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Identification and Fine Mapping of a Quantitative Trait Locus Controlling the Total Flower and Pod Numbers in Soybean

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Abstract: Total flower and pod numbers (TFPN) and effective pod numbers per plant (PNPP) are among the most important agronomic traits for soybean production. However, the underlying genetic mechanism remains unclear. In this study, we constructed a recombinant inbred line population derived from a cross between JY73 (high TFPN) and TJSLH (low TFPN) to map loci for the two traits. In total, six QTL for TFPN and five QTL for PNPP were identified, among which a QTL on chromosome 4, named *qFPN4*, explained 9.2% and 9.6% of the phenotypic variation of TFPN and PNPP, respectively. Analysis of residual heterozygous lines for *qFPN4* indicated that TFPN or PNPP was controlled by a single dominant gene at this locus and delimited the QTL into a ~2.62 Mb interval which tightly linked to an Indel marker C1-5. This mapping result was further confirmed by bulked segregant analysis (BSA) of the near isogenic lines. The genome-sequencing-based BSA also identified eight candidate genes carrying nonsynonymous SNPs and/or Indels; two genes, *Glyma.04G176600* and *Glyma.04G178900*, were nominated as the most promising genes for *qFPN4* based on additional expression and function analysis. These results improve our understanding of the genetic mechanism of TFPN and PNPP and indicate the potential for soybean yield improvement.

Keywords: total flower and pod numbers; pod numbers per plant; *qFPN4*; fine mapping; near isogenic lines; bulked segregant analysis

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1. Introduction

Soybean (*Glycine max* (L.) Merrill) is an important seed crop containing approximately 40% editable oil and 20% high-quality protein in seeds. The protein contains a complete amino acid profile including eight amino acids essential for human health; a wide range of cultivation and high production make soybean a sustainable crop to secure the increasing need for plant protein based diets worldwide [1]. Therefore, yield has been a major goal for soybean improvement. However, soybean germplasm has a narrow genetic basis [2,3] and the yield increase does not keep pace with the growing need [4]. Better molecular strategies with practical markers/genes are needed to explore soybean full genetic potential [5]. A possible approach to increase yield is through trait dissection by breaking down the yield complex trait into major yield components.

Yield is a very complex trait, and many different components contribute to it such as pod numbers per plant (PNPP), seed numbers per pod, and hundred-grain weight, among which PNPP is an especially important factor in determining soybean yield [6], because it is strongly correlated with grain yield per plant (r = 0.84). For example, among the agronomic traits the number of three-seed pods showed the highest correlation with the yield per plant, suggesting the strong indirect effect on the total number of pods [7]. PNPP is mainly determined by multiple factors such as total flower and pod numbers (TFPN), pods produced per plant, and the abscission rate of flowers and pods. Studies have shown

that the total number of flowers or pods or both could greatly enhance soybean yields [8,9]. Therefore, understanding the genetic mechanism of TFPN and PNPP will be helpful to elucidate how these traits genetically contribute to the yield, which is important for the development of high-yield soybean cultivars.

Many studies have mapped quantitative trait loci (QTL) for the related traits of flower or pod numbers with an aim to increase the breeding efficiency for higher yields. In all, five QTL for the number of one-seed pods (NOP), two QTL for the number of two-seed pods (NTP), three QTL for three-seed pods (NThP), six QTL for the number of four-seed pods (NFP), and nineteen QTL for PNPP have been mapped on chromosomes 1–4, 6–7, and 9–20, respectively, reflecting the complex mechanism of soybean yield traits. Previously, two QTL for PNPP were identified using the F_7 and F_8 RIL population that were derived from the cross between the varieties BARC-8 and Garimpo; the QTL were located on chromosomes 4 and 9 [9]. Another study identified 12 QTL for the number of main stem pods on chromosomes 1, 3, 6, 11, 13, and 16 using a $F_{2:10}$ RIL population derived from a cross between Charleston and Dongnong 594 [10]. Zhang et al., (2010) detected three major QTL (*qfn-Chr18-2*, *qfn-Chr19*, and *qfn-Chr20-1*) on chromosomes 18, 19, and 20 for flower numbers and two main QTL (*qpn-Chr11* and *qpn-Chr20*) chromosomes 11 and 20 for pod numbers [11]. About 10 QTL were detected on chromosomes 2, 3, 6, 7, 12, 14, 15, 17, and 19 in the F_2 progenies of the diverse cross combinations between wild soybean (*G. soja*) and cultivated soybean (*G. max*) [12]. A further 12 pod-number-related QTL were identified from a population of introgression lines derived from Charleston and Dongnong 594 [13], including one QTL on chromosome 12 for NOP, one QTL on chromosome 2 for NTP, two QTL on chromosomes 5 and 9 for NThP, six QTL on chromosomes 11, 13, and 15 for NFP, and one QTL on chromosome 11 for PNPP. A similar study using two RIL populations found nine pod-number-related QTL, including four QTL on chromosomes 2, 4, 7, and 20 for NOP, one QTL on chromosome 18 for NTP, one QTL on chromosome 18 for NThP, three QTL on chromosomes 2, 14, and 18 for NFP [6]. Four QTL on chromosomes 4, 7, and 11 were detected in a RIL population of the cross between Zhonghuang 24 and Huaxia 3 in 2 years using the high-density genetic map [14]. To the best of our knowledge, none of these studies have been followed up to report the underlying genes; therefore, the underlying genes and the mechanism are largely unclear. Additionally, little information has been reported for QTL or genes controlling TFPN; therefore, a lack of available information for the molecular genes or markers limited soybean yield improvement from the perspectives of TFPN and PNPP in soybean.

As mentioned above, understanding the genetic mechanism of TFPN and PNPP is important for the development of molecular tools that can facilitate the breeding of high-yield soybean cultivars. Therefore, the objectives of this study are: (1) to detect high confident QTL associated with TFPN or PNPP using linkage mapping, (2) to finely map one major locus and identify the tightly linked molecular markers using the residual heterozygous lines (RHLs) strategy [15], and (3) to propose candidate gene(s) for major QTL. The results increase our understanding of the genetic mechanisms controlling TFPN and PNPP, which would be helpful for soybean yield improvement.

2. Materials and Methods

2.1. Plant Materials and Field Experiments

A segregating population of RILs consisting of 100 individuals was developed from the cross between soybean cultivar JY73 with an average of 232 flowers and pods at the R3 stage (more flowers and pods, MFP) and landrace TJSLH with an average of 154 flowers and pods at the R3 stage (less flowers and pods, LFP) (designated JT population for simplicity). The seeds of F_2 or F_5 RILs along with the parental lines were sown in the field at the Heilongjiang Academy of Agricultural Sciences, Mudanjiang Branch, Mudanjiang, China (44°60′ N, 129°58′ E), during the growing seasons (May–October) in 2015 and 2018, respectively. The F_6 seeds for the RILs were developed by the single seed descent method [15]. The RHLs segregating family for the *qFPN4* locus (Family #5) were selected

from F_6 RILs and planted in the growing season of 2018; the progenies and NILs were planted in rows on 14 May 2019, each line per row. Single seeds for all the hybrids or lines were planted in a 20 cm interval in 5 m rows and spaced 60 cm apart. All trials followed the standard management to control insects and weeds [15].

2.2. Construction of Linkage Map

The linkage map was constructed using Simple Sequence Repeats (SSRs) that were selected from an integrated soybean genetic linkage map [16,17] and insertion and deletion (Indel) markers (Supplementary Table S1) that were preserved from the Key Laboratory of Soybean Molecular Design and Breeding, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences. The total DNA of the two parental lines and the derived F_2 and F_6 population were extracted from freeze-dried leaf tissues using the cetyltrimethylammonium bromide (CTAB) method [18]. The PCR procedure and reaction setup followed the methods as described by Yang et al., (2013) [13] and was run on the MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) in a 20 µL reaction volume. The denatured PCR products were separated on 6% (w/v) denaturing polyacrylamide gel and visualized by ethidium bromide straining (Trigizano and Caetano-Anolles 1998). A genetic map was constructed using Map Manager QTXb20 [19] and JoinMap3 [20].

2.3. QTL Analysis

QTL were identified using multiple QTL model mapping of the MapQTL5 package [21]. The LOD threshold for declaring significant QTL was determined using a permutation test with a significance level of $p < 0.05$ ($n = 1000$).

2.4. Fine Mapping

A total of 19 Indel markers (Supplementary Table S2) were developed to identify the RILs carrying critical recombination and the derived NILs for *qFPN4* locus. The Indel markers (C1-1–C1-10) were used to determine the genotype of the parental lines and recombinants at the locus.

2.5. Genome Resequencing Analysis

Based on a phenotypic investigation, DNAs from 30 individuals with MFP or LFP were pooled separately to build an MFP bulk and a LFP bulk, respectively. Each bulk was used to construct a library followed by whole genome sequencing on the Illumina HiSeq™ 2500 platform at the Shanghai Personal Biotechnology Co., Ltd., (Shanghai, China). The sequencing depth was approximately 30×.

For quality control, low-quality reads with quality scores <20 were filtered out and the adapters were trimmed. The saved reads were aligned to the Williams 82 reference genome (Wm82.a2.v1) using the Burrows–Wheeler Aligner [22]. The SAMtools [23] was used to mark duplicates and the GATK [24] was used for local realignment and base recalibration. An SNP was determined by the identification with both GATK and SAMtools via SNP calling using default parameters. The SNPs identified between the two BSA pools were used for association analysis using the Euclidean distance (ED) method [25]; in theory, the higher the ED value the closer to the QTL site [26]. The associated threshold value was determined based on ED + 3SD (standard deviation) [25]. The Indel-associated regions were obtained in a similar method as SNP-associated regions.

2.6. Identification of the Candidate Gene for qFPN4

The genes within the QTL interval were determined using the Phytozome 13.0 database (https://phytozome-next.jgi.doe.gov/info/Gmax_Wm82_a2_v1, accessed on 1 June 2020) and used as the source for candidate gene search. Function annotations for all candidate genes were identified using the soybean annotation file retrieved from the Phytozome database using default parameters.

2.7. Phenotype Statistics

Five R1-stage plants per row were surrounded with gauze cloth [27], ensuring that all the flowers and pods per plant were shed in a closed bag. The flowers and pods collected in the cloth were counted at the R3 stage to determine TFPN. PNPP, seed number per plant, and yield per plant were investigated at the R8 stage [10]. TFPN was calculated as the sum of the number of abscised flowers and pods and the number of pods per plant.

Chi-square ($\chi 2$) tests were performed to detect segregation distortion. A one-way analysis of variance (ANOVA) was used to identify significant marker–phenotype associations between polymorphic DNA markers and the investigated phenotypes.

3. Results

3.1. Phenotypic Identification of the Mapping Population

The parental lines TJSLH and JY73 exhibited significant differences in four traits including TFPN, PNPP, seed number per plant, and yield per plant. Briefly, the four phenotypic values were higher for JY73 than TJSLH (Figure 1). Correlation analysis among these traits (Table 1) indicates that TFPN showed the strongest positive correlation with PNPP (r = 0.903, *p*-value < 0.01), followed by the yield per plant and the seed number per plant (0.833 and 0.807, respectively, *p*-value < 0.01). Consistently, PNPP showed significant positive correlation with seed number per plant and yield per plant (r = 0.868 and 0.903, respectively, *p*-value < 0.01). These results showed that both TFPN and PNPP are important components for soybean yield, which may contribute to soybean production.

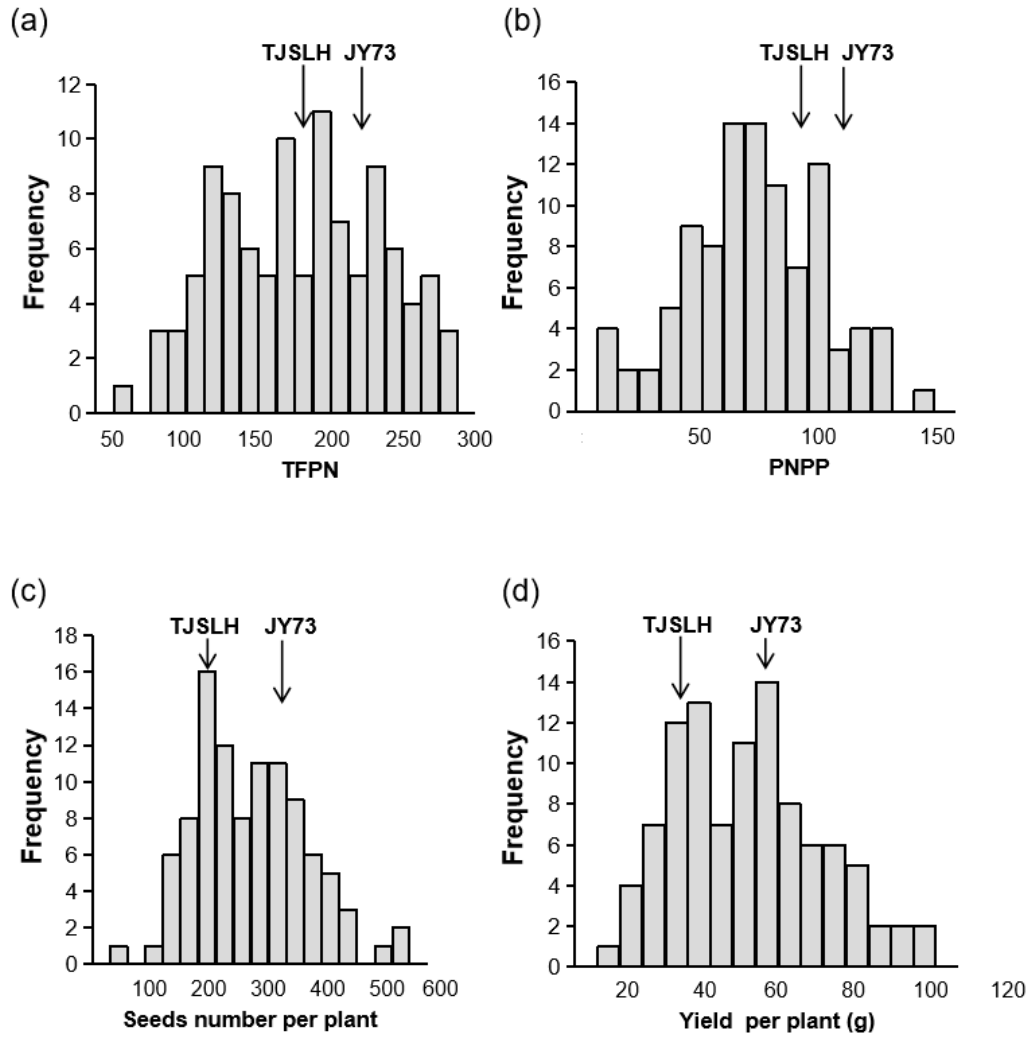


Figure 1. Distribution of TFPN (**a**), PNPP (**b**), and other two agronomic traits (**c**,**d**) in the F_2 population.

Table 1. Correlation coefficient among TFPN, PNPP, and yield-related traits in soybean.

	PNPP	Seed Number per Plant	Yield per Plant
TFPN	0.903	0.807 **	0.833 **
PNPP	-	0.868 **	0.903 **

** means significant differences at $p < 0.01$, student's *t*-tests.

With one MFP cultivar (JY73) and one LFP landrace (TJSLH) as parental lines, we developed a RIL population consisting of 100 individuals in an effort to locate QTL controlling the flower- and pod-associated traits. The distribution of phenotypic values for the four traits are shown in Figure 1. Within the JT population, TFPN at the R3 and PNPP at the R8 stage varied greatly ranging from 50 to 300 and from 10 to 160, respectively. All the phenotypic values showed a wide range of distribution, suggesting that all the four traits are quantitatively controlled by multiple genes. Moreover, strong transgressive segregations for all four traits were observed in the population, suggesting that alleles with positive effects on the measured traits are distributed among the parents.

3.2. Multiple QTL Control TFPN and PNPP

A genetic linkage map consisted of 151 markers (138 SSRs, 13 Indels) was constructed and it covered all 20 chromosomes with a coverage length of 1351.5 cm and an average distance of 11.5 cm in the F_2 TJ RILs. Based on 1000 permutations, a LOD score of 2.0 [28] was used as the threshold to determine QTL controlling TFPN and PNPP.

Using this genetic map and flower/pod number-related phenotypes in the JT population, a total of six QTL controlling TFPN were identified on chromosomes 2, 3, 4, 7, and 10, designated *qFPN2*, *qFPN3*, *qFPN4*, *qFPN7*, *qFPN10-1*, and *qFPN10-2*; five QTL for PNPP were detected on chromosomes 2, 4, 5, 7, and 10, named *qPN2*, *qPN4*, *qPN5*, *qPN7*, and *qPN10* (Table 2, Figure 2). Among the QTL, *qPN2*, *qPN7*, and *qPN10* were also identified in Kuroda et al., (2013) [12] and Ning et al., (2018) [6]. *qPN5* was detected in Yang et al., (2013) [13], which was supportive of the results. Interestingly, *qPN4* was evaluated in the interval of Satt190–Satt195, coinciding with the QTL on chromosome 4 detected by Vieira et al., (2006) [9], Liu et al., (2017) [14], and Ning et al., (2018) [6]. Identification of the QTL in multiple studies with different populations or environment conditions confirmed the robust expression of *qPN4* and its important role for PNPP.

Table 2. QTL information for TFPN and PNPP.

Traits	QTL	Chromosome	Flanking Marker	Genomic Region (Mb)	LOD	PVE (%)	Additive Effect	Re-Identification
TFPN	*qFPN2*	2	Sat_069—PSI0295	43.2–48.3	2.28	14.4	23.04	
	qFPN3	3	Sat_266—GMABAB	34.0–37.9	2.67	13.9	26.10	
	qFPN4	4	Satt190—Satt195	16.7–44.4	2.10	9.2	16.81	
	qFPN7	7	Satt201—Satt245	2.0–9.4	4.28	18.1	33.09	
	qFPN10-1	10	Sat_321—Satt653	2.4–4.6	2.28	11.3	23.34	
	qFPN10-2	10	Satt653—Satt259	4.6–4.9	2.02	8.9	20.06	
PNPP	*qPN2*	2	Sat_069—PSI0295	43.2–48.3	2.38	15.9	17. 55	[6,12]
	qPN4	4	Satt190—Satt195	16.7–44.4	2.19	9.6	13.89	[6,9,14]
	qPN5	5	Sat_407	34.0	2.00	8.8	14.18	[13]
	qPN7	7	Satt201—Satt245	2.0–9.4	3.19	13.7	21.21	[6,12]
	qPN10	10	Sat_321—Satt653	2.4–4.6	2.15	10.3	17.17	[6,12]

PVE, phenotypic variation explanation ratio.

Furthermore, *qPN4* explained 9.6% of the phenotypic variation of PNPP with an additive effect of 13.89; it also controlled TFPN and was designated *qFPN4* that accounted for 9.2% of the observed variation of TFPN with an additive effect of 16.81 (Table 2). Therefore, the locus *qPN4*/*qFPN4* controlled two highly correlated traits: PNPP and TFPN. To our best knowledge, no known genes in this interval controlling flower and pod related

traits has been previously reported in soybean; therefore, *qFPN4* is a novel locus for TFPN and we continued to study this locus.

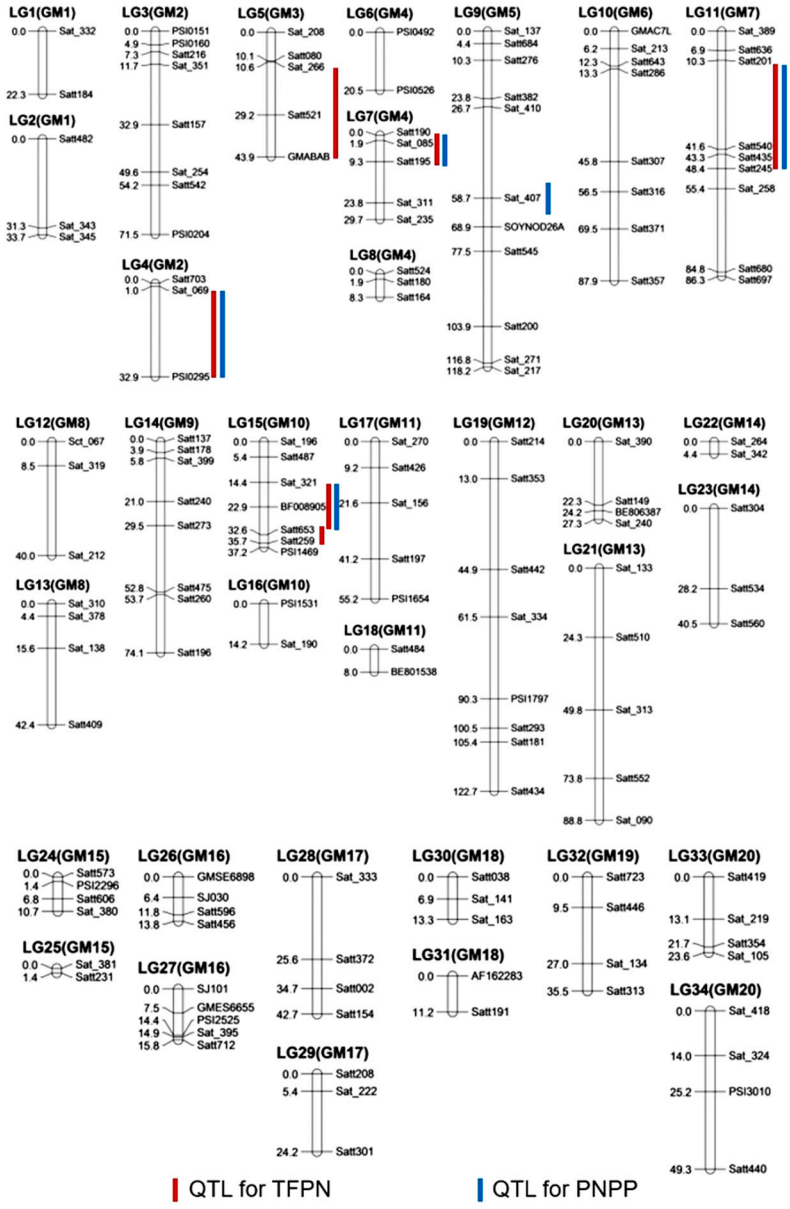


Figure 2. The genetic map of the RIL population and the QTL for TFPN and PNPP.

3.3. Fine Mapping of qFPN4

To confirm *qFPN4*, 11 Indel markers polymorphic between the two parents were developed and used to screen the derived F_6 RILs (Supplementary Table S2). As shown in Supplementary Table S3, the highest LOD score (4.64) for TFPN was detected near C1-7 (Chr.4: 44,383,419 bp), and the highest LOD score (5.05) for PNPP was detected near C1-5 (Chr.4: 44,330,411 bp). C1-7 and C1-5 accounted for 13.1% and 14.2% of the total phenotypic variance in the TFPN and PNPP, respectively. These results indicated that *qFPN4* was likely located close to C1-7 and C1-5. The additive effects for the TJ allele for TFPN and PNPP was 16.2 and 17.0, respectively. Based on the two Indel markers and the nearby markers (ID41040 (Chr.4: 30,260,000 bp), C1-1 (Chr.4: 35,948,983 bp), C1-3 (Chr.4: 41,559,265 bp), C1-8 (Chr.4: 44,458,681 bp) and C1-10 (Chr.4: 45,438,062 bp)), one RHL family #5 heterozygous for *qFPN4* was identified. The family #5 that produced 110 individuals that showed segregation for both TFPN and PNPP were used for fine mapping of *qFPN4*. Specifically, the progeny exhibited a bimodal distribution for both

TFPN and PNPP and the population was clearly divided into two groups: a group with more TFPN and PNPP (n = 81) and a group of low TFPN and PNPP (n = 29). The observed frequency fits a monogenic 3:1 ratio ($\chi 2$ = 0.70, p = 0.98) (Supplementary Figure S1). These results confirmed the role of *qFPN4* for both TFPN and PNPP and further indicated that a single gene in *qFPN4* controls both traits.

In total, six recombinants that carried critical recombination within the QTL interval of *qFPN4* were identified (#5–87, #5–40, #5–46, #5–69, #5–52, and #5–60). Combined analysis with the segregation patterns of the markers in the recombinants and the correlation with the TFPN and PNPP values and *qFPN4* was delimited to a genomic interval of ~2.62 Mb between markers C1-4 (Chr.4: 41,754,458 bp) and C1-6 (Chr.4: 44,375,173 bp) (Figure 3). Among the markers, the marker C1-5 (Chr.4: 44,330,411 bp) co-segregated with TFPN and PNPP, representing the most tightly linked molecular marker for TFPN and PNPP. Overall, these results confirmed the physical region of *qFPN4* in a ~2.62-Mb genomic region (Chr.4: 41,754,458–44,375,173 bp) on chromosome 4.

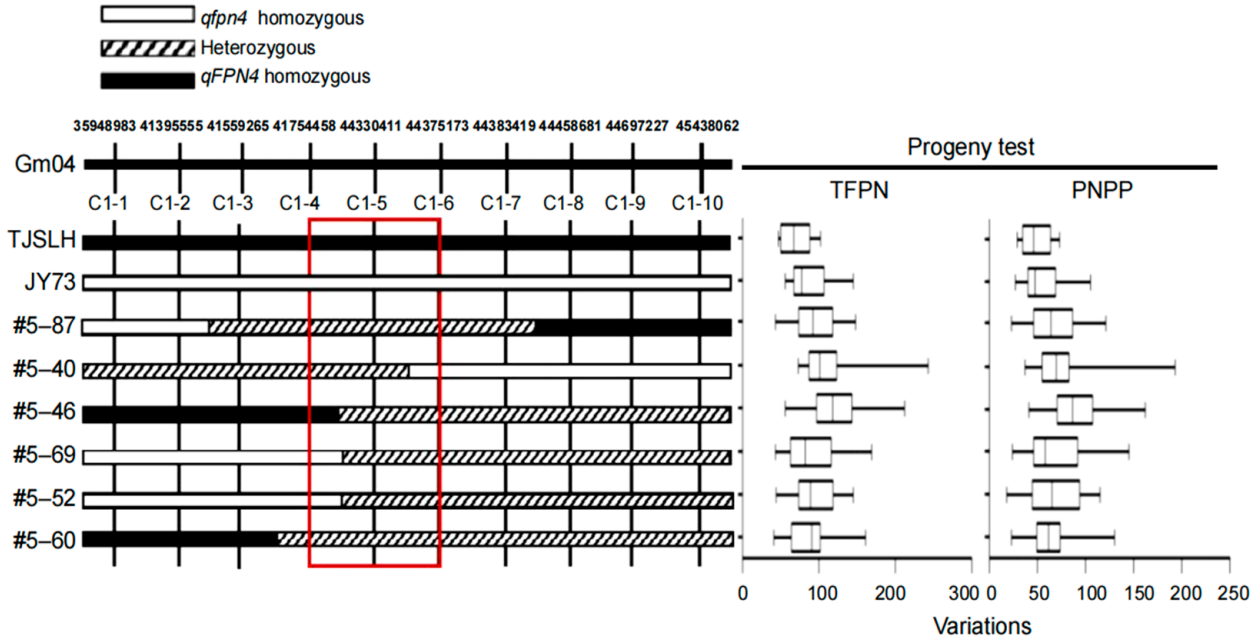


Figure 3. Fine mapping of *qFPN4*. Graphical genotypes of soybean recombinants carrying crossovers at *qFPN4*. The white bars represent the homozygote for the *qFPN4* allele from TJSLH, the black solid bars represent the homozygote for the qfpn4 allele from JY73, and the crosshatched bars represent the heterozygote. All the recombinants were derived from RHL family #5. Six recombinants were genotyped with ten newly developed Indels. Physical coordinates of the markers were obtained from the soybean reference genome and indicated at the top of the markers. TFPN and PNPP for the recombinants were evaluated for at least 30 of their respective progeny and shown in the right panels.

3.4. qFPN4 Controls Low Number of Flowers and Pods

To further confirm the association between the genotype at *qFPN4* and TFPN or PNPP, we identified a set of near-isogenic lines (NILs) from the homozygous progenies of RHL family #5 carrying the TJSLH allele (NIL-*qFPN4*) or the JY73 allele (NIL-*qfpn4*). As shown in Figure 4, field investigation indicated that TFPN and PNPP for NILs-*qFPN4* individuals were on average 50 and 13 lower ($p < 0.05$) than NILs-*qfpn4* individuals, respectively. This analysis confirmed that *qFPN4* plays an important role in the regulation of TFPN and PNPP; of the two alleles, *qFPN4* allele controls low TFPN and PNPP, whereas *qfpn4* is responsible for high TFPN and PNPP.

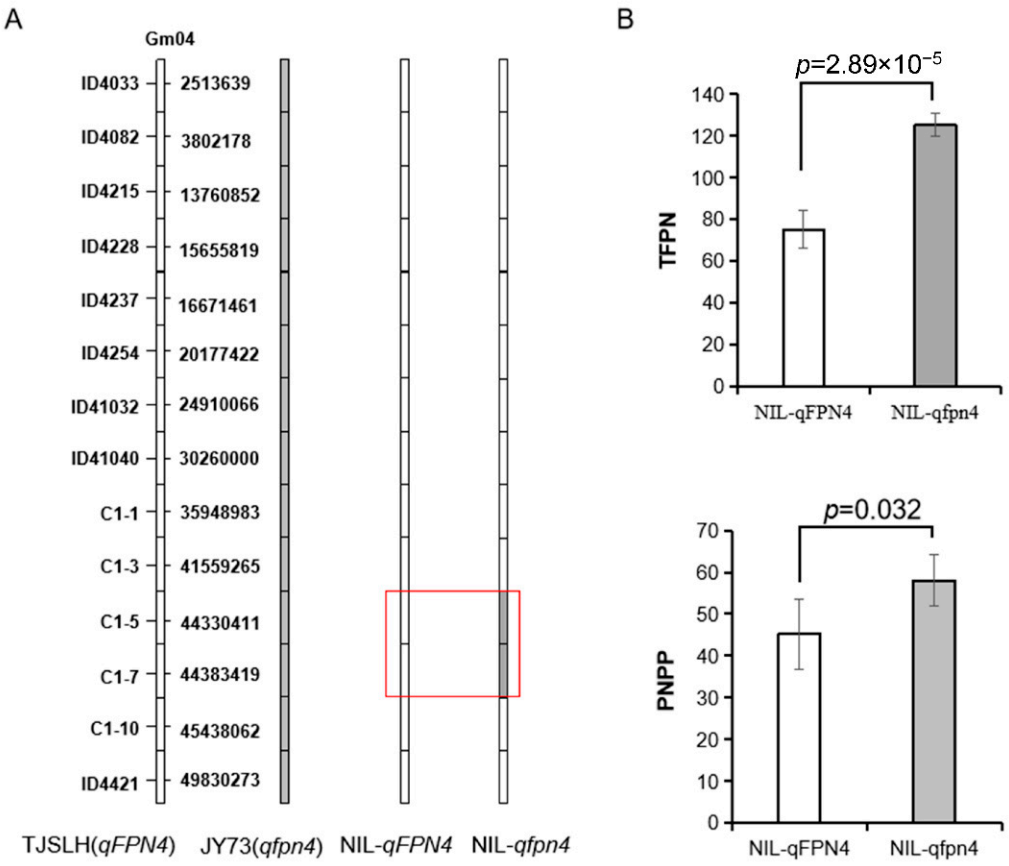


Figure 4. Development of NILs for *qFPN4* and phenotypes comparison. (**A**) The genotype of NIL-*qFPN4* and NIL-*qfpn4* on chromosome 4; (**B**) TFPN and PNPP between NIL-*qFPN4* and NIL-*qfpn4*.

3.5. Genome Sequencing Based BSA Analysis Confirmed qFPN4

To identify candidate genes for *qFPN4*, the progeny of NILs exhibiting extreme phenotypes for the NIL-*qFPN4* and NIL-*qfpn4* were used for BSA. To do this, DNA from 30 individuals with high TFPN and PNPP was pooled into the MFP bulk. Similarly, DNA from low TFPN and PNPP -individuals was mixed to build LFP bulk. To reveal critical variants at this locus, we applied high throughput whole genome sequencing for the two bulks. The sequencing produced a total of 33.28-Gb raw data with 93.44% of high-quality reads after quality control. Overall, 98.34% (208,615,358) and 98.67% (213,395,128) for MFP and LFP reads were mapped to the reference genome, respectively. The mapped reads covered an average coverage of 95.44% in the reference genome with an average sequencing depth of 26×.

Sequence variation analysis identified a total of 1,697,533 SNPs and 352,416 Indels (size < 15 pb) between the two groups, which were used for association analysis. An ED algorithm [29] was used to determine the significant SNP/Indel trait association region between MFP and LFP. With the association threshold of 1.0, one genomic region with significant signals was identified on Chr.4: 39,889,438–45,442,849 bp, which was coincident with the physical location of *qFPN4* (Chr.4: 41,754,458–44,375,173 bp) as determined by RHLs (Figure 5, Supplementary Figure S2). The variation as revealed in this region provides useful variation information to determine candidate genes.

3.6. Promising Genes for qFPN4

The SNP/Indel-based BSA analysis and fine-mapping result (Figures 3 and 5) identified an intersecting region for *qFPN4*, which covered ~2.62 MB region on Gm04: 41,754,458–44,375,173 bp. According to the Williams 82 genome sequence, this region contained 127 genes, of which eight were identified carrying nonsynonymous-SNP or/and Indel muta-

tions between the two bulks. The eight genes included one gene encoded fucosyltransferase 1 (*Glyma.04G166600*), one gene for photosynthetic NAD(P)H dehydrogenase subcomplex B4 (*Glyma.04G167400*), one gene for DDT domain superfamily protein (*Glyma.04G176500*), one gene for galactosyltransferase family protein (*Glyma.04G176600*), one gene for formin 8 (*Glyma.04G177800*), one gene for transducin/WD40 repeat-like superfamily protein (*Glyma.04G178800*), and two genes with unknown function (*Glyma.04G176800* and *Glyma.04G178900*) (Table 3). Especially, the one-base insertion of Glyma.04G178900 led to the frameshift from the 5th amino in CDS region, resulting in a lack of domain (Supplementary Figure S3). The base mutations in the remaining seven genes caused amino acid replacement or deletion, which did not cause frameshift.

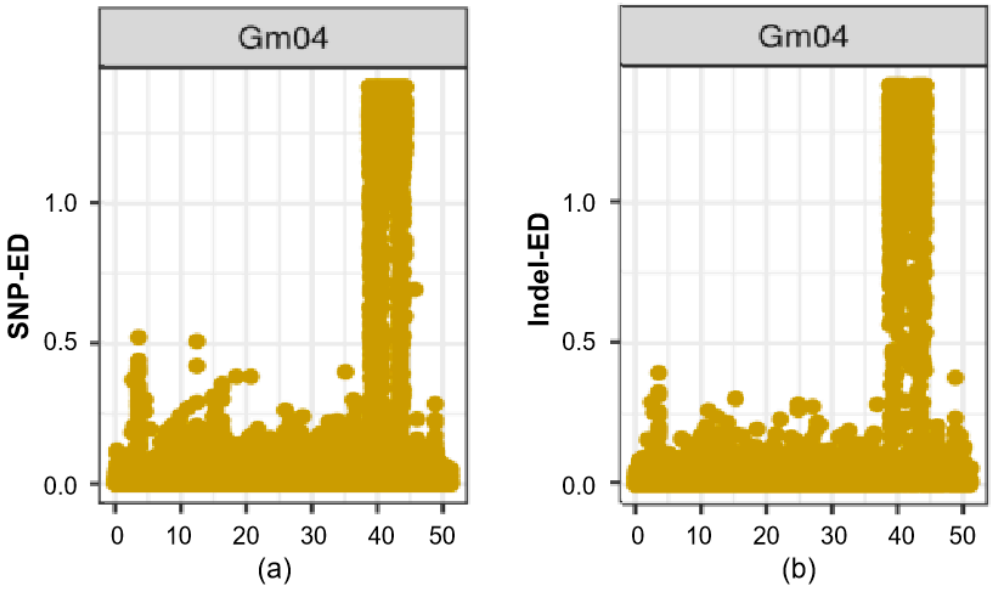


Figure 5. The distribution of ED-associated values on chromosome 4. Note: The color dots represent the ED value of each SNP (**a**) or Indel (**b**) locus. The higher the ED value the better the correlation.

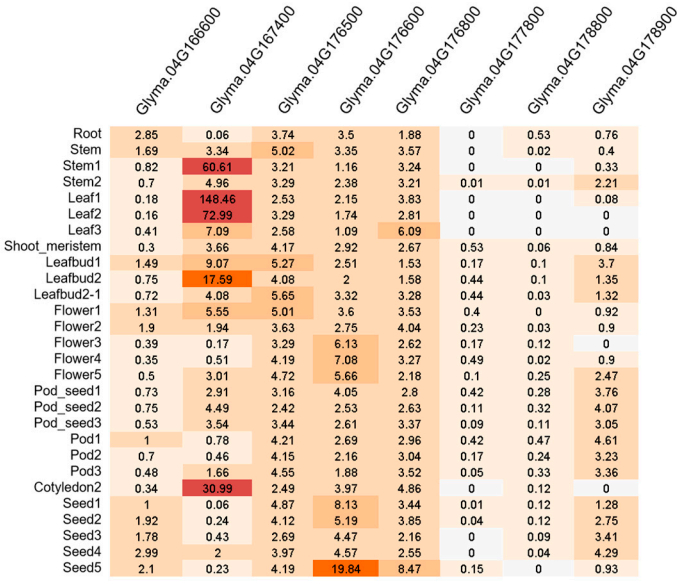
Table 3. Predicted genes within ~2.62 Mb region of *qFPN4* locus in the reference Williams82 sequence.

Gene Name	SNP Position	CDS	Allele for MFP	Allele for LFP	NSSNP	Homolog in *Ath.*	Protein Family
Glyma.04G166600	41834809	exon2	C	T	A78V	AT2G03220.1	Fucosyltransferase 1
Glyma.04G167400	42009788	exon1	G	T	R32I	AT1G18730.1	Photosynthetic NAD(P)H dehydrogenase subcomplex B4
Glyma.04G176500	43949063	exon6	A	C	K259T	AT1G18950.1	DDT domain superfamily
Glyma.04G176600	43971465	exon1	C	A	W11L	AT5G62620.1	Galactosyltransferase family protein
Glyma.04G176800	44006761	exon2	A	G	N154D	AT3G47850.1	-
Glyma.04G177800	44202428	exon2	C	G	K600N	AT1G70140.1	Formin 8
Glyma.04G178800	44358968	exon1	AACG	A	N146del.3	AT5G50120.1	Transducin/WD40 repeat-like Superfamily protein
Glyma.04G178900	44372615	exon1	C	CT	T5ins.1	-	-

NSSNP, substitution or insertion–deletion of amino acids because of the variation of the base.

We also examined the expression patterns in different tissues that were collected from the different developmental stages including roots, stems, leaves, buds, developing pods, and seeds [30]. The result showed distinct expression patterns in the tissues. *Glyma.04G176400* showed the highest expressions in stems, leaves, and cotyledons compared to the other seven genes, implying the important role for the vegetative growth period. Of the remaining genes, *Glyma.04G176600* and *Glyma.04G178900* were highly expressed in the florescence stage (Flower5) and early-stage pods (Pod_seed1, Pod_seed2,

Pod_seed3, Pod1, Pod2, and Pod3) relative to other tissues (Figure 6), indicating that the two genes were likely the candidates for *qFPN4* with roles involved in the regulation of flower and pod development.



	Glyma.04G166600	Glyma.04G167400	Glyma.04G176500	Glyma.04G176600	Glyma.04G176800	Glyma.04G177800	Glyma.04G178800	Glyma.04G178900
Root	2.85	0.06	3.74	3.5	1.88	0	0.53	0.76
Stem	1.69	3.34	5.02	3.35	3.57	0	0.02	0.4
Stem1	0.82	60.61	3.21	1.16	3.24	0	0	0.33
Stem2	0.7	4.96	3.29	2.38	3.21	0.01	0.01	2.21
Leaf1	0.18	148.46	2.53	2.15	3.83	0	0	0.08
Leaf2	0.16	72.99	3.29	1.74	2.81	0	0	0
Leaf3	0.41	7.09	2.58	1.09	6.09	0	0	0
Shoot_meristem	0.3	3.66	4.17	2.92	2.67	0.53	0.06	0.84
Leafbud1	1.49	9.07	5.27	2.51	1.53	0.17	0.1	3.7
Leafbud2	0.75	17.59	4.08	2	1.58	0.44	0.1	1.35
Leafbud2-1	0.72	4.08	5.65	3.32	3.28	0.44	0.03	1.32
Flower1	1.31	5.55	5.01	3.6	3.53	0.4	0	0.92
Flower2	1.9	1.94	3.63	2.75	4.04	0.23	0.03	0.9
Flower3	0.39	0.17	3.29	6.13	2.62	0.17	0.12	0
Flower4	0.35	0.51	4.19	7.08	3.27	0.49	0.02	0.9
Flower5	0.5	3.01	4.72	5.66	2.18	0.1	0.25	2.47
Pod_seed1	0.73	2.91	3.16	4.05	2.8	0.42	0.28	3.76
Pod_seed2	0.75	4.49	2.42	2.53	2.63	0.11	0.32	4.07
Pod_seed3	0.53	3.54	3.44	2.61	3.37	0.09	0.11	3.05
Pod1	1	0.78	4.21	2.69	2.96	0.42	0.47	4.61
Pod2	0.7	0.46	4.15	2.16	3.04	0.17	0.24	3.23
Pod3	0.48	1.66	4.55	1.88	3.52	0.05	0.33	3.36
Cotyledon2	0.34	30.99	2.49	3.97	4.86	0	0.12	0
Seed1	1	0.06	4.87	8.13	3.44	0.01	0.12	1.28
Seed2	1.92	0.24	4.12	5.19	3.85	0.04	0.12	2.75
Seed3	1.78	0.43	2.69	4.47	2.16	0	0.09	3.41
Seed4	2.99	2	3.97	4.57	2.55	0	0.04	4.29
Seed5	2.1	0.23	4.19	19.84	8.47	0.15	0	0.93

Figure 6. The expression patterns and relative levels of the eight genes in different tissues.

Furthermore, we performed genetic diversity analysis in a more diverse soybean panel comprising of 302 representative soybean accessions including wild soybean, landrace, and cultivars that have been sequenced [31]. As shown in Figure 7 and Table 4, genotyping analysis showed the differences in the distribution and frequency for these nonsynonymous-SNP or Indels of eight candidate genes in the population. Alleles of the eight genes may play a function in the development of floral and pod numbers during soybean domestication and development, implying the importance of these genes capable of meeting human needs for higher soybean production.

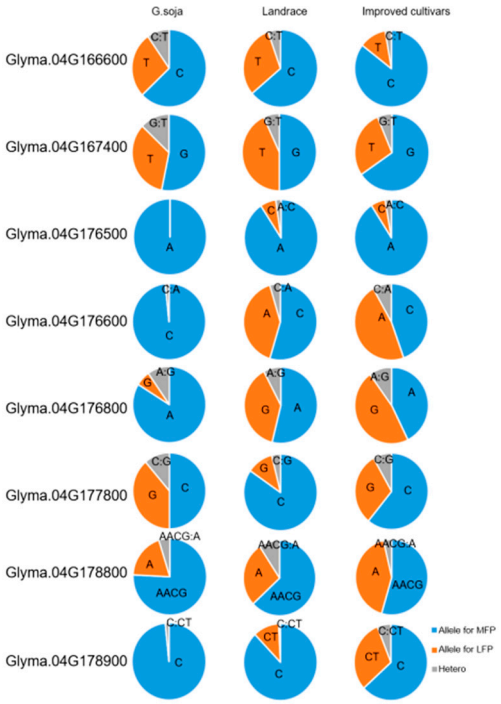


Figure 7. Allelic frequencies of the eight genes in *G. soja*, landrace, and improved cultivars.

Table 4. Variation information and percentage of the eight genes in the population.

Gene Name	* Variation Position	Variation	*G. soja*	Landrace	Improved Cultivar
Glyma.04G166600	233	C	62.90%	63.85%	85.45%
		T	27.42%	30.77%	11.82%
		Y	9.68%	5.38%	2.73%
Glyma.04G167400	95	G	53.23%	50%	65.45%
		T	33.87%	43.08%	28.18%
		K	12.90%	6.92%	6.36%
Glyma.04G176500	776	A	100%	90.77%	90.91%
		C	0	6.92%	6.36%
		M	0	2.31%	2.73%
Glyma.04G176600	32	C	98.39%	54.62%	44.55%
		A	0	40.77%	47.27%
		M	1.61%	4.61%	8.18%
Glyma.04G176800	460	A	83.87%	53.85%	42.73%
		G	6.45%	38.46%	47.27%
		R	9.68%	7.69%	10%
Glyma.04G177800	1800	C	50%	84.61%	60.91%
		G	38.71%	11.54%	30.91%
		S	11.29%	3.85%	8.18%
Glyma.04G178800	437	AACG	75.81%	63.08%	54.54%
		A	19.35%	27.69%	41.82%
		H	4.84%	9.23%	3.64%
Glyma.04G178900	16	C	98.39%	89.23%	67.27%
		CT	0	11.54%	32.73%
		H	1.61%	0.77%	6.36%

* means the position of allele within CDS of gene.

Combined with the above results, *Glyma.04G176600* and *Glyma.04G178900* were not only highly expressed in flower development and early-stage pods, but also accumulated some variants during soybean domestication and development, which were the most likely promising genes for *qFPN4*. Although the function of their homologous genes has been reported that *Glyma.04G176600* might be involved in flower development [32,33], functional characterization of these genes should be performed to further validate their functions on the soybean flower and pod numbers in future studies.

4. Discussion

Soybean has been an important oilseed crop worldwide and the yield represents almost all the economic value in the market. The TFPN of soybean determines the effective number of flowers and pods that is also a main factor leading to effective PNPP, which were all especially important in determining soybean yield [34]. Studies have shown that high pod setting number per plant and PNPP greatly increased soybean yield [8,9,13,35]; therefore, dissecting the genetic basis of TFPN and PNPP is critically important to understand the genetic mechanism of soybean yield. Previous studies have been mainly dedicated to the identification of QTL for related traits associated with the pod number [9–14] with rare reports on the total number of flowers and pods, an important yield component as indicated in this study.

In this study, we selected two parental lines, TJSIH and JY73, with contrasting TFPN and PNPP to develop an F_2 RIL population to dissect the genetic mechanism. Our study identified multiple QTL that control both traits: six QTL for TFPN and five QTL for PNPP. We observed that four QTL on chromosomes 2, 4, 7, and 10 were shared by TFPN and PNPP (Table 2; Figure 2), suggesting that both traits were controlled by the common mechanism. Of the identified QTL, *qPN2*, *qPN7,* and *qPN10* have been identified in Kuroda et al., 2013 [12] and Ning et al., (2018) [6] and *qPN5* was also detected in Yang et al., (2013) [13]. *qPN4* was coincident with the QTL in different studies with different populations or environmental conditions in Vieira et al., (2006) [9], Liu et al., (2017) [14], and Ning et al., (2018) [6], verifying that *qPN4* is a highly confident QTL that deserves to be pursued.

Indeed, our follow-up assays using RHLs and NILs confirmed that *qPN4/qFPN4* controlled both PNPP and TFPN and a single gene controlled the two traits. Thus far, no known genes for flower- and pod-related traits in this region have been reported in soybean; therefore, *qFPN4* is a novel gene controlling the total number of flowers and pods.

It is known that soybean yield is a very complex trait controlled by multiple genes, the majority of which have small effect [1]. Therefore, the observed soybean yield is the accumulative result from a number of genes/QTL; however, few have been identified [36,37]. Here, we confirmed that *qFPN4* is among the QTL that plays a contributing role for soybean yield, enriching the candidate QTL to explore the usefulness for soybean improvement. We further identified a tightly linked marker C1-5 to the QTL, which is approximately 360 kb from the proposed candidate gene *Glyma.04G176600*. This marker will be used for the trait test for the marker-aided selection of MFP for soybean yield improvement.

Next generation sequencing (NGS) based BSA approaches have been proven successful in rapidly mapping genes for various traits in plant species [38]. This strategy is useful for the analysis of the traits controlled by a single gene. For the first time, we showed that a single gene *qFPN4* controls the flower and pod numbers using the RHL strategy. The NGS-based BSA is a very helpful approach to delimit the interval and to identify candidate genes for *qFPN4*; the resulting interval for TFPN in Chr.4: 39,889,438–45,442,849 bp (Figure 5) overlapped with the region of fine-mapping results (Chr.4: 41,754,458–44,375,173 bp) (Figure 3), confirming the robustness of our QTL mapping results. The BSA-seq also enabled the identification of eight candidate genes with nonsynonymous-SNP or/and Indel mutations (Table 3), paving the way for a follow-up investigation.

Among the eight genes, *Glyma.04G166600* encodes fucosyltransferase 1. Several reports have found that glycosyltransferases are involved in the biosynthesis of cell wall polysaccharides, the addition of N-linked glycans to glycoproteins, and the attachment of sugar moieties to various small molecules such as hormones and flavonoids [39–41]. It has shown that an increase in alpha-(1,4)-fucosyltransferase activity was only observed during anther development. Higher activity was detected in mature pollen grains and pollen tubes, especially during pollen tube elongation [42]. The anther was curved in the *Osfuct* mutant, which lacks the α1,3-fucosyltransferase gene [43]. These data suggest that *Glyma.04G166600* might be involved in pollen development but does not impact the number of seed or pod sets. *Glyma.04G167400* encodes a thylakoid protein, NDH dependent flow 6, specific to terrestrial plants and it is essential for activity of chloroplastic NAD(P)H dehydrogenase involved in cyclic electron transport around photosystem I [44]. *Glyma.04G176500* encodes DDT domain superfamily protein, which is involved in different transcription and chromosome remodeling factors [45]. *Glyma.04G176600* encodes galactosyltransferase family protein, which is involved in the formation of several classes of glycoconjugates and in lactose biosynthesis [46]. Several reports have shown that β-(1,3)-galactosyltransferase was required for pollen exine development in Arabidopsis and maize [32,33]. The mutation in the β-(1,3)-galactosyltransferase caused reduced fertility as indicated by shorter fruit lengths and lower seed sets compared to the wild type. These data demonstrate that *Glyma.04G176600* may be important for fertility that is directly related to the number of pod sets in soybean. *Glyma.04G177800* encodes formin 8 and overexpression of *AtFH8* caused dramatic changes in root hair cell development and its actin organization, indicating the involvement of *AtFH8* in polarized cell growth through the actin cytoskeleton [47,48]. *Glyma.04G178800*, a member of transducin/WD40 protein superfamily, whose homologs controls seed germination, growth, and biomass accumulation through ribosome biogenesis protein interactions in Arabidopsis [49]. The remaining two genes (*Glyma.04G176800* and *Glyma.04G178900*) have unknown functions. Therefore, based on the function of the homologous gene, *Glyma.04G176600* is the most likely to control the flower and pod numbers. In addition, the one-base insertion of *Glyma.04G178900* led to the frameshift from the 5th amino of CDS region in allele for LFP (Supplementary Figure S3). Frameshift mutations often result in loss of function, which may reduce both TFPN and PNPP; therefore, *Glyma.04G176600* and *Glyma.04G178900* could be the candidate genes for

qFPN4.With the application of public data, it provides some clues for the characterizing of gene function. Using the expression data in different tissues that were collected from the different developmental stages, including roots, stems, leaves, buds, developing pods and seeds [30], we found that *Glyma.04G176600* and *Glyma.04G178900* were highly expressed in developing flower and early-stage pods relative to other tissues (Figure 6), indicating that the two genes were likely the candidates for *qFPN4* with roles involved in the regulation of flower and pod development; among them, the allelic variation of *Glyma.04G176600* for LFP caused amino acid replacement. Especially, the allelic variation of *Glyma.04G178900* for LFP led to the frameshift from the 5th amino in CDS region, resulting in the missing four amino acids (Supplementary Figure S3). Moreover, the distribution and frequency of these two variations in the *Glyma.04G176600* and *Glyma.04G178900* were different in the *G. soja* (wild soybean), landrace, and improved cultivars, implying that the different haplotypes of *Glyma.04G176600* and *Glyma.04G178900* may play different roles on the floral and pod numbers during soybean domestication and development.

These results improved our understanding of the genetic mechanism of the effective flower and pod numbers. Further analysis of the QTL would allow the identification of markers closely linked to all the QTL, especially the *qFPN4*, which could be used in marker-assisted selection for increased soybean flower and pod numbers and yields in soybean. Further dissection of *qFPN4* could also lead to the identification of causal genes or variants for the precise improvement of TFPN and PNPP in soybean.

5. Conclusions

In this study, we identified that an important QTL, *qFPN4*, for both TFPN and PNPP is controlled by a single dominant gene, where *qFPN4* allele controls low TFPN and PNPP, whereas *qfpn4* is responsible for high TFPN and PNPP. *qFPN4* was delimited into a ~2.62 Mb interval (Gm04: 41,754,458–44,375,173). Within the interval, eight genes carry nonsynonymous SNPs and/or Indels between parental lines. of which *Glyma.04G176600* and *Glyma.04G178900* were nominated as the promising candidate genes for *qFPN4* based on expression analysis. Additionally, we developed an Indel marker C1-5 tightly linking to the QTL, which may facilitate soybean improvement through the marker-aided selection. Our findings confirmed that *qFPN4* plays a contributing role for soybean yield, enriching the candidate QTL to explore the usefulness for soybean improvement.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040790/s1. Figure S1: The observed frequencies of TFPN (A) and PNPP (B) in RHL family #5; Figure S2: The distribution of Euclidean distance (ED)-associated values on 20 chromosomes; Figure S3: The protein sequence alignment of Glyma.04G178900 and its mutant (Glyma.04G178900-mut); Table S1: Indel markers used for mapping QTL for TFPN and PNPP; Table S2: Indel markers used for fine mapping *qFPN4* locus and detecting NILs; Table S3: Mapping QTL for TFPN and PNPP in the F_6 JT RIL population.

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