

QTL Mapping And Candidate Gene Mining of Flag Leaf Size Traits In *Japonica* Rice Based On Linkage Mapping And Genome-Wide Association Study

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Research Article

Keywords: Japonica rice, QTLs, Flag Leaf, Linkage mapping, Genome-wide association study

Posted Date: September 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-866138/v1>

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Abstract

As one of the most important part of the ideal plant type of *japonica* rice, leaf shape affects the photosynthesis and carbohydrate accumulation directly. Mining and using new leaf shape related genes/QTLs can further enrich the theory of molecular breeding and accelerate the breeding process of *japonica* rice. In the present study, 2 RILs and a natural population with 295 *japonica* rice varieties were used to map QTLs for flag leaf length (FL), flag leaf width (FW) and flag leaf area (FLA) by linkage analysis and genome-wide association study (GWAS) through 2 years. A total of 64 QTLs were detected by 2 ways, and pleiotropic QTLs *qFL2* (Chr2_33,332,579) and *qFL10* (Chr10_10,107,835; Chr10_10,230,100) consisted of overlapping QTLs mapped by linkage analysis and GWAS through 2 years were identified. The candidate genes *LOC_Os02g54254*, *LOC_Os02g54550*, *LOC_Os10g20160*, *LOC_Os10g20240*, *LOC_Os10g20260* were obtained, filtered by linkage disequilibrium (LD), and haplotype analysis. *LOC_Os10g20160* (*SD-RLK-45*) showed outstanding characteristics in quantitative real-time PCR (qRT-PCR) analysis in leaf development period, belongs to S-domain receptor-like protein kinases gene and probably to be a main gene regulating flag leaf width of *japonica* rice. The results of this study provide valuable resources for mining the main genes/QTLs of *japonica* rice leaf development and molecular breeding of *japonica* rice ideal leaf shape.

Introduction

Rice is one of the most important food crops on the planet, bearing the lifeline of human food security (Pane et al 2021; Barboza et al 2018; Timmer 2014). Leaf is an important part of rice plant histogenesis and morphogenesis, and also the main organ for photosynthesis and respiration (Adachi et al 2014; Zhu et al 2014; Gu et al 2012). The photosynthetic energy storage and normal life function of rice are directly affected by leaf (He et al 2017). Meanwhile, rice leaf size is an important part of rice ideal plant type, as well as an important character of rice yield formation (Rahman et al. 2013). To be the top functional leaf of rice, flag leaf has significant effects on plant related physiological characters and field population structure (Zhu et al 2012; Giuliani et al. 2013). Therefore, it is an effective way to improve the quality and yield of rice by shaping and screening the leaf morphology in the process of breeding.

Although leaf size has a great influence on high photosynthetic efficiency, the genetic mechanism of leaf morphological characteristics is still unclear (Hoang et al 2019). Mining QTLs by linkage mapping in RILs and GWAS mapping in natural populations to find the candidate genes might be the most efficient way to analyze the genetic basis of rice leaf shape.

In recent years, many QTLs and regulatory genes related to rice leaf morphology have been discovered (<http://www.gramene.org/>) (Fujino et al. 2008; Farooq et al. 2010; Chen et al. 2012; Zhang et al. 2014). These QTLs and genes can change the physiological function of plants by regulating leaf morphology, and have important impacts on the coordination of light energy utilization and "sink-source" relationship. At the same time, their potential impact on rice yield has also been gradually discovered. Tang et al. (2018) Using CSSL population with 143 individuals, and obtained 14 leaf length and 19 leaf width QTLs, and further obtained the rice leaf size gene *Ghd7.1* by fine mapping, mutation test, and allelic variation analysis. Zhang et al. (2015a) mapped the flag leaf width QTL *qflw7.2* to 27.1kb by recombinant inbred lines derived from 93 – 11 and Peiai 64s, and has identified 2 candidate genes *LOC_Os07g41180*, *LOC_Os07g41200*. A natural population of 532 individuals was used for genome-wide association analysis and high-throughput leaf scoring, 73 QTLs associated with rice leaves were mapped as a result (Yang et al. 2015). At present, we've got a clear understanding of the cloned gene *NAL 1* located on chromosome 4, which affects the growth of lateral leaf (Jiang et al. 2015). The *NAL 1* mutant is characterized by dwarf and narrow

leaf, and the expression level of the genes related to the polar transport of auxin and leaf development in the mutant changed (Li et al. 2019a). It was highly expressed in vascular tissue, which played an important role in cell division and cell size regulation, promoting the lateral growth of leave. *OsFLW7* regulated the width of flag leaf, increased photosynthetic leaf area (Xu et al., 2017). At the same time, *OsFLW7* is an allele of *GL7/ GW7*, which may be related to the regulation of grain traits and the mutant was found that the grain length, grain plumpness and yield increased significantly.

GWAS and linkage analysis are both accurate and effective tools for QTL detection of complex rice traits (Famoso et al. 2011). The breadth and accuracy of QTL detection could be significantly improved by combining these 2 methods. In this study, 2 sets of RILs populations with high-density bin-map and 295 re-sequenced *japonica* rice accessions were used to conducted linkage/GWAS mapping of flag leaf length, width and area of *japonica* rice. Two novel QTL *qFL2*, *FL10* and five candidate genes related to flag leaf size in *japonica* rice were discovered, providing important references for molecular breeding of *japonica* rice with ideal leaf size and plant type.

Methods

Populations for QTL Mapping

Natural population is composed of 295 rice varieties, most of which are temperate *japonica* rice, widely collected from Heilongjiang, Jilin and Liaoning provinces, while foreign materials mainly come from Japan, Korea, the Democratic People's Republic of Korea and Russia. This population has been used in previous studies (Li et al. 2019b). RILsA contains 195 individuals, obtained from crossing the narrow erect leaf *japonica* rice variety K131 and wide curved leaf upland rice variety HDB. RILsB contains 189 individuals, which was derived from a cross between the long wide flag leaf *japonica* rice variety WD20342 and short narrow flag leaf variety Caidao. All materials were planted in Acheng rice experimental base of Northeast Agricultural University from 2019 to 2020. Each individual was planted in 8 rows with 20 plants in each row by single plant transplanting and the spacing of rows and plants was 30 cm × 3 cm. The field management of water and fertilizer followed the basic method of conventional field production.

Phenotypic Identification of Flag Leaf Size

In order to reduce the influence of marginal effect on phenotype, five continuous plants in the middle of the fourth row of each variety were selected as the research objects, and the average value of five plants for each line was calculated. The flag leaf length (FL), width (FW) and area (FLA) were investigated at full heading stage using Tuopu YMJ-D Living Leaf Area Meter (Tuopu Yunnong Technology Co., Ltd). Population phenotypic correlation analysis for QTL mapping was performed by SPSS.

Linkage Analysis and Genome-wide Association Study

Two linkage maps for linkage analysis were constructed through 10K array genotyping by targeted sequencing (GBTS) supported by MOLBREEDING Biotechnology Co., Ltd (Shijiazhuang, China). The RILsA population has been used in previous study (Li et al. 2020). Nine hundred and seventy-eight bin markers covered 2465.32 cM of the rice genome with an average distance of 2.52 cM constructed the linkage map (Fig. S1). The linkage map of RILsB contains 527 bin markers covered 1874.85 cM of the rice genome with an average distance of 3.56 cM (Fig. S2). The IciMapping Ver.4.2 (Meng et al. 2015) based on inclusive composite interval mapping (ICIM) was used to detected the QTLs for rice flag leaf traits. The walking speed was set as 1cm, and the LOD threshold of ICIM was set as 2.5. To ensure the accuracy of mapping results, we controlled the type 1 error of whole genome detection below 5% by

1000 permutation tests. The natural population for genome-wide association study (GWAS) was deep re-sequenced by Beijing Genomics Institute (BGI www.genomics.org.cn). A total of 788, 396 SNPs meeting the criteria (minimum allele frequency $\geq 5\%$, deletion rate $\leq 20\%$) were selected for follow-up analysis, and 295 *japonica* rice varieties' population structure analysis, genetic relationship analysis and linkage disequilibrium analysis have been completed in the previous study of our laboratory (Li et al. 2019b). The mixed linear model (MLM) of TASSEL 5.0 (Bradbury et al. 2007) was used for genome-wide association analysis and setting the threshold of SNPs significantly associated with flag leaf traits as 5.46×10^{-6} which was calculated by GEC software (<http://statgenpro.psychiatry.hku.hk/gec/>). If 2 or more SNPs were located in the same LD interval, they were regarded as the same QTL, and the SNP with the smallest p value was treated as the lead SNP. QQman package in R was used to create the Manhattan and Q-Q plots (Turner 2014).

Haplotype Analysis of Candidate Genes

Considering the LD decay of the whole genome was confirmed in previous study by 109 kb (Li et al. 2019b), we selected 218 kb upstream and downstream of the SNP as the target interval to screen candidate genes. Non-synonymous SNPs of all exons were extracted from the "Rice SNP-Seek Database" of The International Rice Informatics Consortium (IRIC) (<https://snp-seek.irri.org/>), which were then used for haplotype analysis of candidate genes with DnaSP software (Julio et al. 2017).

RNA Extraction and qRT-PCR Analysis

The flag leaves of four parents (K131, HDB, WD20342, Caidao) of RILs populations took 4–6 days to fully extended. Five repetitions of flag leaves for each parent were sampled 4 times from the flag leaves begin to drew out to fully extended. The total RNA was extracted with TranZol Up RNA Kit (TransGen Biotech). HiFiScript cDNA synthesis kit (CoWin Biosciences, Beijing, China) was used to synthesize cDNA. qRT-PCR was performed on Bio-Rad CFX96 system using 2×Fast qPCR Master Mixture with 3 biological replicates for each sample. House-keeping gene *Actin1* was used to measure the mRNA levels of candidate genes (Li et al. 2019b) as an internal control. The primers used for qRT-PCR in this study are all shown in Table S1. Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Data shown in figures and tables are mean values of three replicates.

Results

Phenotypic Analysis

The phenotypic data of flag leaf size of RILs populations and natural population from 2019 to 2020 are shown in Table S2; Table S3, and the general trends in 2 years were basically the same. The 3 leaf size traits of parents of RILs populations showed great phenotypic differences in 2 years and the RILs showed significant variation in FL, FW and FLA. FL and FLA in 2 RILs populations with standard deviation from 4.38 to 7.25, presented stronger variation than FW with the standard deviation from 0.17–1.44. The variation characteristics of flag leaf phenotypic in natural population were similar to those of RILs in 2 years. Most of the absolute values of kurtosis and skewness were near 1, which basically conformed to the normal distribution and showed a typical genetic model of quantitative traits, was suitable for linkage analysis and genome-wide association analysis.

Linkage Mapping for Flag Leaf Size in Japonica Rice

We conducted QTL linkage analysis for FL, FW and FLA of 2 RILs populations in 2019 and 2020. A total of 28 QTLs were detected, which were distributed on chromosome 1, 2, 3, 4, 6, 7, 10 and 11 of rice (Table S4). The phenotypic variation explained by a single QTL ranged from 4.97–20.88%. *qFLr7-2* and *qFWr2-3* in RILsA; *qFLr3*, *qFWr2-1*, *qFLAr4-2* and *qFWr10* in RILsB were detected in 2 years simultaneously, represents the stable expression of genetic effects in the corresponding interval. At the same time, *qFLr6-1*, *qFLAr6-1* in RILsA and *qFLr3*, *qFLAr3-2* in RILsB located in the same interval respectively, but detected to control different traits in different populations and years, which were likely to be pleiotropic QTLs.

GWAS for Flag Leaf Size in Japonica Rice

A natural population which has been deeply re-sequenced and its 788, 396 high quality SNP markers were used to conducted GWAS. Manhattan and Q-Q plots for the GWAS are shown in Fig. 1, Fig. 2. Table 1, Table 2 show that a total of 36 SNPs were detected under the threshold of 5.46×10^{-6} which were significantly associated with flag leaf size of *japonica* rice. These SNPs were distributed on all chromosomes of rice except chromosome 11 with the R^2 ranged from 8.87–12.81%. The GWAS results showed that Chr10_10,230,100 (*qFWn10-2*), Chr10_10,107,835 (*qFLAn10-1*) located in one LD interval was detected in both years associated with FW and FLA separately. Chr2_33,332,579 (*qFWn2-2*, *qFLAn2-5*) and Chr7_20,475,568 (*qFWn7*, *qFLAn7-2*) were detected in 2019 associated with FW and FLA simultaneously. The above-mentioned results are consistent with what we obtained in phenotypic analysis.

Table 1
Significant SNPs associated with flag leaf size related traits in natural population in 2019

Traits	QTLs	Peak SNPs	Chr.	Position	Pvalue	R^2 (%)	known genes
FL	<i>qFLn1</i>	Chr1_3505761	1	3505761	3.79E-06	8.87	
FL	<i>qFLn10</i>	Chr10_15440761	10	15440761	6.35E-07	10.02	
FW	<i>qFWn1-2</i>	Chr1_18340871	1	18340871	1.88E-06	9.37	
FW	<i>qFWn2-2</i>	Chr2_33332579	2	33332579	3.18E-08	12.45	
FW	<i>qFWn3</i>	Chr3_18626	3	18626	5.19E-07	10.38	
FW	<i>qFWn7</i>	Chr7_20475568	7	20475568	4.39E-06	12.81	<i>OsFLW7</i>
FW	<i>qFWn8</i>	Chr8_5133437	8	5133437	2.15E-07	10.04	<i>OsBAK1</i>
FW	<i>qFWn10-2</i>	Chr10_10230100	10	10230100	1.67E-06	10.32	
FLA	<i>qFLAn2-2</i>	Chr2_24488823	2	24488823	4.21E-06	8.84	
FLA	<i>qFLAn2-3</i>	Chr2_31463696	2	31463696	1.28E-06	9.60	
FLA	<i>qFLAn2-4</i>	Chr2_32571605	2	32571605	5.55E-07	9.50	
FLA	<i>qFLAn2-5</i>	Chr2_33332579	2	33332579	1.84E-06	9.33	
FLA	<i>qFLAn6-1</i>	Chr6_1083411	6	1083411	1.46E-07	9.63	
FLA	<i>qFLAn7-2</i>	Chr7_20475568	7	20475568	2.29E-06	9.90	

Table 2
Significant SNPs associated with flag leaf size related traits in natural population in 2020

Traits	QTLs	Peak SNPs	Chr.	Position	Pvalue	R ² (%)	known genes
FL	<i>qFLn3-1</i>	Chr3_7448808	3	7448808	3.94E-06	8.87	
FL	<i>qFLn3-2</i>	Chr3_8333379	3	8333379	5.01E-06	8.77	
FL	<i>qFLn3-3</i>	Chr3_8556595	3	8556595	8.66E-07	10.00	
FW	<i>qFWn1-1</i>	Chr1_6294146	1	6294146	6.96E-07	10.12	
FW	<i>qFWn1-3</i>	Chr1_33883944	1	33883944	6.21E-07	10.21	
FW	<i>qFWn2-1</i>	Chr2_30499110	2	30499110	1.60E-06	9.50	
FW	<i>qFWn5-2</i>	Chr5_17982797	5	17982797	7.93E-07	10.03	<i>SNFL1</i>
FW	<i>qFWn6-1</i>	Chr6_3907011	6	3907011	7.32E-07	10.09	
FW	<i>qFWn6-2</i>	Chr6_10031161	6	10031161	1.63E-06	9.49	
FW	<i>qFWn7</i>	Chr7_20345187	7	20345187	2.56E-06	9.29	
FW	<i>qFWn9-1</i>	Chr9_16271846	9	16271846	1.20E-06	9.72	
FW	<i>qFWn9-2</i>	Chr9_16890634	9	16890634	4.80E-06	8.69	
FW	<i>qFWn10-1</i>	Chr10_2188025	10	2188025	3.91E-06	8.88	
FW	<i>qFWn12-1</i>	Chr12_478610	12	478610	4.30E-06	8.82	
FLA	<i>qFLAn1-1</i>	Chr1_3318081	1	3318081	5.09E-07	10.39	
FLA	<i>qFLAn1-2</i>	Chr1_33977764	1	33977764	2.05E-06	9.34	<i>OsDET1</i>
FLA	<i>qFLAn2-1</i>	Chr2_12992454	2	12992454	1.28E-06	9.69	
FLA	<i>qFLAn3-1</i>	Chr3_15386980	3	15386980	1.00E-06	10.12	
FLA	<i>qFLAn3-2</i>	Chr3_16106964	3	16106964	4.33E-07	10.51	
FLA	<i>qFLAn4</i>	Chr4_6025108	4	6025108	3.01E-06	9.06	
FLA	<i>qFLAn7-1</i>	Chr7_5494006	7	5494006	1.50E-06	9.57	
FLA	<i>qFLAn10-1</i>	Chr10_10107835	10	10107835	4.32E-06	8.79	

Identification of Pleiotropic QTLs for Flag Leaf Size in Japonica Rice

In this study, different materials and different analysis methods were used to detect QTLs related to flag leaf size for *japonica* rice in 2 years. QTLs detected by the two methods and whose physical positions of chromosomes coincided were defined as co-location QTLs (pleiotropic QTLs) (Table 3). In co-location QTL *qFL2*, *qFWr2-3* located in C2_33,142,844-C2_35,004,908 was detected in RILsA in both 2019 and 2020. This region contains the lead SNP Chr2_33,332,579 (*qFLAn2-5*, *qFWn2-2*) detected by GWAS and significantly associated with both FW, FLA of *japonica* rice. The other co-location QTL (pleiotropic QTL) *qFL10* contains linkage analysis QTL *qFWr10* located in

C10_9,054,066 - C10_10,570,732 interval, which was repeatedly detected in 2019 and 2020. According to the physical location of rice chromosome, *qFLAn10-1* and *qFWn10-2* were both located in *qFWr10* interval, and their physical locations are partially coincident.

These 2 pleiotropic QTLs *qFL2* and *qFL10* were the most important ones that stable expressed through linkage analysis and GWAS in the same interval in this study, indicating that the corresponding interval probably contain the candidate genes of flag leaf size of *japonica* rice. Thus, further researches will conduct on them.

Table 3
The name and distribution of the co-location QTLs

QTL name	Chr.	GWAS				Linkage mapping			
		Original QTL	Peak SNP	P	R ² (%)	Original QTL	QTL interval	LOD	R ² (%)
<i>qFL2</i>	2	<i>qFLAn2-5</i>	Chr2_33332579	1.84E-06	9.33	<i>qFWr2-3</i>	C2_33142844 - C2_35004908	4.65	9.46
		<i>qFWn2-2</i>	Chr2_33332579	3.18E-08	12.45		C2_33142844 - C2_35004908	2.52	7.27
<i>qFL10</i>	10	<i>qFLAn10-1</i>	Chr10_10107835	4.32E-06	8.79	<i>qFWr10</i>	C10_9054066 - C10_10570732	2.59	8.15
		<i>qFWn10-2</i>	Chr10_10230100	1.67E-06	10.32		C10_9054066 - C10_10570732	2.86	11.26

Candidate Gene Screening and Haplotype Analysis

The P of lead SNP Chr2_33, 332, 579 of *qFWn2-2* in *qFL2* is the smallest, which is 3.18E-08. Considering that the LD of the whole genome is 109kb (Fig. 3A), we selected 109kb upstream and downstream of this SNP as the target interval to screen candidate genes. There are 37 genes in the target region, including 22 function annotated genes, 5 expression proteins with unknown function, 5 hypothetical proteins and 5 retrotransposon proteins (Table S5). We used SNPs with nonsynonymous mutations in exons to analyze the haplotypes of these genes and found that there were 2 functional annotation genes *LOC_Os02g54254*, *LOC_Os02g54550* had significant differences in FW among different haplotypes, and the differences in 2019 and 2020 were basically the same (Fig. 4). Table 4, showed that 2 haplotypes of *LOC_Os02g54254* (G/A) and *LOC_Os02g54550* (C/T) had significant differences in FW.

qFL10, pleiotropic QTL either was composed of stable QTLs detected by linkage analysis and significant SNPs detected by GWAS. There is an overlapping interval between the two ways results, that is, the 10.12Mb-10.22Mb interval of chromosome 10 (overlapping interval of *qFLAn10-1*-*qFWn10-2*) to be the target interval (Fig. 3B, C). Fifteen genes exist in the target region, including 5 function annotated genes, 4 expression proteins with unknown function and 6 retrotransposon proteins (Table S6, Fig. 3D). SNPs with nonsynonymous mutations in exons were used to analyze the haplotypes of the genes, and 3 functional annotation genes

LOC_Os10g20160, *LOC_Os10g20240* and *LOC_Os10g20260* were found to have significant differences in FW among different haplotypes, and the differences in 2019 and 2020 were basically the same (Fig. 4). Haplotype analysis revealed that significant differences for FW were observed between hap1 (TGT), hap2 (TAT) and hap3 (CGT) in *LOC_Os10g20160*. *LOC_Os10g20240* and *LOC_Os10g20260* both had 2 haplotypes as hap1 (GA), hap2(AC) and hap1 (CCC), hap2(CCG) which showed significant difference for FW.

Table 4
Candidate gene haplotypes and the number of varieties corresponding to each haplotype

Gene	Hap1/Number	Hap2/Number	Hap3/Number
<i>LOC_Os02g54254</i>	G/274	A/17	
<i>LOC_Os02g54550</i>	C/275	T/14	
<i>LOC_Os10g20160</i>	TGT/217	TAT/30	CGT/18
<i>LOC_Os10g20240</i>	GA/279	AC/11	
<i>LOC_Os10g20260</i>	CCC/255	CCG/17	

Identification of candidate genes based on qRT-PCR

According to the results of haplotype analysis, 5 candidate genes were analyzed by qRT-PCR using 4 RILs parents (K131, HDB, WD20342, Caidao) as templates in 4 growth periods from the flag leaf begin to drew out to fully extended. The candidate genes and quantitative primers are shown in Table S1. Expressions of *LOC_Os02g54254*, *LOC_Os02g54550*, *LOC_Os10g20240*, *LOC_Os10g20260*, had no obvious regularity and did not increase significantly in a certain period (Fig. 5). On the other hand, *LOC_Os10g20160* presented different expression type, and the expression level of the RILs parents in 4 periods was significantly higher than that of other genes. *LOC_Os10g20160* (*SD-RLK-45*) belongs to S-domain receptor-like kinases (SD-RLK) family shows preferential in leaf, shoot and seeds (Aya et al. 2011). Thus, *SD-RLK-45* is probably the candidate gene of *qFL 10*.

Discussion

Leaf type improvement is one of the important ways to increase rice yield (Dastan et al. 2020). By rationally controlling leaf type traits could enhance lodging resistance, photosynthetic utilization rate, and what's more, increase yield per plant (Ya et al. 2015; Clerget et al. 2016). *Japonica* rice has better cooking and eating quality due to higher amylose content, which cultivated and consumed in East Asia as the major variety (https://en.wikipedia.org/wiki/Japonica_rice), and is obviously worth to conduct researches on the genetic variation of leaf type (size) of *japonica* rice. Although some genes or QTLs regulating flag leaf size were identified by classical mapping and reverse genetics, the number of studies on leaf genetic variation about *japonica* rice is still relatively small (Jiang et al. 2010; Yang et al. 2015; Hu et al. 2012). In this study, three *japonica* rice populations were used for linkage mapping and GWAS in 2 years, and 64 leaf shape QTLs were mapped, some of which were overlapped or similar to that in previous studies, and some were novel QTLs. Interval of *qFLr6-2* and *qFLAr3-1* detected by linkage mapping overlapped with *qFLL6* and *qLA3-1* (Shen et al, 2011) regulating leaf length and leaf area respectively. *qFLL1.2* mapped by Zhang et al, (2015b) using an RIL population and resequencing genetic map was found to be located within *qFLr1* possessing same function. The recognized narrow leaf gene *NAL1* (Li et al, 2019a) on chromosome 4 of rice was found to be located within *qFLAr4-4* in this study. *qFWn7* located in the same interval with *OsFLW7* identified by Xu et al (2017). Known genes *OsBAK1* (Li et al, 2009), *SNFL1* (He et al, 2018) and *OsDET1* (Zang et al, 2016), were found to be located within or nearby the LD interval of *qFWn8*, *qFWn5-2*, *qFLAn1-2* in this study. Among these genes, *OsBAK1* controlling leaf angle and length by regulating brassinosteroid (BRs), and has been observed significantly reduce plant height. The study of *SNFL1* mutant indicated that the length of epidermal cells and the number of longitudinal veins in flag leaves decreased remarkably. *OsDET1* has been proved to regulate ABA signal transduction and play an important role in maintaining rice growth and plant type development.

Through 2 years of GWAS and linkage analysis, we found pleiotropic and stable QTLs *qFL2* and *qFL10*, representing stable genetic effects. In view of the genome-wide LD decay of GWAS, we selected 218 kb upstream and downstream to screen candidate genes, and also served as the overlap region of linkage analysis and GWAS (Li et al. 2019b). Based on haplotype analysis, we obtained 5 candidate genes: *LOC_Os02g54254* (*OsLKR/SDH*), *LOC_Os02g54550* (*OsFBX63*), *LOC_Os10g20240* (*OsKNOLLE*), *LOC_Os10g20260* (*CSIF7*), *LOC_Os10g20160* (*SD-RLK-45*). *OsLKR/SDH* was proved to be a bifunctional lysine degrading enzyme (Takaiwa et al. 2010), *OsFBX63* is a F-box family gene, the function of which hasn't been studied. Syntaxin-related protein *OsKNOLLE* probably play an important role in regulating abiotic stress resistance (Wang et al. 2011). *CSIF7* was confirmed as a Cellulose Synthase family gene (Julian et al. 2015). *SD-RLK-45* belongs to *SD-RLK* family and supposed to be a novel functional gene. Characteristics of expression during leaf development period make *SD-RLK-45* the most likely candidate gene for *qFL10*.

Plant receptor-like protein kinases (*RLKs*) comprise one of the largest and most diverse superfamily of plant proteins with 610 and 1131 members in the *Arabidopsis* and rice genomes, respectively (Zou et al. 2015). The *RLKs* gene superfamily played fundamental roles in hormone perception, developmental regulation, innate immunity, adaptation to abiotic stresses, and quantitative yield components (Melissa et al. 2009; Herder et al. 2012, pan et al. 2019). S-domain RLKs (*SD-RLKs*) belongs to a subfamily of *RLKs*, with 147 members in rice. (Morillo et al. 2006) Recent studies on rice have confirmed that *OsSRK1* regulates leaf width by promoting cell division in the leaf primordium and *OsSRK1*-overexpression plants exhibited enhancing ABA sensitivity and salt tolerance compared with wild type (Zhou et al. 2020).

In the present study, there were significantly differences in flag leaf width of the haplotypes of 5 candidate genes *OsLKR/SDH*, *OsFBX63*, *OsKNOLLE*, *CSIF7* and *SD-RLK-45*. Only the expression of *SD-RLK-45* showed excellent characteristics during flag leaf development, and it's most likely to be the candidate gene of *qFL10*. As a novel gene that has not been systematically studied, additional data are needed to verify the function of *SD-RLK-45* in controlling flag leaf width or size. The overexpression, construction of CRISPR-Cas9 and omics experiments will be the focus of our future studies.

Conclusions

Two RILs and 295 *japonica* rice varieties were collected to identify the flag leaf size phenotypic. Two pleiotropic QTLs *qFL2*, *qFL10* consisted of overlapping QTLs mapped by linkage analysis and GWAS were identified. Based on LD decay distance and pleiotropic interval overlapping, 2 intervals of 218-kb and 100-kb were selected for candidate gene screening. *LOC_Os02g54254*, *LOC_Os02g54550*, *LOC_Os10g20160*, *LOC_Os10g20240*, *LOC_Os10g20260* were identified by haplotype analysis as candidate genes, and qRT-PCR showed *LOC_Os10g20160* probably to be a novel functional gene contributing flag leaf size by regulating flag leaf width of *japonica* rice. The results provide resources for leaf type improvement breeding.

Declarations

Funding

This research was supported by the Heilongjiang Provincial government Postdoctoral Foundation of China (LBH-Z16188), the Natural Science Foundation Joint Guide Project of Heilongjiang (LH2019C035) and the Province-Academy Science and Technology Cooperation Project of Heilongjiang (YS20B05). Application R & D Project of Heilongjiang Academy of Agricultural Science (2021YYYF037).

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Ethical Statement

The authors of this paper declare that we have no conflict of interest. Euphytica is the only journal we submitted. The submitted work is original and haven't been published elsewhere in any form or language. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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Figures

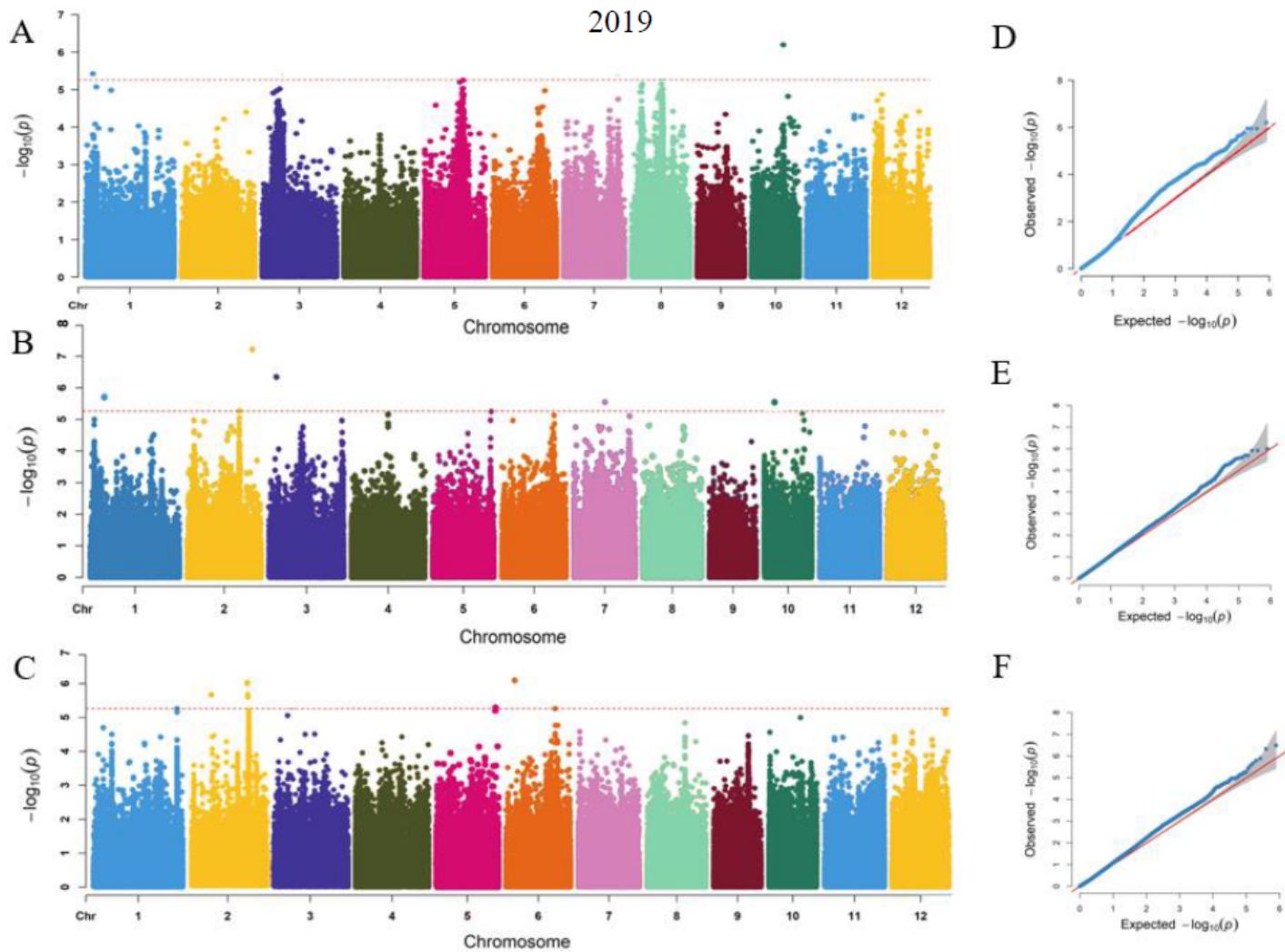


Figure 1

Manhattan plots and quantile-quantile (Q-Q) plots of genome-wide association studies for FL, FW and FLA in 2019. A, Manhattan plot for FL. B, Manhattan plot for FW. C, Manhattan plot for FLA. D, Q-Q plot for FL. E, Q-Q plot for FW. F, Q-Q plot for FLA

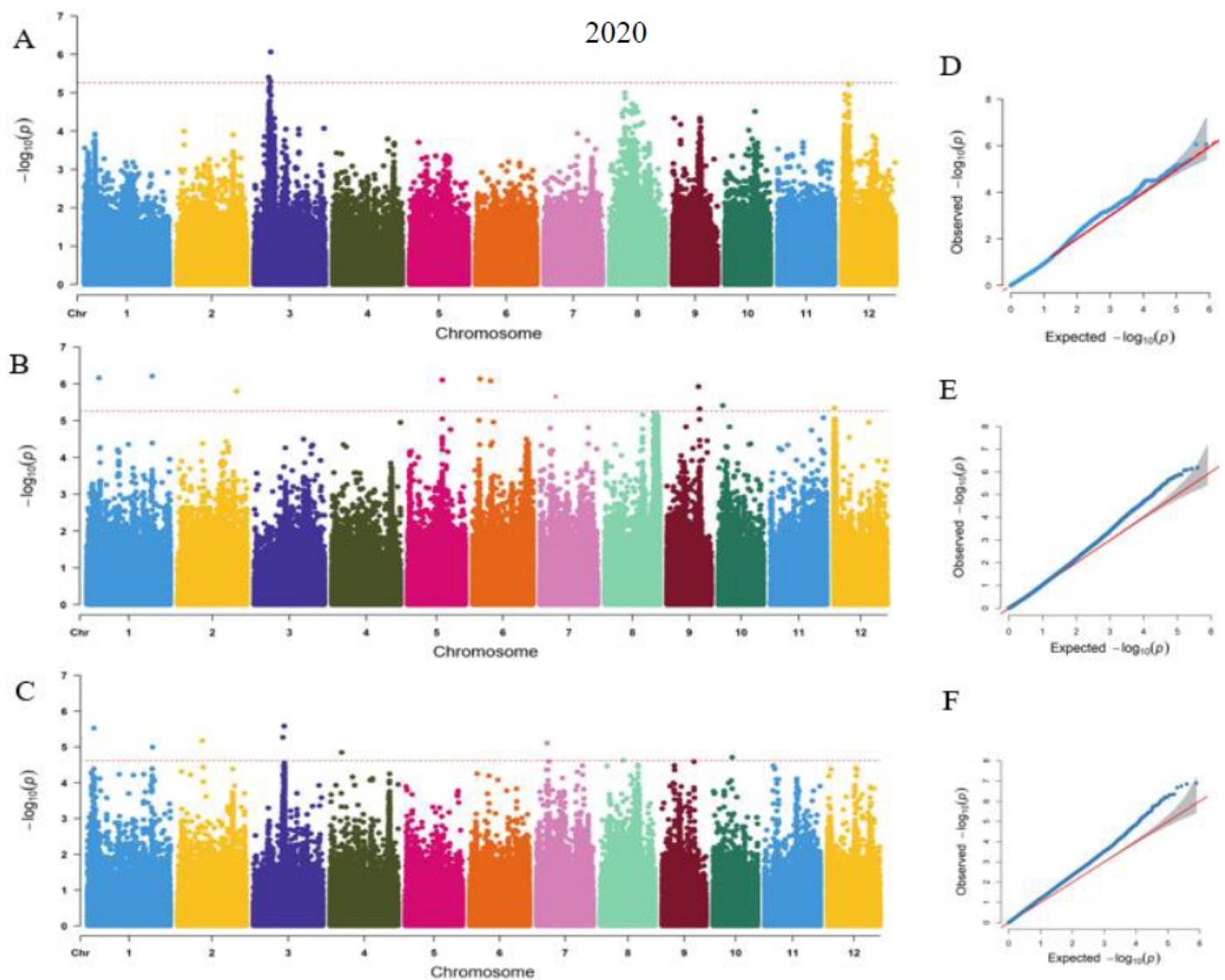


Figure 2

Manhattan plots and quantile-quantile (Q-Q) plots of genome-wide association studies for FL, FW and FLA in 2020. A, Manhattan plot for FL. B, Manhattan plot for FW. C, Manhattan plot for FLA. D, Q-Q plot for FL. E, Q-Q plot for FW. F, Q-Q plot for FLA

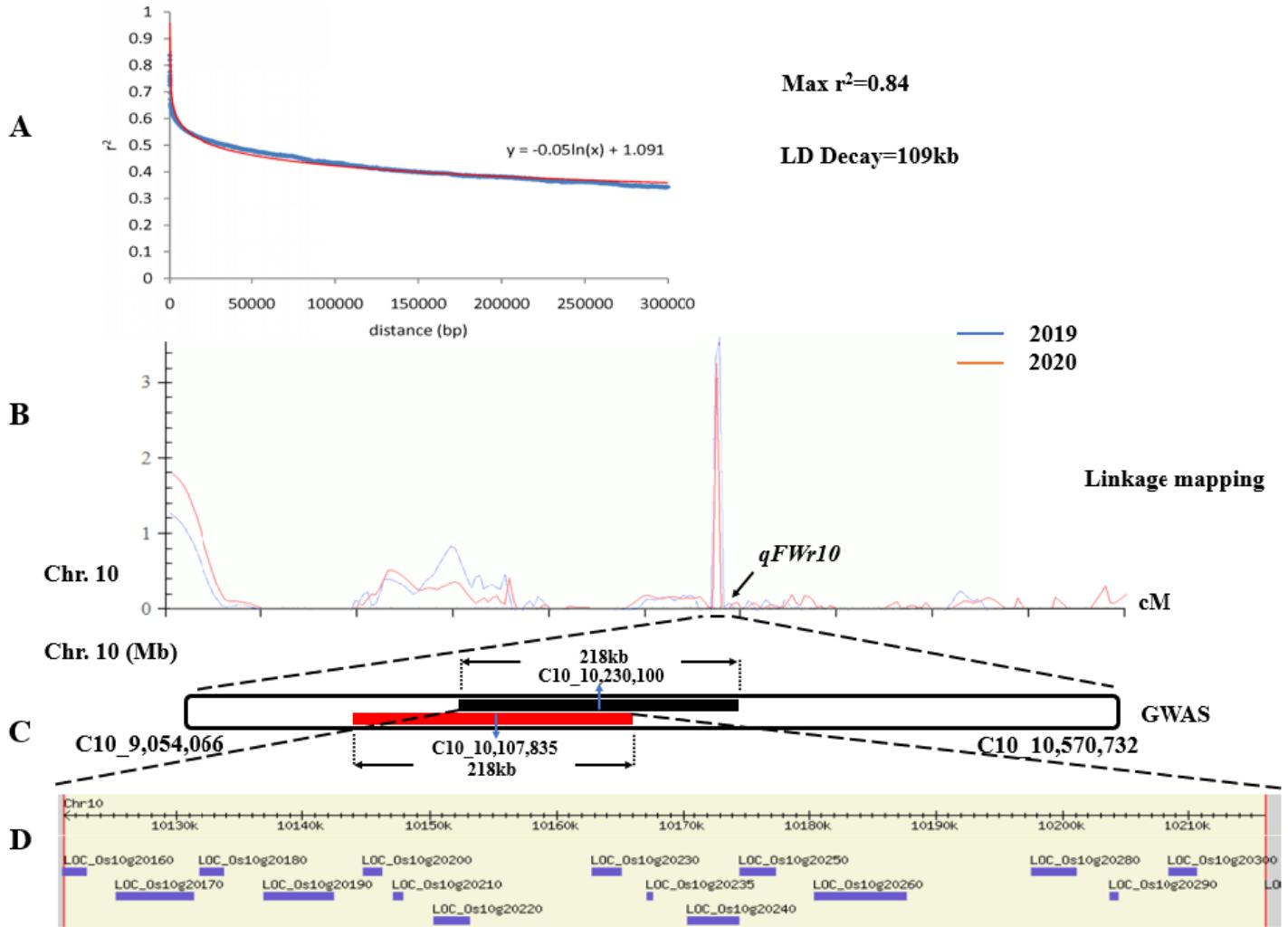


Figure 3

Identification of candidate genes by linkage mapping and GWAS. A LD decay of the whole genome in 295 japonica rice varieties. When r^2 decays to the half, the corresponding physical distance (109 kb) is recorded as the LD attenuation distance of the whole genome. B qFWr10 located in C10_9,054,066 - C10_10,570,732 interval, which was repeatedly detected in 2019 and 2020. C Overlapping physical location of the lead SNP (C10_10,107,835, C10_10,230,100) on chromosome 10 detected by GWAS. D 15 genes in the 218 kb region.

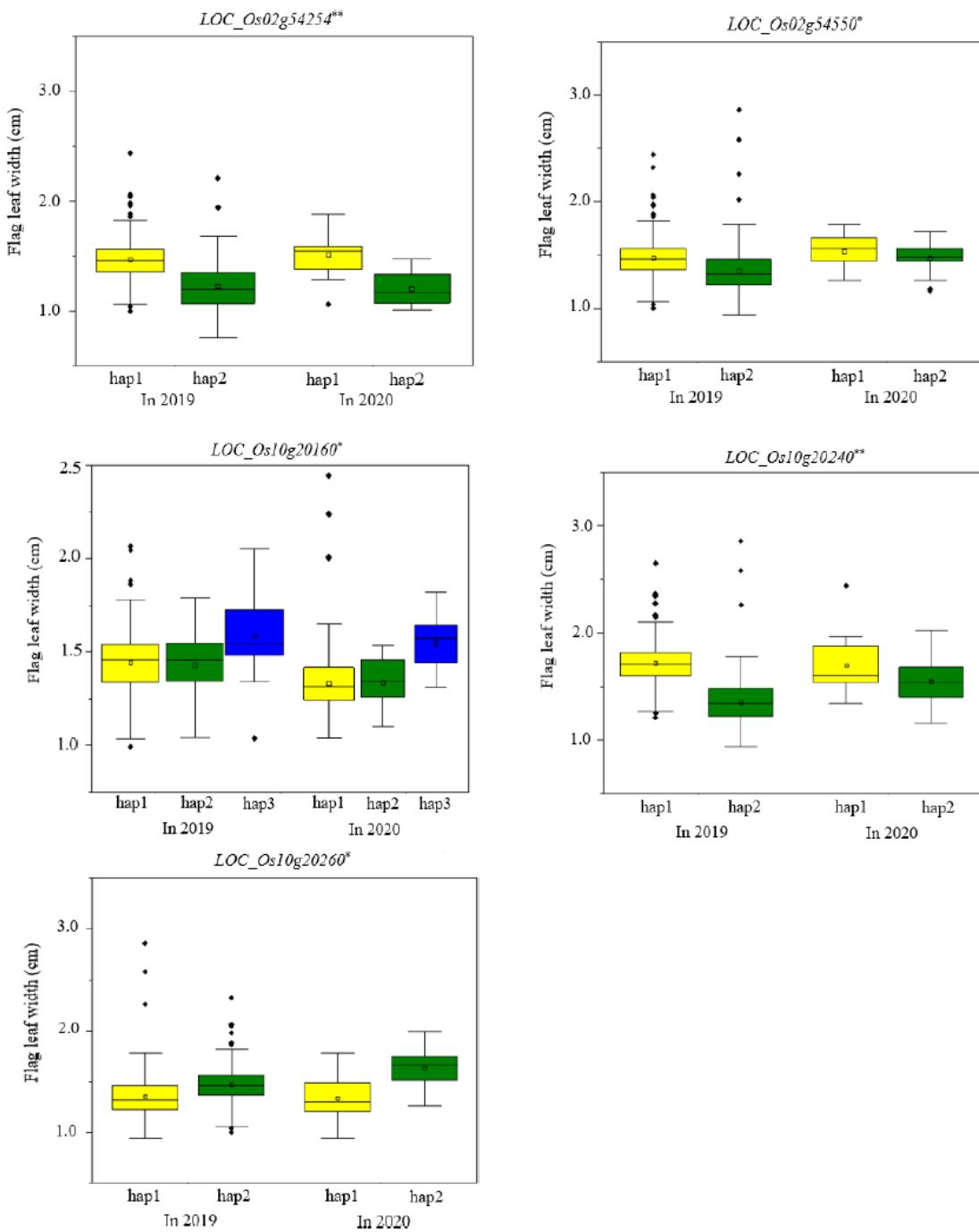


Figure 4

Boxplot for Flag leaf width based on the haplotypes (green, yellow blue indicate the phenotypic result for hap1, hap2 and hap3, respectively (The *and ** suggested significance of ANOVA at $P < 0.05$ and $P < 0.01$, respectively)

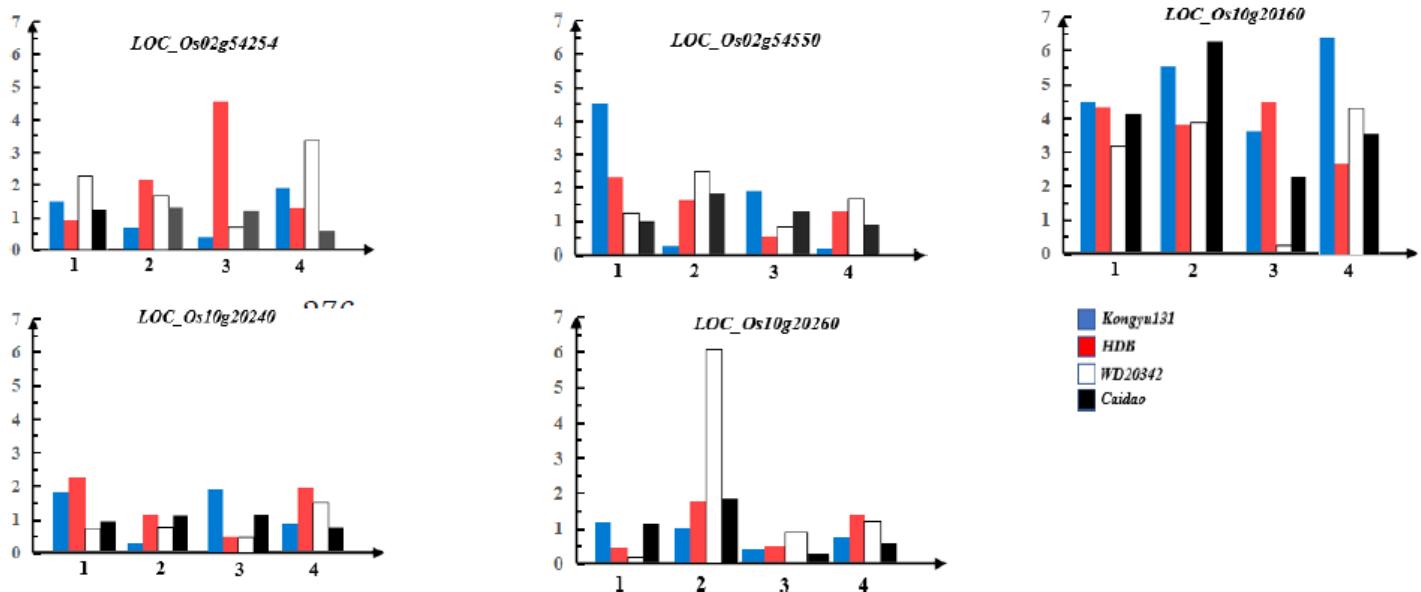


Figure 5

Expression patterns of 5 genes in 4 growth periods

Supplementary Files

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- SupplementaryFigS2GeneticlinkagemapofRILsB.png
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