nal1-2* lines from the 0th day to the 49th day after sowing (**B**). Comparison of the total tiller number per plant (**C**). Data in **C** is presented as means ± SE ($n = 4$). Different lower-case letters denote significant differences between lines according to Duncan's test ($p < 0.05$). Scale bar is 5 cm. in (**A**)

To validate the association between these loci and tiller number, we developed an allele-specific KASP marker targeting a functional SNP in *NAL1* and a TaqMan probe marker targeting a functional SNP in *OsOFP19*. Marker trait analysis in the F_2 population showed that the *NAL1* marker explained a substantial proportion of the phenotypic variance (PVE = 22.07%), while the *OsOFP19* marker contributed a smaller but significant effect (PVE = 3.65%). When both markers were analyzed together, an additive effect was observed, with the PTT1 allele in *OsOFP19* enhancing the effect of the PTT1 allele in *NAL1*. This suggests that pyramiding these alleles could be beneficial for breeding high-tillering lines. Additive effects between QTLs have long been recognized as important for the improvement of complex traits, as they provide the opportunity for cumulative genetic gains across loci [44]. The development of robust KASP and TaqMan Probe markers linked to these QTLs provides a practical and scalable tool for marker-assisted selection (MAS). KASP and TaqMan genotyping is known for

its specificity, cost-effectiveness and usefulness in large-scale breeding pipelines [45, 46], making it well suited for improving upland rice varieties to enhance their performance in waterlogged lowland environments.

In summary, our study identified *NAL1* and *OsOFP19* as important genes associated with variation in tiller number in rice. The functional significance of *NAL1* was supported by CRISPR-Cas9 knockout lines that showed significantly increased number of tiller and narrow leaf phenotype. These results provide new insights into the genetic control of tillering in rice and offer molecular tools to improve shoot architecture in future breeding programs. Further studies of the molecular mechanisms underlying the function of *NAL1* and *OsOFP19* are essential to fully elucidate their role in meristem regulation and yield optimization.

Conclusion

In this study, two major QTLs, *qTN4* and *qTN5*, associated with tiller number in rice were successfully identified by QTL-seq analysis. These loci, located on chromosomes 4 and 5, were used to develop functional KASP or TaqMan Probe markers that effectively track genotype segregation in the F_2 population. The validated markers exhibited a strong association with the trait and showed an additive effect when combined. These results highlight the potential application of *NAL1*- and *OsOFP19*-based markers in marker-assisted selection (MAS) and provide valuable tools to improve tillering capacity and increase productivity of upland rice varieties in future breeding programs.

Abbreviations

BSA	Bulk segregant analysis
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9
DAS	Days after sowing
InDel	Insertion/Deletion
KASP	Kompettitive Allele-Specific PCR
MAS	Marker-assisted selection
NAL1	Narrow leaf 1
NDCMP49	Niaw Dam Chaw Mai Pai 49
OFP19	Ovate family protein 19
PTT1	Pathum Thani 1
QTL-seq	Quantitative trait loci-sequencing
SNP	Single nucleotide polymorphism
TN	Tiller number

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-07239-6.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements

We thank the Rice Department, the Ministry of Agriculture and Cooperatives, for providing the plant materials (NDCMP49 and PTT1) used in this study.

Author contributions

S.A., T.Toojinda, J.L.S. and S.W. conceived the study. S.A., M.S., V.R., R.X. and S.W. designed the experiments. T.R., N.P., T.Thianthavon, R.D. and P.M. performed experiments. T.R., N.P. and W.A. analyzed data. T.R., N.P., and M.K.P. wrote the manuscript. S.A., S.W., J.E.G. reviewed the manuscript. All authors read and approved the final manuscript.

Funding

This work was partially financially supported by the Kasetsart University Research and Development Institute, KURDI (Grant No. FF (KU) 55.67), the Office of the Ministry of Higher Education, Science, Research and Innovation, and the Thailand Science Research and Innovation through the Kasetsart University Reinventing University Program 2024, the National Research Council of Thailand (NRCT Grant Number: NRCT-RTA/812/2563), the Thailand Rice Science Research Hub of Knowledge (NRCT Grant Number: N35E680080), and the National Science, Research and Innovation Fund, Thailand Science Research and innovation (TSRI Grant Number: FFB680075/0337) through the National Science and Technology Development Agency (NSTDA).

Data availability

The datasets generated and analyzed in this study are available in the GenBank of NCBI with the project accession of PRJNA1287108. Submitted data will remain private until related manuscript has been accepted. All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Rice Science Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[2]National Center for Genetic Engineering and Biotechnology (BIOTEC), 113 Thailand Science Park, Pahonyothin Road, Khlong Nueng, Khlong Luang 12120, Pathum Thani, Thailand
[3]Cell and Developmental Biology Department, School of Biological Sciences, University of California San Diego, La Jolla, San Diego, CA 92093-0116, USA
[4]Research Center for Applied Botany, National Research and Innovation Agency, Jl. Raya Jakarta - Bogor KM 46, Cibinong, Jawa Barat 16911, Indonesia
[5]State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Horticulture, South China Agricultural University, Guangzhou 510640, China
[6]Institute for Sustainable Food, School of Biosciences, University of Sheffield, Sheffield S10 2TN, UK
[7]Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

Received: 6 July 2025 / Accepted: 18 August 2025
Published online: 02 September 2025

References

1. Abbas S, Haider A, Kousar S, Lu H, Lu S, Liu F, et al. Climate variability, population growth, and globalization impacting food security in Pakistan. Sci Rep. 2025;15:4225.

2. Alam M, Lou G, Abbas W, Osti R, Ahmad A, Bista S et al. Improving Rice Grain Quality Through Ecotype Breeding for Enhancing Food and Nutritional Security in Asia-Pacific Region. Rice (N Y). 2024;17:47.
3. Joshi R, Singh B, Shukla A. Evaluation of elite rice genotypes for physiological and yield attributes under aerobic and irrigated conditions in Tarai areas of Western Himalayan region. Curr Plant Biology. 2018;13:45–52.
4. Xia H, Luo Z, Xiong J, Ma X, Lou Q, Wei H, et al. Bi-directional selection in upland rice leads to its adaptive differentiation from lowland rice in drought resistance and productivity. Mol Plant. 2019;12:170–84.
5. Purugganan MD. The evolution of rice: molecular vignettes on its origins and spread. Archaeol Anthropol Sci. 2010;2:61–8.
6. Aesomnuk W, Ruengphayak S, Ruanjaichon V, Sreewongchai T, Malumpong C, Vanavichit A, et al. Estimation of the genetic diversity and population structure of thailand's rice landraces using SNP markers. Agronomy. 2021;11:995.
7. Lyu J, Li B, He W, Zhang S, Gou Z, Zhang J, et al. A genomic perspective on the important genetic mechanisms of upland adaptation of rice. BMC Plant Biol. 2014;14:160.
8. Saito K, Linquist B, Keobualapha B, Phanthaboon K, Shiraiwa T, Horie T. Cropping intensity and rainfall effects on upland rice yields in Northern Laos. Plant Soil. 2006;284:175–85.
9. Wang B, Smith SM, Li J. Genetic regulation of shoot architecture. Annu Rev Plant Biol. 2018;69:437–68.
10. Chang S, Chang T, Song Q, Wu J, Luo Y, Chen X, et al. Architectural and physiological features to gain high yield in an elite rice line YLY1. Rice (N Y). 2020;13:60.
11. Yu J, Xuan W, Tian Y, Fan L, Sun J, Tang W, et al. Enhanced OsNLP4-OsNiR cascade confers nitrogen use efficiency by promoting tiller number in rice. Plant Biotechnol J. 2021;19:167–76.
12. Zha M, Imran M, Wang Y, Xu J, Ding Y, Wang S. Transcriptome analysis revealed the interaction among strigolactones, auxin, and cytokinin in controlling the shoot branching of rice. Plant Cell Rep. 2019;38:279–93.
13. Yan Y, Wei M, Li Y, Tao H, Wu H, Chen Z, et al. MiR529a controls plant height, tiller number, panicle architecture and grain size by regulating SPL target genes in rice (Oryza sativa L). Plant Sci. 2021;302:110728.
14. Li X, Qian Q, Fu Z, Wang Y, Xiong G, Zeng D, et al. Control of tillering in rice. Nature. 2003;422:618–21.
15. Wang Y, Li J. Molecular basis of plant architecture. Annu Rev Plant Biol. 2008;59:253–79.
16. Wang Y, Li J. Branching in rice. Curr Opin Plant Biol. 2011;14:94–9.
17. Oikawa T, Kyozuka J. Two-Step regulation of LAX PANICLE1 protein accumulation in axillary meristem formation in rice. Plant Cell. 2009;21:1095–108.
18. Tanaka W, Ohmori Y, Ushijima T, Matsusaka H, Matsushita T, Kumamaru T, et al. Axillary meristem formation in rice requires the WUSCHEL ortholog TILLERS ABSENT1. Plant Cell. 2015;27:1173–84.
19. Lu Z, Shao G, Xiong J, Jiao Y, Wang J, Liu G, et al. MONOCULM 3, an ortholog of WUSCHEL in rice, is required for tiller bud formation. J Genet Genomics. 2015;42:71–8.
20. Mjomba FM, Zheng Y, Liu H, Tang W, Hong Z, Wang F, et al. Homeobox is pivotal for Oswus controlling tiller development and female fertility in rice. (Bethesda). 2016;G3:6:2013–21.
21. Jiang D, Fang J, Lou L, Zhao J, Yuan S, Yin L, et al. Characterization of a null allelic mutant of the rice NAL1 gene reveals its role in regulating cell division. PLoS ONE. 2015;10:e0118169.
22. Jiang L, Liu X, Xiong G, Liu H, Chen F, Wang L, et al. DWARF 53 acts as a repressor of Strigolactone signalling in rice. Nature. 2013;504:401–5.
23. Zhou F, Lin Q, Zhu L, Ren Y, Zhou K, Shabek N, et al. D14-SCF(D3)-dependent degradation of D53 regulates Strigolactone signalling. Nature. 2013;504:406–10.
24. Takagi H, Abe A, Yoshida K, Kosugi S, Natsume S, Mitsuoka C, et al. QTL-seq: rapid mapping of quantitative trait loci in rice by whole genome resequencing of DNA from two bulked populations. Plant J. 2013;74:174–83.
25. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for illumina sequence data. Bioinformatics. 2014;30:2114–20.
26. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754–60.
27. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The sequence alignment/map format and samtools. Bioinformatics. 2009;25:2078–9.
28. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO et al. Twelve years of samtools and BCFtools. Gigascience. 2021;10(2):giab008.
29. Edwards MD, Stuber CW, Wendel JF. Molecular-marker-facilitated investigations of quantitative-trait loci in maize. I. Numbers, genomic distribution and types of gene action. Genetics. 1987;116:113–25.
30. Thianthavon T, Aesomnuk W, Pitaloka MK, Sattayachiti W, Sonsom Y, Nubankoh P et al. Identification and validation of a QTL for bacterial leaf streak resistance in rice (Oryza sativa L.) against Thai Xoc strains. Genes. 2021;12(10):1587
31. Dumhai R, Wanchana S, Saensuk C, Choowongkomon K, Mahatheeranont S, Kraithong T, et al. Discovery of a novel CnAMADH2 allele associated with higher levels of 2-acetyl-1-pyrroline (2AP) in yellow Dwarf coconut (Cocos nucifera L). Sci Hortic. 2019;243:490–7.
32. Khush GS. What it will take to feed 5.0 billion rice consumers in 2030. Plant Mol Biol. 2005;59:1–6.
33. Hittalmani S, Shashidhar HE, Bagali PG, Huang N, Sidhu JS, Singh VP, et al. Molecular mapping of quantitative trait loci for plant growth, yield and yield related traits across three diverse locations in a doubled haploid rice population. Euphytica. 2002;125:207–14.
34. Liu G, Zeng R, Zhu H, Zhang Z, Ding X, Zhao F, et al. Dynamic expression of nine QTLs for tiller number detected with single segment substitution lines in rice. Theor Appl Genet. 2009;118:443–53.
35. Liu G, Zhu H, Liu S, Zeng R, Zhang Z, Li W, et al. Unconditional and conditional QTL mapping for the developmental behavior of tiller number in rice (Oryza sativa L). Genetica. 2010;138:885–93.
36. Li Z, Xu Y. Bulk segregation analysis in the NGS era: a review of its teenage years. Plant J. 2022;109:1355–74.
37. Lin L, Zhao Y, Liu F, Chen Q, Qi J. Narrow leaf 1 (NAL1) regulates leaf shape by affecting cell expansion in rice (Oryza sativa L). Biochem Biophys Res Commun. 2019;516:957–62.
38. Qi J, Qian Q, Bu Q, Li S, Chen Q, Sun J, et al. Mutation of the rice narrow leaf1 gene, which encodes a novel protein, affects vein patterning and Polar auxin transport. Plant Physiol. 2008;147:1947–59.
39. Takai T, Adachi S, Taguchi-Shiobara F, Sanoh-Arai Y, Iwasawa N, Yoshinaga S, et al. A natural variant of NAL1, selected in high-yield rice breeding programs, pleiotropically increases photosynthesis rate. Sci Rep. 2013;3:2149.
40. Fujita D, Trijatmiko KR, Tagle AG, Sapasap MV, Koide Y, Sasaki K, et al. NAL1 allele from a rice landrace greatly increases yield in modern indica cultivars. Proc Natl Acad Sci USA. 2013;110:20431–6.
41. Zhang G-H, Li S-Y, Wang L, Ye W-J, Zeng D-L, Rao Y-C, et al. LSCHL4 from Japonica cultivar, which is allelic to NAL1, increases yield of indica super rice 93 – 11. Mol Plant. 2014;7:1350–64.
42. Taguchi-Shiobara F, Ota T, Ebana K, Ookawa T, Yamasaki M, Tanabata T, et al. Natural variation in the flag leaf morphology of rice due to a mutation of the NARROW LEAF 1 gene in Oryza sativa L. Genetics. 2015;201:795–808.
43. Yang C, Ma Y, He Y, Tian Z, Li J. OsOFP19 modulates plant architecture by integrating the cell division pattern and brassinosteroid signaling. Plant J. 2018;93:489–501.
44. Staub JE, Serquen FC, Gupta M. Genetic markers, map construction, and their application in plant breeding. Horts. 1996;31:729–41.
45. Neelam K, Brown-Guedira G, Huang L. Development and validation of a breeder-friendly KASPar marker for wheat leaf rust resistance locus Lr21. Mol Breed. 2013;31:233–7.
46. Steele K, Quinton-Tulloch M, Vyas D, Witcombe J. Thousands of trait-specific KASP markers designed for diverse breeding applications in rice (Oryza sativa). (Bethesda). 2025;G3(1):jkae251.

Publisher's Note