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RESEARCH ARTICLE

Genetic mapping for flag leaf shape in new plant type based recombinant inbred lines in rice (*Oryza sativa* L.)

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Abstract

The flag leaf, a pivotal element in rice plants' photosynthesis process, holds great significance in rice breeding initiatives aimed at optimizing plant traits. The dimensions of the flag leaf play a critical role in photosynthesis, thereby exerting a considerable influence on the potential yield of rice. In this study, we utilized an NPT-based mapping population comprising PR126 (a green super rice cultivar) and Pusa NPT34 (a new plant type line) recombinant inbred line (RIL) population consisting of 175 lines, evaluated across three distinct locations. A total of seventeen QTLs were detected for flag leaf length (seven), width (five), and area (four) distributed across chromosomes 2, 3, 4, 5, and 6, observed across different locations. Among these 17 QTLs, 8 were found to colocalize on two genomic regions. Validation of these QTLs was performed using $F_{2:3}$ families obtained from the cross between Pusa Basmati 1509 and Pusa NPT34. One marker, RM190, successfully validated the QTLs *qFLW6.1* and *qFLA6.1* at ADT. Remarkably, both validated QTLs are situated within the same marker interval on chromosome 6 and genetic contribution for both QTLs is from 'Pusa NPT34'. Consequently, further refinement through fine mapping of the marker intervals holds promise for narrowing down the genomic region and pinpointing candidate genes. This will facilitate more precise marker-assisted selection strategies for enhancing flag leaf shape attributes.

Keywords: New Plant Type, flag leaf shape, qtl mapping, rice, RILs

Introduction

Rice cultivation spans across more than 114 countries on six continents, with India alone contributing over twenty percent of the world's rice production (Singh et al. 2021; Singh et al. 2022; Kumar et al. 2019). Meeting the rising food demand necessitates a projected 40% increase in rice production by 2025 (Fahad et al. 2018). In India, rice is grown in an area of 44mh with an average production of 105 mt of milled rice (Shidenur et al. 2019). Despite ranking first in acreage, India holds the second position in production, trailing behind China.

Photosynthesis is the main process of producing food in crops and the primary source of grain yield in rice (Zhang et al. 2015). The upper few leaves produce a maximum amount of carbohydrates predominantly by the flag leaf. It has been reported that the flag leaf's size and shape greatly impact photosynthesis and thereby may affect the yield (Yue et al. 2006). The size of the flag leaf in rice is positively correlated with yield-related traits such as thousand grains weight, panicle weight and other traits (Wang et al. 2020). Therefore, flag leaf traits are one among the major traits for increasing grain yield (Rahman et al. 2014). Researchers have suggested that flag leaf with large, wide and long architecture is ideal for high yield because it can capture more radiation (Saitoh et al. 2002). These leaf traits are determining factors for desired plant type (Tsukaya 2006). In recent years, some *indica* hybrid cultivars with these leaf characteristics have

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been recognized as 'super rice' (Wani et al. 2011). Therefore, it is important to introduce the leaf characteristics of high-yielding *indica* rice to improve the rice grain yield further. This excites breeders to search novel gene(s)/QTLs for flag leaf architecture.

Rice flag leaf size-related traits such as flag leaf length (FLL), flag leaf width (FLW) and flag leaf area (FLA) are typical quantitative traits controlled by polygenes and have a profound influence of the environment. Understanding the genetics of flag leaf and identification of genetic variation for the flag leaf traits plays prime importance in developing high-yielding varieties. In the recent past, several developments took place toward understanding the genetics of flag leaf architecture. Several genes/QTLs have been identified by different researchers like, *NAL1* (Jiang et al. 2015) for flag leaf size, *OsFLW7* (Xu et al. 2017) for flag leaf width, *qFL2* and *qFL10* for flag leaf length (Wang et al. 2022). Similarly, Chen et al. 2012 and Xiao et al. 2007, mapped five QTLs for flag leaf width on chromosomes 1, 4, 7 and 10 and eight QTLs for flag leaf size in backcross recombinant inbred lines, respectively. During 2015, Zhang et al. mapped QTL, *qFLW7.2* for FLW on chromosome 7 and identified two putative candidate genes. Recently Wang et al. 2022, have identified two QTLs, namely, *qFL2* and *qFL10* for flag leaf width and further analysis able to identify one putative candidate gene for FLW. The *NARROW LEAF1 (NAL1)* gene was mapped on chromosome 4 and later cloned to identify a single SNP which contributes for the variation in flag leaf width (Zhao et al. 2011).

The practical application of mapped QTLs has become clear in breeding, as the markers linked to these QTLs can serve as foreground markers in marker-assisted selection (MAS). The success of linkage-based mapping relies on selecting parental lines with significant phenotypic variation for the desired trait. This approach allows for the association of the segregating trait with allelic variation between two homozygous parents, which can be easily traced within their offspring. Therefore, identification and mapping genes/QTLs for flag leaf is the first step in understanding the genetic basis. Hence, the current study was undertaken to understand the genetics of flag leaf size-related traits in recombination inbred lines (RILs) derived from Pusa NPT34 and to identify the QTLs responsible for variation in the flag leaf size.

Materials and methods

Plant materials and population development

Recombinant inbred lines (RILs) in F_6 generation derived from the cross, PR126/Pusa NPT34 were used in the present study. PR126 is a short-duration *indica* rice variety that matures in 120 days. PR126 was bred in China and developed originally as Huanghuazhan (HHZ), having the parentage of Fenghuazhan/Huangxinzhang (Zhou et al. 2010; Chen et al. 2017). It is one of the most popular green super rice (GSR) varieties distributed across the globe by the International Rice Research Institute (IRRI). Being widely grown in southern China, HHZ shows wide adaptation, high-yielding potential, narrow and longer flag leaf (Zhou et al. 2016; Yu et al. 2020). The other parent, Pusa NPT 34 is an advanced breeding line, derived from new plant type (NPT) breeding, having high broader and shorter flag leaf.

Hybridization of the parents, PR126 and Pusa NPT34 was carried out during the *Kharif* season of the year 2017 at the Division of Genetics, ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi. The F_1 plant was grown during *Rabi* (Nov-April) season of 2017-18 at the Rice Breeding and Genetics Research Centre (RBGRC), IARI, Aduthurai, Tamil Nadu. After the hybridity test using RM8094, the selected F_1 plant was selfed and the subsequent generation was developed by single seed descent method. By *Kharif* 2020, 175 recombinant inbred lines (RILs) in the F_6 generation were raised along with parental lines and evaluated at three locations namely New Delhi, Karnal and Aduthurai. The recommended package of practice was followed in all the seasons at all the locations.

Multisite evaluation of recombinant inbred lines (RILs)

Three sites spread across India, namely, New Delhi (DEL), Karnal (KAR) and Aduthurai (ADT) were selected for the evaluation of RILs. These sites are part of the shuttle breeding chain of ICAR-IARI, which is exclusively used for rice crop improvement (Nagarajan et al. 2013) and represents diverse agroecology. The evaluation site includes research station, Division of Genetics, ICAR-IARI, New Delhi located at 28°38'N; 77°09' E; 220m (DEL), IARI-Regional Station, Karnal, Haryana, located at 29°42' N; 76°59' E; 249m (KAR) and Research farm, IARI-RBGRC, Aduthurai, Tamil Nadu located at 11°00'N; 79°28' E; 20m (ADT). The soil types were sandy loam at DEL and KAR, and alluvial clay at ADT. The experiment was conducted under irrigated transplanted ecosystems at all the sites, and the nursery was raised on elevated beds for 21 days and then transplanted into a puddled field with a spacing of 20 x 15 cm. The experiments followed a common design, augmented randomised complete block (ARCB) design with five checks viz., PR126, Pusa NPT34, Pusa Basmati 1509, Rasi and PKF_8-218. Each entry was transplanted in two rows, each having ten plants. Experimental layout and randomization were carried out using PBTools v1.4 (IRRI 2014). The genotypes were divided into eight blocks and checks were replicated in all the blocks.

Phenotyping

Five plants were randomly tagged from each family at physiological maturity, and data was recorded on the targeted traits. The traits observed were flag leaf length, and flag leaf width. The data on flag leaf area was derived from

above observations using the formula FLA = FLL x FLW x 0.75 (Palaniswamy and Gomez's, 1972). Three flag leaf of main tillers were selected from each plant to record the data on flag leaf size-related traits. Each family's mean of five plants is considered for further data analysis. Border plants were excluded to reduce the error.

Parental polymorphism and linkage map construction

Genomic DNA was isolated from each RIL using freshly collected leaves from the field. After DNA isolation, 1083 SSR markers were used for parental polymorphism and the amplicons were resolved using 3.5 % agarose gel electrophoresis (Archana et al. 2021). The resolved PCR products were classified as PR126 type (P1), Pusa NPT34 type (P2) and heterozygotes and scored as A, B, and H, respectively. All these steps were done manually in our laboratory. The final genotypic data was curated by excluding all monomorphic data and markers that showed segregation distortion. A linkage map was constructed using the software QTL IciMapping v4.2 (Meng et al. 2015) by multipoint analysis using the Kosambi mapping function (Kosambi 1944; Vinod 2011) and a likelihood of odds (LOD) value of 3.0 (Lander et al. 1987). The map distance was measured in centimorgans (cM).

Statistical testing and QTL mapping

The recorded observations on FLL, FLW and FLA were subjected to analysis of variance (ANOVA) individually as well as across sites. Best linear unbiased predictors (BLUPs) were generated using a restricted maximum likelihood (REML) approach using the *lme4* package integrated with the software PBTools v1.4 (IRRI 2014). The box plots were drawn in Microsoft excel 2019 and a histogram was constructed using the *rcompanion* package in R statistical environment. Correlation coefficients were calculated and graphically visualized using the *corrplot* package.

QTLs were mapped for all the traits using a one-dimensional composite interval mapping (Li et al. 2007; Wang 2009) strategy for additive traits implemented in QTL IciMapping v3.2. Threshold LOD values for each trait were determined at an alpha of 0.05 using a permutation test running 1000 iterations. The walk distance was set at 1cM distance with a probability of inclusion at 0.01 for forward stepwise regression. The genome-wide linkage map was graphically drawn using GGT v2.0 software (Van Berloo 1999).

Validation of identified quantitative traits loci

A validating population was developed to validate the QTLs by hybridizing Pusa Basmati 1509 with Pusa NPT34. Pusa Basmati 1509 is a Basmati cultivar with extralong slender grains with a narrow and long flag leaf when compared to Pusa NPT34. The F_1 was developed at DEL, during *kharif* of 2020. The F_1 seeds were harvested at physiological maturity and dried to optimum moisture content to avoid fungal infection and were grown during *Rabi* season of 2020-21 at IARI-RBGRC, Aduthurai, Tamil Nadu. The hybridity of the F_1 plants was checked using polymorphic SSR markers, such as RM190. True F_1s were selfed and from the harvested seeds of one F_1 plant, a total of 163 F_2 plants were raised along with the parental lines at ICAR-IARI, New Delhi during the *Kharif* season of 2021. The F_2 plants were selfed and harvested separately. The $F_{2:3}$ harvest from F_2 plants was used for a multisite evaluation conducted at two locations like, IARI-RBGRC, Aduthurai in Tamil Nadu and Mandya (MND) in Karnataka. The $F_{2:3}$ seeds of each F_2 plant were partitioned into two and used for raising evaluation trials at ADT and MND during the *rabi* of 2021-22.

The parental polymorphism was tested for the QTL linked SSR markers between PB1509 and Pusa NPT34. Polymorphic markers were checked for their segregation ratio using the *chi-square* test and those showing a distorted segregation were removed from the study. Phenotypic data from the multisite evaluation were tested for ANOVA and best linear unbiased predictors (BLUPs) were generated using the REML approach using the *lme4* package integrated with the software PBTools v1.4 (IRRI 2014). The genotypes were treated as random variables in the model. The F_2 genotypic data was regressed on the phenotypic BLUPs of $F_{2:3}$ and the corresponding individual F_2s to perform single marker analysis. The marker that showed significant variation among the parental classes for the target trait was considered validated for the corresponding linked QTL.

Results and discussion

Phenotypic evaluation of RILs

Flag leaf is one of the major parts of the plant for the synthesis of photoassimilates during the active reproductive stage and it decides the final yield of majority of the cereal crops (Borill et al. 2015). Hence FLL, FLW and FLA are the major factors in determining the leaf morphology and desirable plant type (Fan et al. 2015). Therefore, it is very important to study the natural variation for flag leaf and identify the genomic region for flag leaf size. The phenotypic evaluation of parental lines of the recombinant inbred lines (RILs) showed that the parents were completely contrasting for FLL, FLW and FLA. The PR126 exhibited an average FLL of 37.83 cm, FLW of 1.53 cm and FLA of 40.69 cm^2. Another parent, Pusa NPT34 showed an average FLL of 26.88 cm, FLW of 2.28 cm and FLA of 43.55 cm^2. The mapping population developed from the contrasting parents for the traits under study is good for genetic study and mapping QTLs (Khan 2015). The RILs developed from these parents were evaluated at three locations. The site-wise analysis of variance (ANOVA) showed that the variation for FLL, FLW and FLA was highly significant (p-value <0.01) across the sites (Supplementary

Table 1. Descriptive statistics of PR126, Pusa NPT34 and RIL population across sites during *Kharif* 2020

Parameter	DEL			KAR			ADT		
	FLL	FLW	FLA	FLL	FLW	FLA	FLL	FLW	FLA
PR126*	38.45	1.52	41.46	37.54	1.57	41.99	36.16	1.50	38.61
Pusa NPT34*	28.20	2.40	48.11	26.57	2.23	42.16	25.86	2.20	40.38
Mean ± SE	38.14 ± 0.37	2.17 ± 0.02	58.99 ± 0.93	38.01 ± 0.42	2.27 ± 0.02	61.57 ± 1.04	36.27 ± 0.38	2.24 ± 0.02	57.68 ± 0.86
SD	4.91	0.3	12.42	5.66	0.3	13.92	5.12	0.31	11.53
Min	23.78	1.51	22.74	24.01	1.53	29.92	22.04	1.54	25.05
Max	56.55	2.74	89.34	53.58	3.14	100.51	49.76	2.91	86.27
Genetic variability parameter									
	FLL	FLW	FLA	FLL	FLW	FLA	FLL	FLW	FLA
PV	22.06	0.08	136.79	28.75	0.07	169.42	25.77	0.07	109.28
GV	18.52	0.05	116.74	24.75	0.05	157.98	19.03	0.04	87.87
EV	3.54	0.02	20.05	4.01	0.02	11.45	6.74	0.03	21.4
GCV	11.28	10.76	18.32	13.09	10.29	20.41	12.03	8.72	16.25
PCV	12.32	12.9	19.83	14.11	11.81	21.14	14	11.53	18.12
ECV	4.94	7.12	7.59	5.27	5.79	5.5	7.16	7.54	8.02
hBS	83.94	69.53	85.34	86.07	75.95	93.24	73.85	57.24	80.41
GA	8.13	0.4	20.59	9.52	0.42	25.04	7.73	0.3	17.34

Table 1). The phenotypic variation for flag leaf size present among the RILs is shown in Fig. 1. The pooled analysis of variance (ANOVA) showed that the effect of environment on the FLL, FLW, FLA was significant and the GxE interaction (GEI) was found significant for all the flag leaf related traits (Supplementary Table 2). The variation in the RILs for flag leaf size related traits is beacuse of segregation and fixation of the dispersed genomic regions from both parents.

The frequency distribution curve and box plots for FLL, FLW and FLA showed normal distribution across all the locations (Fig. 2 & Supplementary Fig. 1). The distribution curve and descriptive statistics of the RILs across the location showed the presence of extreme values for all the traits across the sites. Many of the previous reports showed that flag leaf size is a quantitative trait, shows continuous variation and is controlled by polygenes (Kobayashi et al. 2003; Bing et al. 2006; Bian et al. 2014). The presence of extreme phenotypes beyond the parental range indicates the presence of transgressive segregants for flag leaf traits. The recovery of the transgressive segregants indicates the gene dispersion among the parent for FLL, FLW and FLA (Mackay et al. 2021). The phenotypic evaluation of RILs across the sites showed that at DEL the minimum and maximum FLL, FLW and FLA was 23.78 to 56.55 cm, 1.51 to 2.74 cm and 22.74 to 89.34 cm^2, respectively. Similarly, at KAR 24.01 to 53.58 cm, 1.53 to 3.14 cm, and 29.92 to 100.51 $cm^{2,}$ whereas at ADT it was found to be 22.04 to 49.76 cm, 1.54 to 2.91 cm, and 25.05 to 86.07cm^2. The mean performance for FLL was

Fig. 1. Phenotypic variation for flag leaf length (FLL), flag leaf width (FLW) and flag leaf area (FLA) among the RILs and parents during kharif 2020 at ICAR-IARI

higher at DEL and KAR. For FLW highest mean performance was seen in KAR and ADT. Similarly, for FLA highest mean performance was recorded at KAR (Table 1). It indicates that DEL and KAR region supports the expression of the flag leaf traits better than ADT and confirms the presence of GxE interactions (Wu et al. 2019). Genetic variability analysis showed that the phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV%) for FLL was high in KAR, for FLW in DEL and for FLA in KAR. The broad sense heritability (h^2bs) was high for FLA (86.33%) followed by FLL (81.29%) and FLW (67.60%) across sites (Table 1). The presence of high hBS indicates that most of the variations

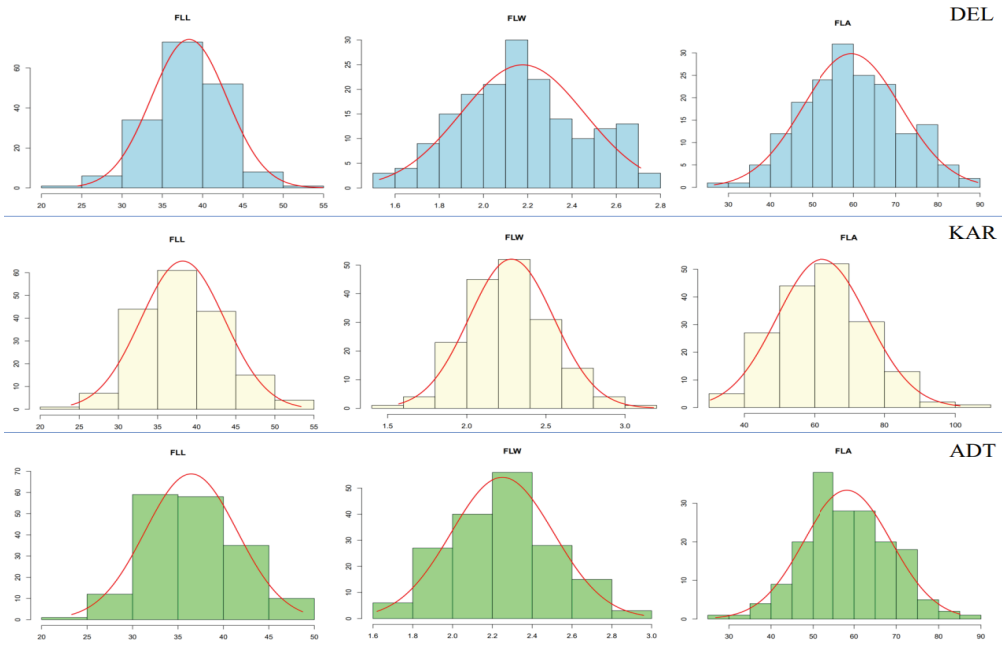

Fig. 2. Phenotypic frequency distribution pattern of Flag leaf length (FLL), Flag leaf width (FLW) and Flag leaf area (FLA) in RIL population across the sites during *kharif* 2020

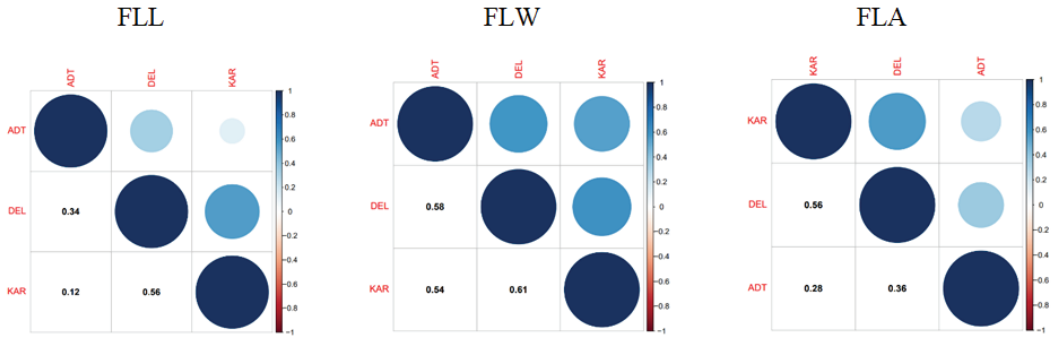

Fig. 3. Correlation coefficient between the sites for Flag leaf length (FLL), Flag leaf width (FLW) and Flag leaf area (FLA) using combined BLUP of *kharif* 2020

present among the RILs were genetically controlled and that selection among the families will be more effective (Bassuony and Zsembeli, 2021). The correlation analysis between the sites showed that DEL and KAR have more correlation for FLL, FLW and FLA followed by DEL and ADT. Low correlation was observed between KAR and ADT for all the traits (Fig. 3). This explains the comparable performance of the RILs for flag leaf traits in DEL and KAR than the ADT.

Linkage map construction

The parental polymorphism survey was carried out using 1083 SSR markers with an average density of 90.25 markers per chromosome. A total of 126 markers have been found to be polymorphic, indicating an 11.63% genomic diversity. Among the polymorphic markers, 23 showed distortion after being tested for segregation patterns, and those were eliminated, leaving a final set of 103 markers that could be used for map construction, which accounted for 9.51% of the original set. The parents showed a low level of marker diversity, indicating a great deal of genomic similarity between the parental lines. The most apparent commonality between PR126 and Pusa NPT34 was their *indica* background, although a pedigree-level comparison was not possible. Moreover, the percentage of marker outturn (9.5%) for map construction was well comparable with SNP systems, which stood at a range of 3.0 to 10% of the total markers. The availability of uniformly distributed markers throughout the genome is one of the important factors for linkage map construction and mapping (Bernardo. 2013). The polymorphic markers graphical genotype revealed a uniform distribution of the markers across the 12 chromosomes, which is depicted in (Supplementary Fig. 2). Chromosome 11 showed the highest

Table 2. Chromosome-wise number of the markers used for the construction of the linkage map

Chromosome	Markers used for polymorphism survey	Markers	Polymorphism (%)	Length (in cM)	Marker density (cM)
1	105	8	7.62	177.95	22.24
2	93	10	10.75	215.34	21.53
3	89	10	11.23	212.66	21.27
4	99	10	10.10	229.76	22.98
5	101	12	11.88	222.59	18.55
6	78	7	8.97	230.41	32.92
7	81	5	6.17	63.75	12.75
8	87	7	8.05	224.01	32.00
9	113	6	5.31	234.57	39.10
10	77	5	6.49	219.01	43.80
11	93	14	15.05	222.59	15.90
12	67	9	13.43	162.43	18.05
Total	1083	103		2415.07	

number of polymorphic markers, while chromosomes 7 and 10 exhibited the lowest (Table 2).

QTL mapping

The agronomic data were subjected to analysis of variance (ANOVA) individually as well across sites. Best linear unbiased predictors (BLUPs) were generated and environment-wise BLUPs were used for QTL mapping for all the traits. A total 17 QTLs were identified for flag leaf size-related traits (Table 3).

Flag leaf length (FLL)

QTL analysis using ICIM approach able to identify seven site-specific QTLs for FLL, namely, *qFLL2.1, qFLL2.2, qFLL3.1, qFLL4.1*, *qFLL5.1, qFLL6.1*, and *qFLL6.2* (Fig. 4 and Table 3). One Major site-specific QTL, *qFLL5.1*, was mapped between RM146 and RM164 on chromosome 5 with LOD value of 5.96. The PVE was found to be 10.50% with an additive effect of 1.32, indicating that the positive allele was contributed from PR126. The marker interval was previously reported for possessing QTLs for flag leaf width (*qflw5*), and flag leaf size traits (Jiang et al. 2004). The remaining 6 were site-specific minor QTLs of which *qFLL6.1*, and *qFLL6.2* were mapped in the adjacent marker interval on chromosome 6. The *qFLL6.1,* was bracketed between RM190 and RM204 with LOD value of 3.04 and PVE of 7.07, whereas other QTL, *qFLL6.2*, was bracketed between RM204 and RGNMS2221 with LOD value of 3.20 and PVE of 8.75%. The additive effect of the former was -1.08, and later, it was -1.76, which indicates that the positive allele was contributed from Pusa NPT34. The QTLs *qFLL6.1* and *qFLL6.2* were co-localized with the earlier report for leaf length and width (Yan et al. 2003; Mei et al. 2005). The genomic region is also a site for yield and many yield-attributing traits (Zhuang et al. 2002). A genomic region flanked between the markers HvSSR02-59 and RM13672 possessed a minor QTL, *qFLL2.1* on chromosome 2. The QTL had the LOD values of 3.26 and PVE of 5.46 % at Delhi with an additive effect of 0.95. Another QTL, *qFLL2.2*, was mapped on the same chromosome bracketed between RM6 and RM207. The LOD value was 2.99 and PVE of 5.05% with an additive effect of 0.91. Both the QTL mapped on chromosome 2 had positive sign for additive effect, indicating that a positive allele was contributed from PR126. The QTL *qFLL3.1*, mapped between RM168 and RM520 on chromosome 3, showed a LOD of 4.3 and PVE of 9.73% with an additive effect of -1.26. Similarly, *qFLL4.1*, was mapped between HvSSR04-19 and RM5709, which had a LOD of 3.67. The PVE was 6.39 with an additive effect of -1.03. Both the QTLs had a negative sign for additive effect, indicating that the positive allele was contributed from Pusa NPT34. Many QTLs associated with the flag leaf size have been identified and fine mapped namely, *qfll1* (Yan et al. 1999), *qFLLnpt-2*(Farooq et al. 2010), qFLW7.2 (Zhang et al. 2015). Though no novel QTLs are identified for flag leaf length, the mapped QTLs in the present study co-localised with previously reported QTLs for flag leaf development, thus validating the earlier QTLs.

Flag leaf width (FLW)

QTL mapping for FLW detected five QTLs namely, *qFLW2.1, qFLW3.1, qFLW4.1, qFLW5.1,* and *qFLW6.1,* on chromosomes 2, 3, 4, 5, and 6, respectively (Fig. 4 and Table 3). Among the five QTLs detected, two were major and three were minor QTLs. The QTL, *qFLW4.1*, was consistent across the sites and mapped between RM335 and nksssr04-11. The QTL showed LOD values ranged between 3.81 to 8.16 and PVE of 9.85% at Delhi, 13.02% at Karnal and 7.21% at Aduthurai with an additive effect of 0.074, 0.068 and 0.036, respectively. The QTL had positive sign for additive effect across the sites, indicating positive allele contribution from PR126.

These stable QTLs provide useful information for genetic improvement of flag leaf morphological traits in rice through QTL pyramiding (Swamy and Sarla. 2008; Dhawan et al. 2021). Another major QTL was mapped on chromosome 6 bracketed between RM204 and RGNMS2221 at DEL and ADT. The LOD values were 2.95 and 8.55, with PVEs of 9.48 and 16.43% at ADT and DEL, respectively. The additive effect of the QTL was found -0.10 at DEL and -0.041 at ADT, indicating positive allele contribution from Pusa NPT34. This marker interval also possessed QTL for FLL (*qFLL6.2*) and the adjacent marker interval also possessed QTL for FLL (*qFLL6.1*). The marker interval possessing *qFLW4.1*, is also reported to harbors QTLs for flag leaf length and width (Xu et al. 2009; Roja et al. 2016). It indicates the present marker interval rich in QTLs that are important for leaf development. The remaining three QTLs were site-specific minor QTLs. The remaining minor site-specific QTL, *qFLW2.1, qFLW3.1* and *qFLW5.1* were mapped between RM13672-RM6, RM168-RM520 and HvSSR05-21-RM146, respectively. The LOD value of the QTLs ranged from 3.12 to 4.41 with PVE of 4.56 to 5.48%. The additive effect of all the QTL had a negative sign, indicating the positive allele contribution from Pusa NPT34. No novel QTLs were detected for FLW in the current study.

Flag leaf area (FLA)

Five site-specific QTLs were mapped for FLA, namely, *qFLA3.1*, *qFLA4.1*, *qFLA4.2, qFLA6.1*, and *qFLA6.2* on chromosomes 3, 4, 4, 6, and 6, respectively (Fig. 4 & Table 3). Among five QTLs, 4 QTLs were major QTL having PVE of more than 10%. Two QTLs, *qFLA6.1* and *qFLA6.2*, were mapped at adjacent marker intervals RM190-RM204 and RM204-RGNMS2221 on chromosome 6. The former QTL had LOD value of 7.64 with PVE of 15.27 and later had LOD value of 4.52 with PVE of 14.99%. Both QTLs had negative additive effects, indicating the positive allele contribution from 'Pusa NPT34'. Both the marker intervals were also possessed QTLs for FLL (*qFLL6.1*, *qFLL6.2*) and FLW (*qFLL6.1, qFLL6.2*). As this marker interval possessed QTLs for FLL, FLW and FLA, and the positive allele contribution is from the 'Pusa NPT34', this genomic region provides an added advantage to the breeder for transferring the genes/QTLs. Similarly, two QTLs, *qFLA4.1* and *qFLA4.2*, were mapped on chromosome 4 between HvSSR04-19-RM5709 and nksssr04-11-RM567 respectively. The QTLs had LOD value of 6.76 and 4.58 with PVE of 10.74 % and 7.76 % respectively. The additive effect of *qFLA4.1*, indicates that

Table 3. QTLs mapped for FLL, FLW and FLA in the RIL population of PR126/Pusa NPT34

QTL name	Trait name	Chromosome	Position	Left marker	Right Marker	LOD	PVE (%)	Add
qFLL2.1	FLL_DEL	2	114	HvSSR02-59	RM13672	3.26	5.46	0.95
qFLL2.2	FLL_DEL	2	166	RM6	RM207	2.99	5.05	0.91
qFLL3.1	FLL_DEL	3	130	RM168	RM520	4.30	9.73	-1.27
qFLL4.1	FLL_DEL	4	2	HvSSR04-19	RM5709	3.67	6.39	-1.03
*qFLL5.1**	FLL_DEL	5	114	RM146	RM164	5.96	10.50	1.32
qFLL6.1	FLL_DEL	6	148	RM190	RM204	3.04	7.07	-1.08
qFLL6.2	FLL_KAR	6	167	RM204	RGNMS2221	3.20	8.75	-1.76
qFLW2.1	FLW_DEL	2	145	RM13672	RM6	4.05	5.48	-0.06
qFLW3.1	FLW_DEL	3	131	RM168	RM520	3.12	4.56	-0.05
*qFLW4.1**	FLW_DEL	4	174	RM335	nksssr04-11	8.16	9.85	0.074
	FLW_KAR	4	174	RM335	nksssr04-11	5.30	13.02	0.068
	FLW_ADT	4	175	RM335	nksssr04-11	3.81	7.21	0.036
qFLW5.1	FLW_DEL	5	98	HvSSR05-21	RM146	4.41	5.18	-0.0536
*qFLW6.1**	FLW_DEL	6	163	RM204	RGNMS2221	8.55	16.43	-0.10
	FLW_ADT	6	171	RM204	RGNMS2221	2.95	9.48	-0.041
*qFLA3.1**	FLA_DEL	3	130	RM168	RM520	5.18	10.39	-3.63
*qFLA4.1**	FLA_DEL	4	2	HvSSR04-19	RM5709	6.76	10.74	-3.69
qFLA4.2	FLA_KAR	4	176	nksssr04-11	RM567	4.58	7.76	3.91
*qFLA6.1**	FLA_DEL	6	151	RM190	RM204	7.64	15.27	-4.3942
*qFLA6.2**	FLA_KAR	6	171	RM204	RGNMS2221	4.52	14.99	-5.422

LOD, Logarithm of the odds; RM, Rice microsatellite; HvSSR, highly variable simple sequence repeats; *, Major QTLs.

Table 4. QTL hotspots identified in PR126 x Pusa NPT34 derived RILs during *kharif* 2020

Clusters	Chromosome	Marker interval	Interval length (Mb)	Number of QTLs	Name of the QTLs
I	3	RM168-RM520	2.82	3	*qFLL3.1*, *qFLW3.1* and *qFLA3.1*
II	6	RM190-RGNMS2221	3.47	5	*qFLL6.1*, *qFLL6.2*, *qFLW6.1*, *qFLA6.1*, and *qFLA6.2*

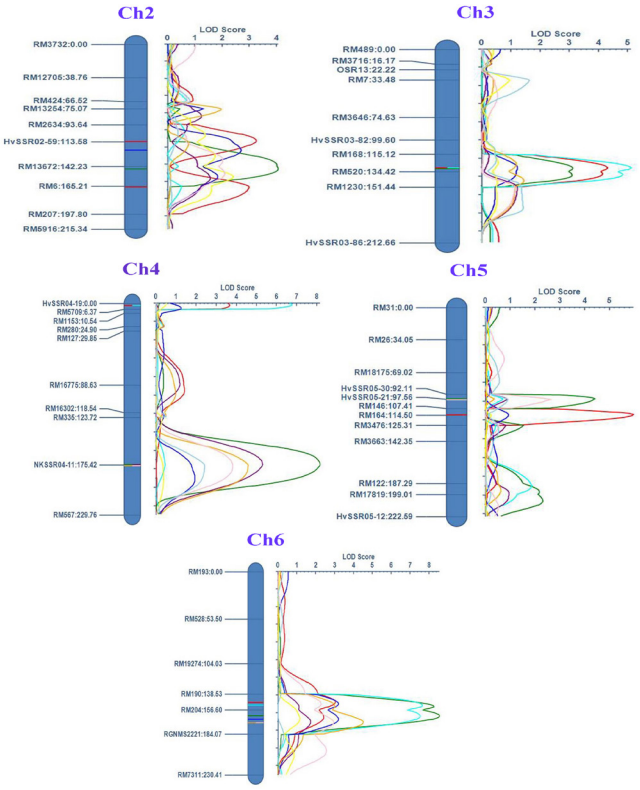

Fig. 4. Map positions of QTLs mapped for Flag leaf length (FLL), Flag leaf width (FLW) and Flag leaf area (FLA) in the 'PR126'/'Pusa NPT34' population across all three sites during *Kharif* season of 2020

Table 5. Validation of QTLs for FLW and FLA in F2:3

QTL	Site	Trait	Marker	Chromosome	R2 (%)	Additive effect
qFLW6.1	ADT	FLW	RM190	6	5.15	0.05
qFLA6.1	ADT	FLA	RM190	6	6.18	2.3

the positive allele contribution from 'Pusa NPT34' whereas *qFLA4.2* was contributed from 'PR126'. Another minor QTL was mapped between RM168 and RM520 with a LOD value of 5.18 and a PVE of 10.39 %. The additive effect was -3.63 and negative sign of the additive effect indicated the positive allele contribution from 'Pusa NPT34'. Flag leaf in cereal crop contribute 45-58% photosynthesis (Liu et al. 2018). Previous studies have identified many QTLs for FLA namely, *qFLWR10*, *qFLA.acs–1B*, *qFLL10* (Zhou et al. 2012, Yang et al. 2016, Zhang et al. 2015). But in the current study no novel QTLs were identified for FLA. Out of five QTLs identified for FLA, one was contributed from PR126 (*qFLA4.2*) and remaining from Pusa NPT34, this confirms that the genes for FLA is dispersed between the parents and this is the reason for the appearance of extreme phenotypes higher and lower than the parental value for FLA. Similarly, in another study, many chromosome segment substitution lines (CSSLs) having lower and higher value than the parents for the leaf morphology was observed (Bian et al. 2014).

Co-localization of QTLs for flag leaf size-related traits

Among 17 QTLs mapped, 8 QTLs were co-localised on two genomic regions (Table 4). A QTL cluster on chromosome 3 contains three QTL, *qFLL3.1*, *qFLW3.1*, and *qFLA3.1*. The QTL hotspot is located at the marker between RM168 and RM520 with an interval length of 2.82 Mb. Similarly, the second QTL hotspot contains five QTLs, *qFLL6.1*, *qFLL6.2*, *qFLW6.1*, *qFLA6.1*, and*qFLA6.2* located on chromosome 6 having a span of 3.47 Mb between RM190 and RGNMS2221. The QTL cluster on chromosome 6 carries QTLs for FLL, FLW and FLA. The conglomeration of QTLs for various flag leaf size-related traits at different hotspot regions indicated that specific regions are particularly rich in genes that are important for the expression of flag leaf developmental traits. This provides an added advantage to the breeder in transferring the haplotypes carrying the hotspot region for flag leaf-related traits. Among the two co-localised regions, the highest number of QTLs (five) were co-mapped on the short arm of chromosome 6 within a 3.47 Mb region between markers RM190 and RGNMS2221. It was further interesting to observe that all the desirable trait-contributing QTLs for FLL, FLW and FLA came from Pusa NPT34. This eventually indicates that the hotspot on chromosome 6 sponsors a longer and wider flag leaf with a higher flag leaf area. Among the five QTLs, *qFLW6.1*, was a consistent major QTL and the QTL hotspot could be an important genomic region for further studies.

Validation of qFLW6.1 and qFLA6.1

An early segregating population from the cross, Pusa Basmati 1509 / Pusa NPT34 was used for QTL validation, because Pusa Basmati 1509 provided an extreme contrast with PusaNPT34 for FLL and FLW. The validation population was developed to understand the QTL effect in the background of Pusa Basmati 1509, and the simultaneous introgression of QTL in Pusa Basmati 1509 through markers-assisted selection. The flanking markers of the consistent QTLs for FLL, FLW and FLA was used for the parental polymorphism survey, followed

by genotyping of the F_2 population using polymorphic markers. RM190 validated for *qFLW6.1*, *qFLA6.1* at ADT. At ADT, the genotypes that carry the homozygous allele of PB1509 type (RM190) showed an average FLW of 1.89cm, whereas genotypes with homozygous Pusa NPT34 allele showed 1.98cm. The R^2 (%) and the additive effect was 5.15 and 0.05, respectively. Similarly, RM190 also validated for *qFLA6.1* at ADT. The genotypes that carry the homozygous allele of PB1509 type (RM190) showed an average FLA 49.88 cm^2 whereas, genotypes that carry the homozygous Pusa NPT34 allele showed 54.42 cm^2. The R^2 (%) and the additive effect was 6.18 and 2.3, respectively. In the current study, we were able to validate two QTLs, *qFLW6.1* and *qFLA6.1* among the $F_{2:3}$ lines of the cross, PB 1509/ Pusa NPT34 using flanking markers identified in QTL mapping (Table 5). Several rice genes/QTLs regulating flag leaf size were identified and validated (Wang et al. 2011; Zhang et al. 2015; Tang et al. 2018; Du et al. 2022), determining length, width, and area. Notably, *qFLW6.1* and *qFLA6.1*, enhancing flag leaf width and area, reside in the same hotspot on chromosome 6 from 'Pusa NPT34'. QTL hotspots, gathering multiple traits, offer insights into gene expression, interaction, and pleiotropy (Wu et al. 2021), making them valuable for fine mapping and breeding for superior flag leaf varieties.

Supplementary material

The Supplementary Tables S1 and S2 are provided, www.isgpb.org

Authors' contribution

Conceptualization of research (PKB, GKS, SKV, AKS); Designing of the experiments (PKB, NS); Contribution of experimental materials (NS, PKB, VJS); Execution of field/lab experiments and data collection (NS, S, SR, BKD, VJS, MN); Analysis of data and interpretation (NS, PKB, KKV, RKE, HB); Preparation of the manuscript (NS, PKB, KKV, VJS).

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Supplementary Table S1. Site wise analysis of variance (ANOVA)

Sites	Source	df	FLA	FLL	FLW
DEL	Check	4	414.05**	144.76**	2.09**
	Genotype vs. Check	1	10577.78**	1342.52**	2.99**
	Genotype	174	136.79**	22.06**	0.08**
	Block	7	24.43	3.52	0.02
	Residuals	28	20.05	3.54	0.02
	CV (%)		7.98	5.07	7.28
	CD		14.21	5.97	0.49
KAR	Check	4	358.32**	148.66**	1.63**
	Genotype vs. Check	1	17907.88**	1830.8**	7.24**
	Genotype	174	169.42**	28.75**	0.07**
	Block	7	58.4**	7.5	0.05*
	Residuals	28	11.45	4.01	0.02
	CV (%)		5.85	5.44	5.99
	CD		10.74	6.35	0.42
ADT	Check	4	264.44**	135.38**	1.48**
	Genotype vs. Check	1	15545.51**	1450.52**	7.89**
	Genotype	174	109.28**	25.77**	0.07**
	Block	7	47.77	3.85	0.07*
	Residuals	28	21.4	6.74	0.03
	CV (%)		8.53	7.37	7.81
	CD		14.68	8.24	0.54

**p≤ 0.01 and *p≤ 0.05

Supplementary Table S2. Pooled analysis of variance for flag leaf size related traits in RILs

Groups	FLL	FLW	FLA
Geno	10.36**	0.05**	71.73**
Env	0.95**	0.01*	3.01*
Geno x Env	11.02**	0.01**	55.8**
Residual	4.79	0.02	20.42