Fine mapping of QFlw-5B , a major QTL for flag leaf width in common wheat (*Triticum aestivum* L.)

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Research Article

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Abstract

Flag leaf width (FLW) is important to production in wheat. In a previous study, a major quantitative trait locus (QTL) for FLW (*QFlw-5B*) was detected on chromosome 5B, within an interval of 6.5 cM flanked by the markers *XwPt-9103* and *Xbarc142*, using a mapping population of recombinant inbred lines derived from a cross between Kenong9204 (KN9204) and Jing411 (J411) (denoted as KJ-RILs). The aim of this study was to fine map *QFlw-5B* and characterize its genetic effects on yield-related traits. Multiple near-isogenic lines (NILs) were developed using a residual heterozygous line (RHL) for *QFlw-5B*. Six recombinants for *QFlw-5B* were identified, and its location was narrowed to a 2.5 Mb region based on phenotypic and genotypic analyses. This region contained 27 predicted genes, two of which were the most likely candidate genes for *QFlw-5B*. An analysis of genetic effect indicated that the FLW of NIL-KN9204 was significantly higher than that of NIL-J411 across all of the environments tested. Meanwhile, significant increases in plant height, grain width and 1000-grain weight were observed in NIL-KN9204 compared with NIL-J411. These results indicated that *QFlw-5B* has great potential for marker-assisted selection in wheat breeding programs designed to improve both plant structure and yield. This study also provides a basis for the map-based cloning of *QFlw-5B*.

Key Message

A major stable QTL for flag leaf width was narrowed down to 2.5 Mb region containing two predicated putative candidate genes, and its effects on yield-related traits was characterized.

Introduction

Wheat (*Triticum aestivum* L.) is a primary food crop, feeding approximately 40% of the world's population. The continuous increase of wheat yields is critical to meeting growing food requirements, owing to increased consumption (Curtis and Halford 2014). The wheat leaf is the main photosynthetic organ, and it provides the plant's main source of energy for plant growth and panicle development (Tian et al. 2015). The top three leaves, particularly the flag leaf, are the main source of carbohydrate production (Zhang et al. 2015), contributing up to 50% of all leaf-based photosynthetic activity and approximately 41–43% of the carbohydrates needed for grain filling after anthesis (Araus et al. 1987; Sharma et al. 2003). Dimensions such as flag leaf length (FLL), flag leaf width (FLW) and flag leaf area (FLA) are important to determining the amount of light that can be captured for photosynthesis. Breeding wheat with flag leaves of optimal size has been regarded as an appropriate method of improving grain yield (Tu et al. 2021). Selection for ideal flag leaf size has been one of the key indicators in traditional wheat breeding strategies designed to improve yield potential. A number of studies have shown that flag leaf size is positively associated with 1,000-grain weight, grain yield per plant and other yield-related traits in cereals such as rice and wheat (Khaliq et al. 2008; Wang et al. 2011; Wang et al. 2020). The FLA is determined by FLL and FLW. If the flag leaf is too long, it drapes, causing reduced photosynthetic efficiency, especially in wheat cultivars containing a large number of spikes in a large population. Therefore, increasing FLW is an effective method of improving the FLA.

FLW is a complex agronomic character, which is regulated by both genotype and environment. With the availability of molecular markers and genetic maps, many QTLs related to FLW have been documented in rice and wheat (Mei et al. 2003; Wang et al. 2012; Wu et al. 2015; Fan et al. 2015; Liu et al. 2018; Zhao et al. 2018; Jin et al. 2020). Genetic research into FLW has led to significant advances in developing improved model rice plants. For example, *qFLW4* for FLW in rice has been fine-mapped to an interval of 74.8 Kb (Chen et al. 2012), while the narrow leaf genes *Nal1*, *Nal7* and *Nal9*, (Fujino et al. 2008; Qi et al. 2008; Li et al. 2013), narrow-curly leaf genes *Nal2* and *Nal3* (Cho et al. 2013; Ishiwata et al. 2013) and *Nrl1* for narrow and rolled leaf (Hu et al. 2010) have all have been cloned in rice. Although there has been less research into locating QTLs for FLW in wheat, a major QTL for FLW, *TaFLW1*, was fine mapped to a 0.2 cM interval in the 5AL12-0.35–0.57 deletion bin, which is closely linked with *Fhb5* (Xue et al. 2013). Moreover, recently, *QFlw-6A* was fine-mapped to a 613 bp segment on chromosome 6A, with 10 candidate genes (Jin et al. 2020). However, no report of the map-based cloning of a QTL for FLW has yet been documented in wheat.

Previously, we identified an environmentally stable QTL (*QFlw-5B*) for FLW on chromosome 5B using a population of recombinant inbred lines (RILs) derived from a cross between Kenong9204 (KN9204) and Jing411 (J411) (KJ-RILs) (Fan et al. 2015). In this work, we used secondary mapping populations to (1) verify and fine-map *QFlw-5B*, (2) characterize the genetic effects of *QFlw-5B* on yield-related traits across multiple environments and (3) identify possible candidate genes for FLW based on RNA-Seq and multi-omics analysis.

Materials And Methods

Plant materials and field trials

The KJ-RILs were created using KN9204, which has a larger FLW than the other parent, J411 (Fan et al. 2015). A residual heterozygous line (RHL) was identified from the F₇ KJ-RILs; then, a secondary mapping population for *QFlw-5B* was developed by self-crossing the RHL. The near-isogenic line (NIL) pairs, NIL-KN9204 and NIL-J411, were planted in four environments: 2018–2019 at Yantai (E1), 2019–2020 at Weifang (E2), 2019–2020 at Yantai (E3) and 2019–2020 at Shijiazhuang (E4). The near-isogenic families were planted in E1 and E3. For the field trials, seeds were sown in five-row plots (2 m long) with 40 seeds in a randomized complete block design with three replicates during the crop seasons. All field trials were well-watered and managed in accordance with standard local practices.

Phenotype evaluation and statistical analysis

FLW and yield related traits of the secondary mapping population for *QFlw-5B* were evaluated in this study. For each plot, the main shoots of 10 plants in the center of each row were randomly selected to measure the FLW at 15 days after flowering and investigate plant height (PH), spike length (SL), spike number per plant (SNPP) and kernel number per spike (KNPS) at physiological maturity. The 1000-grain weight (TGW), grain length (GL) and grain width (GW) were measured after harvest.

The FLW was measured at the widest part of the flag leaf; PH was measured from the base of the plant to the tip (excluding the awns); SL was measured from the base of the spike to the tip (excluding the awns); SNPP was evaluated by calculating the total number of spikes for each plant; KNPS was measured by calculating the total number of kernels per spike of the main shoot for each plant; and TGW was evaluated in grams after harvest by weighing two samples of 500 kernels. Three samples of 10 kernels were lined up length-wise along a ruler with a precision of 0.1 mm, to measure GL, and then were arranged breadth-wise to measure GW. All lengths were reported in centimeters. The average value of each plot represented the phenotype value.

Marker development and genotype identification

DNA was extracted from fresh leaves following a modified cetyltrimethylammonium bromide (CTAB) extraction method (Devi et al. 2013). The SNP flanking sequence nearby *QFlw-5B* on chromosome arm 5B was used to BLAST against KN9204 (unpublished data) and IWGSC RefSeq v2.1 (https://urgi.versailles.inra.fr/blast_iwgsc/blast.php) to obtain the physical position of *QFlw-5B*. High throughput whole-genome re-sequencing (10×) was performed on the two parents KN9204 and J411. We aligned Illumina reads to the KN9204 genome (unpublished data) and IWGSC RefSeq v2.1 according to Chai et al. (2018) and Chen et al. (2020). SNPs and InDels were identified with the aim of developing a saturated genetic map of the target region. Polymorphic sequences containing InDel (>3 bp) were converted to PCR-based markers using PrimerServer (<http://202.194.139.32/PrimerServer/>). Each PCR reaction consisted of 5 µL 2 × Taq PCR Star Mix, 1.5 µL of each primer (at 2 µM), 1 µL DNA template (50–100 ng/µL) and 1 µL H₂O. The PCR conditions were as follows: pre-denaturation for 5 min at 94 °C; 36 cycles of 94 °C denaturation for 30 s, approximately 55–60 °C primer annealing (depending on the annealing temperature for each primer) for 30 s and a 72 °C extension for 30 s, with a final extension of 72 °C for 10 min.

QTL detection for flag leaf related traits based on physical map

All phenotypic values of FLW and FLA of the 188 KJ-RILs in eight environments (Fan et al. 2015) were used to detect QTLs by inclusive composite interval mapping (ICIM) performed by IciMapping 4.1 (<http://www.isbreeding.net/>). The physical position of markers in KN9204, rather than the genetic position, was used for QTL mapping analysis, with an aim to facilitate further genomic and genetic analysis of the target QTL based on the physical map of KN9204. The walking speed chosen for all QTLs was 0.01 Mb, and the *P* value inclusion threshold was 0.001. The threshold of the LOD scores was evaluated using 1,000 permutations with a type I error of 0.05.

Transcriptome profiling analysis and candidate gene predication

We performed transcription profiling for the flag leaf of NIL-KN9204 and NIL-J411 at two stages: the heading (HD) and grain filling (GF) periods. The leaves from NIL-KN9204 and NIL-J411 were placed in separate bottles, immediately frozen in liquid nitrogen and stored in a –80 °C ultra low-temperature freezer for long-term preservation. RNA was isolated from leaves of the NILs using a Quick RNA Isolation Kit TAKARA9769S (Takara Biomedical Technology, Beijing). RNA sequencing was performed using a commercial service (Beijing Biomarker Cloud Technologies, China). After the expression level of each transcript and gene was calculated using the fragments per kb per million fragments (FPKM) method, differential expression analysis was conducted using DESeq2 (V1.6.3) (Love et al. 2014). The differentially expressed genes (DEGs) from KN9204 were associated with the corresponding genes in Chinese Spring according to IWGSC RefSeq v2.1 (CSv2.1) and subjected to gene ontology (GO) term enrichment analysis (<http://wheat.cau.edu.cn/TGT/>). Genes with an adjusted *P* value < 0.05 and |log₂ (fold change)| ≥ 1 were recognized as DEGs. Functional analysis of the candidate genes was performed based on the gene annotations in IWGSC RefSeq v2.1 in Triticeae-Gene Tribe (<http://wheat.cau.edu.cn/TGT/>).

Results

Verification of *QFlw-5B* based on the high-density molecular map

Previously, *QFlw-5B* was mapped onto chromosome 5BL within an interval of 6.5 cM (67.86–74.34 cM), flanked by the markers *XwPt-9103* and *Xbarc142* in multiple environments (Fan et al. 2015). We have developed a high-density genetic map consisting of 119,566 loci spanning 4424.4 cM (Cui et al. 2017). We used BLAST (<http://ftp.ncbi.nlm.nih.gov/blast/executables/release/>) to align the SNP probes to the KN9204 genome (data not shown). Physical position of markers in KN9204, rather than the genetic position, was used for QTL mapping analysis of FLW. *QFlw-5B* was reproducibly identified in six of the eight environments, within an interval of KN5B: 599.42–607.26 Mb flanked by *AX-109386758* and *AX-110429178* (Table 1). In addition, *QFla-5B*, a stable QTL for FLA, was reproducibly identified in six of the eight environments in this chromosome region.

Table 1 QTLs for FLW and FLA on chromosome 5B based on the high-density physical map in eight different environments

Trait-En ^a	Left Marker	Right Marker	LOD	PVE (%)	Add (mm)	Position (Mb)
FLW-E3	<i>AX-109386758</i>	<i>AX-110464594</i>	7.82	12.69	0.04	599.4233
FLW-E4	<i>AX-86178161</i>	<i>AX-110429178</i>	7.81	11.21	0.04	607.2214
FLW-E5	<i>AX-109386758</i>	<i>AX-110464594</i>	4.79	12.05	0.04	599.4233
FLW-E6	<i>AX-110130655</i>	<i>AX-108917956</i>	12.88	16.65	0.05	605.2219
FLW-E7	<i>AX-109386758</i>	<i>AX-110464594</i>	3.70	9.60	0.03	599.4233
FLW-E8	<i>AX-110127489</i>	<i>AX-110633922</i>	3.49	8.36	0.04	599.4233
FLA-E2	<i>AX-109386758</i>	<i>AX-110464594</i>	6.01	9.37	0.77	599.4233
FLA-E3	<i>AX-109386758</i>	<i>AX-110464594</i>	5.44	8.34	0.76	599.4233
FLA-E4	<i>AX-111061703</i>	<i>AX-86178161</i>	10.31	12.45	1.38	606.9214
FLA-E5	<i>AX-110185848</i>	<i>AX-111693079</i>	3.99	9.55	0.74	600.8500
FLA-E6	<i>AX-110130655</i>	<i>AX-108917956</i>	12.41	15.93	1.22	605.2219
FLA-E7	<i>AX-94494539</i>	<i>AX-108763396</i>	2.75	5.02	0.64	602.3226

a: For details of environments from E1 to E8, see Fan et al. (2015). En.: Environment

To verify the results of the primary QTL mapping analysis, preliminary fine-mapping of *QFlw-5B* was performed using the 188 KJ-RILs as secondary mapping populations. Three major stable QTLs, i.e., *QFlw-4B*, *QFlw-5B* and *QFlw-6B*, led to the differences in FLW between KN9204 and J411 (Fan et al. 2015). We divided the 188 KJ-RILs into four groups based on the genotypes of *QFlw-4B* and *QFlw-6B* (Fig. 1). The KJ-RILs within each group could be regarded as QTL near isogenic lines (QNILs) of *QFlw-5B*. The four sets of QNILs positioned *QFlw-5B* between markers *AX-109036733* (KN5B:599.04) and *AX-86178161* (KN5B:607.16), confirming their authenticity and reliability of *QFlw-5B*.

Fine mapping of the *QFlw-5B* locus

A total of 109,322 SNPs and InDels between KN9204 and J411 were identified based on genome re-sequence analysis in the target region. Of these, 2004 InDels (>3 bp) could be used for PCR-based marker development. These InDels were distributed in the target region in clusters rather than uniformly (Supplementary Figures S1). Eight PCR-based InDel markers were developed for further fine mapping analysis (Supplementary Table S1).

To fine map *QFlw-5B*, we identified the RHL for the target region from the KJ-RILs, and one from the F₇ generation was identified. The secondary mapping populations were genotyped using the PCR-based InDel markers in the target region, and six recombinants (RL1–RL6) were detected (Fig. 2). These six recombinants were phenotyped for FLW in E1 and E3. The results showed that the FLW values for RL1, RL2 and RL3 were significantly greater than those for RL5 and RL6. The genotypic scores for RL1, RL2 and RL3 in the chromosomal region between 5B-1D-4 and 5B-ID-6 were identical to KN9204, whereas those for RL5 and RL6 were identical to J411. Based on the fragments represented as graphical genotypes, we finally defined the mapping interval for *QFlw-5B* as approximately 2.5 Mb, corresponding to the physical interval from 598.10 Mb to 600.596 Mb (between markers ID-4 and ID-6) in KN9204.

Genetic effects analysis of *QFlw-5B*

To further clarify the effects of *QFlw-5B* on FLW and other important agronomic traits, the NIL pairs were evaluated in four environments (E1 to E4). Across all of the environments, the average FLW of NIL-KN9204 was 12.8–31% higher than that of NIL-J411 (Fig. 3a, b). To characterize the differences in cellular structure, the flag leaf from each of NIL-J411 and NIL-KN9204 were picked for paraffin sectioning (Fig. 4a). The results indicated that the higher FLW of NIL-KN9204 was caused mainly by the more vascular bundles and bulliform cells (Fig. 4b).

The effects of the *QFlw-5B* locus on other important agronomic traits were analyzed (Table 2). Alleles from KN9204 increased PH, TGW and GW by 9.44–23.24%, 7.42–29.18% and 4.09–10.9%, respectively, compared to those of J411 across all the environments. NIL-KN9204 showed increased GL by 1.65% and 2.15% in E2 and E3, respectively, compared with that of NIL-J411. There was no significant difference in KNPS between the NIL pairs, however, except for E4, in which the NIL-KN9204 had a higher KNPS. NIL-KN9204 had a shortened SL in E1 and E4 compared with NIL-J411, along with a lower SNPP in E3.

Table 2 Comparison of agronomic traits between NIL-KN9204 and NIL-J411 in four environments

Environment	Genotype	PH (cm)	SNPP	SL (cm)	KNPS	TGW (g)	GL (mm)	GW (mm)
E1	NIL-KN9204	85.29±4.7	12.9±3.43	7.97±0.86	44.42±5.11	50.99±5.42	7.01±0.54	3.31±0.28
	NIL-J411	74.56±5.85	13.36±3.46	8.47±0.93	44.53±4.78	47.47±4.46	6.94±0.45	3.18±0.24
	Percentage	14.4%**	3.57% ns	6.27%**	0.25% ns	7.42%**	1.01% ns	4.09%**
E2	NIL-KN9204	90.32±3.85	18.00±4.66	10.04±0.67	57.78±4.02	40.53±3.82	6.76±0.12	3.20±0.13
	NIL-J411	73.29±4.83	22.22±4.32	10.11±0.46	58.50±4.59	33.25±2.62	6.65±0.09	2.97±0.10
	Percentage	23.24%**	23.4% ns	0.70% ns	1.25% ns	21.89%**	1.65%*	7.74%**
E3	NIL-KN9204	92.70±4.32	13.75±3.49	9.61±0.98	57.88±3.83	47.64±4.91	6.66±0.15	3.56±0.22
	NIL-J411	84.20±3.91	18.78±4.41	9.79±0.58	55.50±3.51	36.88±5.29	6.52±0.12	3.21±0.23
	Percentage	10.1%**	36.58%*	1.87% ns	4.29% ns	29.18**	2.15%*	10.9%**
E4	NIL-KN9204	86.02±4.33	28.38±3.89	9.49±0.70	59.13±4.45	-	-	-
	NIL-J411	78.60±4.48	29.75±4.75	10.62±0.75	45.33±5.09	-	-	-
	Percentage	9.44%**	4.83% ns	11.91**	30.44%**			

*P < 0.05, **P < 0.01; ns: indicates not significant; - indicates missing data. E1: 2018-2019 Yantai; E2: 2019-2020 Weifang; E3: 2019-2020 Yantai; E4: 2019-2020 Shijiazhuang. PH: Plant height; SNPP: Spike number per plant; SL: Spike length; KNPS: Kernel number per spike; TGW: 1000-grain weight; GL: Grain length; GW: grain width

Differential gene expression of NIL pairs and candidate gene prediction

To identify DEGs between NIL-KN9204 and NIL-J411, RNA sequencing was performed on the flag leaves of NIL-KN9204 and NIL-J411 during the HD and GF periods. A total of 601 and 796 DEGs were identified between the NIL pairs during the HD and GF periods, respectively. Of these, one DEG, *TraesKN5B01HG44240*, was in the targeted QTL region of 598.10–600.596 Mb (Supplementary Table S2).

Gene ontology (GO) term enrichment analysis (P value < 0.05) indicated that the genes were enriched in various biological processes, especially the carbohydrate metabolic process (GO:0005975), cell wall macromolecule catabolic process (GO:0016998), response to carbohydrate (GO:0009743) and (1→3)-beta-D-glucan catabolic process (GO:0006076) during the HD period (Supplementary Table S3). The molecular functions during the HD period were enriched for genes involved in sucrose 1F-fructosyltransferase activity (GO:0050306), sucrose alpha-glucosidase activity (GO:0004575) and chitinase activity (GO:0004568). During the GF period, genes were mainly enriched in allantoin transport (GO:0015720), uracil transport (GO:0015857) and pyrimidine nucleobase salvage (GO:0043100), in terms of biological processes. The molecular functions were mainly involved in allantoin: proton symporter activity (GO:0005274), uracil: cation symporter activity (GO:0015505) and receptor signaling activity (GO:0038023).

According to the KN9204 genome annotation, the region contained 33 predicted genes (*TraesKN5B01HG44080* to *TraesKN5B01HG44400*), including six low confidence genes (*TraesKN5B01HG44200LC*, *TraesKN5B01HG44260LC*, *TraesKN5B01HG44280LC*, *TraesKN5B01HG44300LC*, *TraesKN5B01HG44310LC* and *TraesKN5B01HG44350LC*) (Supplementary Table S4) and 27 high confidence genes (Supplementary Table S5). We determined the relative expression levels of these 27 genes in the flag leaves from NIL-KN9204 and NIL-J411 by RNA-Seq. The FPKM values of the 27 genes are listed in Supplementary Table S6. Fifteen (*TraesKN5B01HG44090*, *TraesKN5B01HG44100*, *TraesKN5B01HG44130*, *TraesKN5B01HG44190*, *TraesKN5B01HG44220*, *TraesKN5B01HG44270*, *TraesKN5B01HG44290*, *TraesKN5B01HG44320*, *TraesKN5B01HG44330*, *TraesKN5B01HG44340*, *TraesKN5B01HG44360*, *TraesKN5B01HG44370*, *TraesKN5B01HG44380*, *TraesKN5B01HG44390* and *TraesKN5B01HG44400*) were not expressed or expressed at very low levels ($\text{FPKM} \leq 0.14$) during the two stages; therefore, they were excluded from the list of candidate genes. Eight genes (*TraesKN5B01HG44080*, *TraesKN5B01HG44110*, *TraesKN5B01HG44120*, *TraesKN5B01HG44160*, *TraesKN5B01HG44170*, *TraesKN5B01HG44180*, *TraesKN5B01HG44210* and *TraesKN5B01HG44250*) showed similar relative expression levels between NIL-KN9204 and NIL-J411 at the two stages; therefore, these genes may not affect FLW development and they were also excluded as candidate genes. Four genes remained, *TraesKN5B01HG44140*, *TraesKN5B01HG44150*, *TraesKN5B01HG44230* and *TraesKN5B01HG44240*, of which *TraesKN5B01HG44140* and *TraesKN5B01HG44230* were differentially expressed between NIL-KN9204 and NIL-J411 only during one period (GF and HD, respectively). *TraesKN5B01HG44150* and *TraesKN5B01HG44240* were differentially expressed in both of the two flag leaf developmental stages (Fig. 5, Supplementary Table S6), and these presented as the candidate genes with great potential.

Discussion

Comparisons with previously reported QTLs/genes for FLW

FLW is a complex quantitative and important agronomic trait. To date, several QTLs for FLW have been mapped on chromosome arm 5B. Xue et al. (2015) delimited a QTL for FLW to the interval between *Xgwm540* and P3516.1 (about 66.61 Mb) on chromosome arm 5B. Moreover, Yang et al. (2016) placed one QTL associated with FLW on chromosome arm 5B in the interval: 507.92–654.92 Mb, while Lian J et al. (2016) reported a QTL associated

with FLW on chromosome arm 5B (437.80–440.03 Mb). The initial positioning of *QFlw-5B* corresponded to the physical location of 590.07–597.87 Mb in IWGSC RefSeq v1.0 of Chinese Spring. Thus, *QFlw-5B* in this study might be identical to that reported by Yang et al. (2016). To date, there has been no report on the fine mapping or map-based cloning of this QTL.

A large number of genes associated with flag leaf size have been discovered in rice (Supplementary Table S7). We assigned these genes to the wheat genome based on homologous analysis and five were mapped to chromosome 5B, including *NAL2/NAL3* (*TraesCS5B03G0427100*), *Nrl1/DNL1* (*TraesCS5B03G0243400*), *OsIAA3* (*TraesCS5B03G0146700*), *OsGASR1* (*TraesCS5B03G0999500*) and *OsHox32* (*TraesCS5B03G0946900*). After comparison with the genes in KN9204 (Supplementary Table S5), it was found that *QFlw-5B* should harbor a novel gene regulating flag leaf width, but it was not one of those homologous genes.

QTL mapping of FLW and the genetic effects of *QFlw-5B*

Liu et al. (2018) detected that FLW was positively correlated with KNPS and SL, while being negatively correlated with SNPP and PH. Zhao et al. (2018) detected that FLW was positively correlated with KNPS and TGW, and negatively correlated with SNPP. Fan et al. (2015) also found that FLW was positively correlated with SL, KNPS and TGW. In this study, we found that FLW was positively correlated with TGW, KNPS and PH, while being negatively correlated with SNPP and SL; so the results for TGW, KNPS and SNPP were consistent with those from previous studies (Liu et al. 2018; Zhao et al. 2018; Fan et al. 2015), while the results for SL and PH were inconsistent. Combined with the above studies, our findings indicated that a larger FLW provides more photosynthetic benefit for TGW and KNPS, which may lead to increased production.

In our study, the positive allele of *QFlw-5B* increased FLW by 12.8–31%, and the effect was mainly attributed to a greater number of vascular bundles and bulliform cells (Fig. 4). Meanwhile, the positive allele of *QFlw-5B* increased TGW by 7.42–29.18%, and the effect was mainly attributed to the primary effect on grain width (Table 2). Overall, we propose that *QFlw-5B* may be of great value in molecular marker-assisted selective breeding programs.

DEGs and candidate genes analysis in HD and GF period

To better understand the molecular and the biological functions of the phenotypic characterization associated with the 5B QTL, GO term enrichment analysis were performed based on the DEGs between the NILs (Supplementary Table S3). During the HD period, we found that many of the DEGs were associated with the activity of enzymes involved in sugar metabolism such as sucrose alpha-glucosidase activity (GO:0004575) and sucrose 1F-fructosyltransferase activity (GO:0050306). In addition, the carbohydrate metabolic process (GO:0005975), cell wall macromolecule catabolic process (GO:0016998) and response to carbohydrate (GO:0009743) were also enriched during the HD period. As is known, flag leaves are closely associated with photosynthesis, which is related to carbon and sugar metabolism. While these molecular and the biological functions could not be found in GF period. This may be responsible for the diminished photosynthesis in the late reproductive period of wheat (Patterson TG et al. 1980). The cellular component was enriched for chloroplast membrane (GO:0031969) during both the HD and GF periods.

Fine mapping demonstrated that *QFlw-5B* covers a genomic region of approximately 2.5 Mb, including 27 predicted genes. We predicted the candidate genes for *QFlw-5B* by using RNA sequences between NIL-KN9204 and NIL-J411. Combining the log2FoldChange and the *P* values of the genes, we finely predicted two genes, *TraesKN5B01HG44150* and *TraesKN5B01HG44240*, to be candidate genes (Supplementary Table S6). According to the wheat genome annotation information (<http://wheat.cau.edu.cn/TGT/m8/?navbar=Home>), *TraesKN5B01HG44150* encodes a ribosome-binding ATPase, YchF. In *Escherichia coli* YchF binds the 50S and 70S ribosomal particles in a nucleotide-independent manner and hydrolyzes ATP utilizing a potassium-dependent mechanism (Tomar SK et al. 2011). In *Brucella melitensis*, the YchF homologue DugA has been suggested to be involved in iron metabolism and may be a regulator of the Ton system (Danese I, et al. 2004). Koller-Eichhorn R et al (2007) have biochemically characterized the human homologue of YchF and found that it binds and hydrolyzes ATP more efficiently than GTP. *TraesKN5B01HG44240* encodes a ribosome production factor 2 homolog (RPF2), which was shown to more rigidly bind to 5S rRNA by forming a complex with Rrs1. In *Arabidopsis thaliana*, the over expression of ARPF2 (*A. thaliana* homolog of yeast Rpf2: At3g23620) resulted in short height, abnormal leaf morphology and an extended period of reproductive growth (Maekawa S et al. 2018). In the future, the function of the candidate genes will be validated via sequence analysis between parental lines and genetic transformation.

Declarations

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Author Contribution Statement

C Zhao and F Cui designed the research; X Liu, H Liu, Z Zhao and R Qin performed the experiments; W Kong, S Zhang, S Wang and Y Chen conducted the field trials; H Sun and Y Wu analyzed the data; C Zhao and F Cui wrote the manuscript and all authors read and approved the manuscript.

Ethical standards

The authors declare that they have no conflict of interest. All authors contributed to the study and approved the final version for submission. The manuscript has not been submitted to any other journal.

Data Availability Statement

All data generated or analyzed during this study are included in this published article and its electronic supplementary information files, further inquiries can be directed to the corresponding author.

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Figures

Figure 1

Preliminary fine-mapping of *QF/w-5B* using the 188 KJ-RILs as secondary mapping populations. The flag leaf width (FLW) in the 188 KJ-RILs was evaluated in eight environments, where four leaves of each were investigated under low nitrogen (LN) and high nitrogen (HN) conditions, respectively. The rectangles on the left indicate the recombinants at the target region; *QNIL-KN9204* and *QNILJ411* represent alleles from KN9204 and J411, respectively; R1-R7: recombinant type; Arabic numbers on the left of the rectangles represent the number of the corresponding recombinants; AA indicates that the alleles are identical to KN9204, and BB indicates that the alleles are identical to J411. The histogram on the right indicates the average FLW under LN and HN, respectively

Figure 2
 Fine mapping of *QFLW-5B*. (Right) The eight markers used to screen recombinants, and the graphical genotypes of six recombinants. The arrows represent the 2.5 Mb interval of fine mapping. RL1–RL6: recombinant line1–recombinant line 6. (Right) The flag leaf width (FLW) phenotype of each recombinant line; the values of FLW are the means (mean ± SD) of recombinant plants; lowercase and capital letters represent significance at $P < 0.05$ and $P < 0.01$, respectively; the number in brackets represents the number of recombinant plants

Figure 3
 Comparisons of the mean flag leaf width (FLW) between NIL-KN9204 and NIL-J411 in four environments. **a**: Morphological differences flag leaf in E3; bar = 1 cm. **b**: The phenotype of FLW for NIL-KN9204 and NIL-J411 in four environments; E1: 2018-2019 Yantai; E2: 2019-2020 Weifang; E3: 2019-2020 Yantai; E4: 2019-2020 Shijiazhuang. The values of FLW are the means (mean ± SD) of NIL-KN9204 and NIL-J411; asterisks indicate significance determined by ANOVA, * $P < 0.05$; ** $P < 0.01$

Figure 4
 Analysis of the cell morphology of flag leaves in NIL-KN9204 and NIL-J411. **a:** Paraffin sections of flag leaf for NIL-J411 and NIL-KN9204, bar=50μm. **b:** Cell morphology differences of flag leaf in NIL-KN9204 and NIL-J411; ordinates indicate the number of bulliform cell, upper epidermal cell, inferior epidermal cell, big vascular, small vascular and vascular respectively; asterisks indicate significance determined by ANOVA, *P < 0.05, **P < 0.01, ns: not significant

Figure 5
 Analysis of differentially expressed genes in NIL-KN9204 and NIL-J411 within the target region of 598.10–600.596 Mb during the heading date (HD) and grain filling (GF) periods

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