Natural variation reveals that *OsFLA2* controls flag leaf angle in rice (*Oryza sativa* L.)

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Abstract

Flag leaf angle (FLA) is an important outcrossing trait affecting the hybrid seed production in rice (*Oryza sativa* L.). Natural variation of FLA has been reported in

rice, but the molecular basis for this variation is largely unknown. Here we investigated the phenotypic values of FLA in 353 rice natural accessions in six environments, which indicated rich phenotypic variation. We performed a genome-wide association study on FLA using 1.3 million single nucleotide polymorphism (SNPs). A total of 37 SNPs were identified significantly associated with FLA, of which 27 were located in previously reported QTLs/Genes and 10 were novel. We identified two causal gene loci for FLA, *OsFLA6* and *OsFLA2*; *OsFLA6* was co-localized with the gene *OsLIC*. In addition, the accessions with large and small FLA values have corresponding high and low *OsFLA6* expression. We also confirmed that the allele $OsFLA2^{TT}$ increased the FLA compared with that of the isogenic line carrying allele $OsSYL2^{CC}$ by transgenic complementation experiment. The allele frequencies of $OsFLA6^{GG}$ *and* $OsFLA2^{TT}$ decreased gradually with an increase in latitude in the Northern Hemisphere. Our results should facilitate the improvement of FLA of parents of hybrid rice.

Keywords: Flag leaf angle; Genome-wide association mapping; Heterosis utilization; Hybrid seed production; Natural variation; Rice.

1 Introduction

Asian cultivated rice (*Oryza sativa* L.) is one of the most important staple foods feeding more than 3.5 billion people worldwide. With the human population increasing and the arable land decreasing, increasing the rice grain yield per unit area per unit time is an inevitable choice. The utilization of heterosis is one of the effective strategies to enhance rice grain yield. However, it entails the production of F_1 hybrid seeds yearly. For hybrid seed production in *O. sativa*, to remove the barriers to cross-pollination at the initial heading stage, farmers usually cut off one-third or one-half of the flag leaf blade of the parents, which can not only require more labors but also high

operating skills to avoid injuring the young panicle. In addition, the wound caused by leaf clipping also had adverse effect on the normal growth of rice plant. If the flag leaf angle (FLA) in female parents is larger than 90°, leaf clipping can be omitted. Therefore, breeding a male sterile line with larger FLA can not only omits the procedure of flag leaf clipping but also facilitates the mechanization of hybrid rice seed production.

Early research has shown that the FLA trait is controlled by a pair of major genes and a number of minor gene pairs, with a small angle being partially dominant (Shen, 1983). To date, 163 quantitative trait loci (QTLs) for FLA have been identified, including 24, 20, 16, 16, 10, 11, 11, 18, 12, 6, 11 and 8 on chromosomes 1 to 12, respectively (Table S1) (Li *et al*., 1999; Yan *et al*., 1999; Dong *et al*., 2003; Kobayashi *et al*., 2003; Luo *et al*., 2008; Zhang *et al*., 2008; Cai, 2009; Huang *et al*., 2010; Chen *et al*., 2012; Hu *et al*., 2012; Wang *et al*., 2012; Zhang *et al*., 2013; Bian *et al*., 2014; Zou *et al*., 2014; Lu *et al*., 2015; Zhu *et al*., 2016; Dong *et al*., 2018a, b; Ham *et al*., 2019). Among these QTLs, only one QTL *qFla-8-2* has been fine-mapped and predicted the candidate genes (Zhu *et al*., 2016). Several genes for leaf angle (refers to all leaves growing on a stem), such as *lla*, *OsLIC, ILA1* and *OsARF19*, have been cloned (Wang *et al*., 2005, 2008; Ning *et al*., 2011; Zhang *et al*., 2015), but no cloned gene responsible for FLA has been reported thus far. Therefore, it is necessary to discover favorable alleles for FLA to enhance the yield of hybrid seed production in rice.

In this study, we performed a genome-wide association analysis (GWAS) by combining the FLA of 353 rice accessions on six environments with single nucleotide polymorphism (SNP) data and identified significant SNP loci. Further, we identified a novel causative gene *OsFLA2* for FLA by the gene-based association method. The function of $OsFLA2^{TT}$ was validated by transgenic complementation test. These results filled a gap of gene cloning and functional analysis of FLA characteristics. This study sets the stage for further improvement of FLA of the parents of hybrid rice.

2 Results

2.1 Phenotypic statistics of FLA in natural rice accessions

The phenotypic value of FLA was investigated in the 353 rice accessions containing *indica* and *japonica* subspecies across six environments. The distributions of average value over the six environments for FLA in *indica* and *japonica* subspecies were shown in Figure 1a. Compared with *indica* rice, the *japonica* rice population had lower values for FLA (Figure 1a). In the 353 accessions, the mean value of FLA was calculated per environment, ranging from 35.87 ± 20.66° to 38.49 ± 21.38°, with coefficients of variation (CV) across the six environments from 55.55% to 58.48% (Figure 1b, c). These results showed that there existed the abundant phenotype variation in the population studied. Based on the results of joint analysis of variance for FLA, we found that there were significant differences among genotypes, no significant differences among the environments, and significant differences for the

interactions of genotypes with environments (Table S2). These results indicated that although the environment had effect on FLA, the abundant phenotypic variation of FLA was mainly attributable to variation in genotype.

2.2 Genome-wide association mapping for FLA

Based on the mixed linear model with correction of kinship bias, GWAS on FLA trait of 353 accessions with high-quality SNPs (MAF > 0.05) were carried out. We detected a total of 37 significantly associated SNP loci in the 28 LD regions (Table 1). These SNP loci located on chromosomes 1, 2 and 4–10 and explained the total phenotypic variation approximately from 1.50% to 9.27%, which were repeatedly detected in at least four environments, indicating that the SNPs linked with FLA were more stable (Figure S1, Table 1 and Table S3). Eighteen of these SNP loci were detected across six environments and five loci were detected in five environments (Figure S1, Table 1 and Table S3). Next, we further analyzed the major SNP loci relevant to FLA with a significant peak, present in chromosome 6 and 2, respectively.

2.3 Allele *OsFLA6*^{GG} increase FLA

For the association signal in the 30.70-30.92 Mb region on chromosome 6, there were 31 genes for FLA identified (Figure 2a, b). Although 18 genes contain nonsynonymous SNPs, none of these SNPs was found to have a significant association with FLA in GWAS or gene-based association (GBA) (Table S4). Thus, we applied the method reported by Yano *et al*. (2016) and Fang *et al.* (2017) to use the nearby LD block (29.63-30.14 Mb) for further analysis considering allelic heterogeneity. In this region, 42 of 87 genes contain nonsynonymous SNPs (Table S5 and Table S6). Only one nonsynonymous SNP was found to be significantly associated with FLA ($-\log_{10}P \geq 7.0$); it was located within the gene locus *Os06g0704300*. Hereafter, gene *Os06g0704300* is referred to as *OsFLA6*. The full length of *OsFLA6* is 3620 bp, including 11 exons and 10 introns. *OsFLA6* was classified into two haplotypes based on three missense SNPs in the coding region (Figure 2c). Among them, the SNP locus (29,739,644) was significantly associated with FLA (Table 1 and Table S5), which causes a base change from base A to base G at nucleotide (nt) 328 in the coding sequence, resulting in an amino acid change from threonine (T) to alanine (A) at amino acid 110. The average FLA value of 116 accessions carrying the allele *OsFLA6*^{AA} were 26.5 ± 11.8°. The average FLA value of 232 accessions carrying the allele *OsFLA6*^{GG} were 37.5 ± 12.3°. The differences in FLA value between allele *OsFLA6*^{AA} and *OsFLA6*^{GG} was highly significant (Welch's *t*-test; *P*=6.06E-04) (Figure 2d).

Further, we performed qRT-PCR analysis of flag leaf and flag leaf lamina joint at differentiation stage 6, 7, and 8, respectively, sampling from three accessions (A7444, Shenlenuo and Ludao) with large FLA and three accessions (Nipponbare, Kendao 13 and Chenwan 3hao) with small FLA. The results showed that the expression of *OsFLA6*^{AA} was higher than that of *OsFLA6*^{GG} in flag leaf and flag leaf lamina joint at differentiation stage 7, but no significant difference was found at stages 6 and 8

(Figure 2e). We also found that the expression of $OsFLA6^{AA}$ in each of the three accessions with larger FLA was significantly higher than that of $OsFLA6^{GG}$ in each of the three accessions with smaller FLA (Figure 2e). Based on the website of China Rice Data Center (http://www.ricedata.cn/gene/list/286.htm), we found that the gene locus *Os06g0704300* was identical to *OsLIC* (*Oryza sativa* leaf and tiller angle increased controller), which encodes a CCCH-type zinc finger protein and regulates leaf angle and tiller angle through the BR signaling pathway (Wang *et al*., 2008; Zhang *et al*., 2012). Research on the function of *LIC* has been reported and we will no longer study the function of $OsFLA6^{GG}$.

2.4 Introduction of the allele $OsFLA2^{TT}$ increases FLA

For the association signal in the 2.16-2.50 Mb region on chromosome 2, there were 18 genes for FLA identified (Figure 3a, b). Based on SNP information, 16 of the 18 genes contain nonsynonymous SNPs (Table S7 and Table S8). However, only one nonsynonymous SNP was significantly associated with FLA ($-\log_{10}P \geq 7.0$); it was located within the gene locus *Os02g0142875*. Hereafter, gene *Os02g0142875* is referred to as *OsFLA2*. The full length of *OsFLA2* is 2177 bp, including three exons and two introns. Gene *OsFLA2* encodes a 95 amino acid protein. For *OsFLA2,* there was no putative conserved domains detected. *OsFLA2* was classified into two haplotypes based on six SNPs in its cDNA containing one intron and five exon SNPs (Figure 3c). For the five exon SNPs, one SNP site (2,372,278 bp) was synonymous and four was nonsynonymous. Among the four nonsynonymous SNPs, the SNP site (2,372,437) was significantly associated with FLA (Table 1 and Table S7). The SNP site (2,372,437) causes a base change from base C to base T at nt 137 in the cDNA sequence, which results in an amino acid change from serine (S) to phenylalanine (F) at amino acid 46. The average FLA values of 286 accessions carrying the allele $OsFLA2^{CC}$ was 28.5 ± 10.5°. The average FLA values of 24 accessions carrying the allele $OsFLA2^{TT}$ was 51.5 ± 13.1°. The difference in FLA values between the $OsFLA2^{CC}$ and $OsFLA2^{TT}$ genotypes was highly significant (Welch's *t*-test; *P*=4.88E-17904) (Figure 3d).

The qRT-PCR results showed that there were expression for $OsFLA2^{TT}$ and $OsFLA2^{CC}$ in flag leaf and flag leaf lamina joint at differentiation stages 6, 7 and 8 (Figure 3e). The expression of $OsFLA2^{TT}$ was was higher than that of $OsFLA2^{CC}$ in flag leaf and flag leaf lamina joint at differentiation stage 7, but no significant difference was found at stages 6 and 8 (Figure 3e). We also found that the expression of $OsFLA2^{TT}$ in each of the three accessions (A7444, Shenlenuo and Ludao) with large FLA was significantly higher than that of $OsFLA2^{CC}$ in each of the three accessions (Nipponbare, Kendao 13 and Chenwan 3hao) with small FLA (Figure 3e). These results suggested that enhanced expression of $OsFLA2^{TT}$ might increase FLA.

According to the results of GWAS, no SNP loci located in the promoter region of *OsFLA2* were associated with FLA. Based on the website of promoter functional elements

(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/#opennewwindow), we found that there were no SNP loci in the cis-element regulatory region. So, we speculated that phenotypic variation between the accessions with the TT allele and those with the CC allele was caused by SNP loci in the coding sequence region. Next, we conducted transformation of *OsFLA2* gene to confirm it.

The genome sequence of the allele $OsFLA2^{TT}$ and empty vector were introducted into Nipponbare, respectively. Compared with the plants of Nipponbare genome, plants transformed with the allele $OsFLA2^{TT}$ had a larger FLA, whereas those transformed with the empty vector showed no phenotypic change (Figure 3f, g). These results showed that *OsFLA2* was the causal gene for FLA on chromosome 2.

2.5 Allele frequency distribution of *OsFLA2* and *OsFLA6*

To elucidate the allele types of *OsFLA2* and *OsFLA6* loci in wild rice, we analyzed the the sequences of 12 wild rice reported by Dang *et al*. (2020). The sequencing analysis results showed that the alleles of both *OsFLA2* and *OsFLA6* loci were all found in wild rice (Figure 4). We investigated the regional differentiation of diverse alleles on *OsFLA2* and *OsFLA6* gene loci. For the allele $OsFLA2^{TT}$ (large FLA), it mainly distributed in accessions collected from low-latitude regions, such as southeastern Asia. For $OsFLA2^{CC}$ (small FLA), we found that it mainly distributed in accessions collected from high-latitude regions, such as northeastern China, and FLA decreases with the increase of latitude (Figure 4). A similar situation was observed for *OsFLA6*, in which the allele $OsFLA6^{GG}$ was mainly distributed in accessions collected from southern China and southeastern Asia (Figure 4). These results suggested that the large FLA accession with the allele $OsFLA2^{TT}/OsFLA6^{GG}$ was naturally selected during the *indica* rice domestication process and that the smalle FLA accession with the allele $OsFLA2^{CC}/OsFLA6^{AA}$ was naturally retained during the *Japonica* rice domestication process.

3 Discussion

In this study, we investigated the FLA phenotype data in 353 rice accessions and confirmed that there existed a rich phenotypic variances. The CV for FLA ranged from 55.55% (E3) to 58.48% (E4) (Figure 1c). The results of a joint variance analysis indicated that the variations in FLA were the main contribution to diverse genotypes, although significant interactions between genotypes and environments were detected. In conclution, these results provide the basis to mine the elite allels for FLA.

We detected 37 SNP loci significantly associated with FLA, which were located in 28 LD regions (Table 1). By searching the website of Gramene (http://www.gramene.org/markers/) and the China Rice Data Center database (http://www.ricedata.cn/gene/), local LD region harboring the 27 associated SNP sites were overlapped with the flanking regions of eight QTLs and two genes (*OsBRI1* and *OsLIC*) reported previously (Li *et al*. 1999; Yamamuro *et al*. 2000; Dong *et al*. 2003; Luo *et al*. 2008; Wang *et al*. 2008; Hu *et al*. 2012; Zhang *et al*. 2012; Zhang *et al*. 2013; Bian *et al*. 2014; Ham *et al*. 2019) (Table S9), and the remaining 10 associated

233SNP loci were newly identified in this study.

234 Two GWAS signals significantly associated with FLA were identified to nearly 235single-gene resolution. Gene *OsFLA6* coincided with the locations of gene, *OsLIC*. 236Wang *et al*. (2008) reported that inhibition of endogenous *OsLIC* expression resulted 237in significant increase of leaf angle and tiller angle. Zhang *et al*. (2012) confirmed that 238*OsLIC* modulated the leaf angle by acting as an antagonistic transcription factor of 239*BRASSINAZOLE-RESISTANT 1* (*BZR1*) via the brassinosteroids signaling pathway. In 240this study, we further confirmed that *OsFLA6* (*OsLIC*) could regulate the FLA. Gene 241*OsFLA2* is a newly identified gene in this study. The full length of *OsFLA2* is 2177 242bp, including three exons and two introns. Gene *OsSYL2* encodes an 95 amino acid 243protein. We have demonstrated that a base C-to-T nonsynonymous mutation at nt 137 244in the cDNA sequence of *OsFLA2* caused the large FLA phenotype by qRT-PCR and 245the complementation test.

246 For FLA, it is a common feature that *indica* rice (at low latitude) has a large FLA 247and *japonica* rice (at high latitude) has a small FLA in China. A small FLA in 248temperate *japonica* rice, especially in northeastern China, is a trait adaptive to high-249latitude climate conditions (short maturity period) that underwent purification 250selection. The small FLA of temperate *japonica* rice is beneficial for obtaining high 251grain yield in pure-line cultivars (Yang *et al*., 1984) but adverse for hybrid seed 252production due to the requirement of removing the flag leaf of male-sterile plants to 253receive pollen from the male parent (Dong *et al*., 2018b). Therefore, it is necessary to 254increase the FLA to facilitate pollination in the F_1 hybrid seed production. The 255accessions with the two alleles, $OsFLA2^{TT}$ and $OsFLA3^{GG}$, can be used to increase 256FLA in the maintainer lines (pollen parents used for multiplying the CMS lines) of 257hybrid *japonica* rice by crossing and marker-assisted selection breeding method.

2584 Experimental procedures

2594.1 Rice accessions

260In our previously study, the three stigma characteristics of 353 accessions were 261reported (Dang *et al*., 2020). In this study, using the same 353 accessions, we 262investigated the FLA characteristic following the same field plant and management. 263These accessions were grown across six different environment, over 3 years (2014-2642016) and two locations of Nanjing (32°07'N, 118°64'E) and Yuanyang (35°05'N, 265113°96'E).

2664.2 Phenotypic investigation

267The FLA of the plants were measured with a protractor at the stage of the panicle on 268the main stem heading 10cm above the flag leaf lamina joint. For each accession, the 269average FLA value of 10 plants was used as the phenotypic value.

2704.3 Genome-wide association study

271The genotyping data for the accessions are available at EBI European Nucleotie

Archive with the accession number ERP000106, NCBI Sequence Read Archive with the accession number PRJNA171289 and PRJNA554986 (Huang *et al*., 2012; Chen *et al*., 2014; Dang *et al*., 2020). Based on the mixed linear model, the GWAS was conducted using the R package Genomic Association and Prediction Integrated Tool (Lipka *et al*., 2012). We used the R package "LDheatmap" to construct the LD heatmaps surrounding peaks in the GWAS (Shin *et al*., 2006). The manhattan and quantile-quantile plots were drawn by using the R package qqman (Turner, 2014). We used the correction method of Benjamini and Hochberg (1995) to calculated the false discovery rate (FDR) and selected 1.0×10^{-5} as the threshold. The position of SNPs in *OsFLA2* and *OsFLA6* are based on data from the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu).

4.4 RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from flag leaf and flag leaf lamina joint at development stages 5–8 (as per the criterion described by Itoh *et al*., 2005) by using the ultrapure RNA kit (OMEGA BIO-TEK, https://www.omegabiotek.com), respectively, sampled from the six accessions (three accessions with smaller FLA and three accessions with larger FLA). The RNase-free DNase I treatment (Vazyme, http://www.vazyme.com) was used to remove any genomic DNA contamination. And the HiScript II Q RT SuperMix (Vazyme, http://www.vazyme.com) was used to perform the first-strand cDNA synthesis by reverse transcription from 1 μg of RNA. We used the 18S rRNA gene as an internal control. We performed the qRT-PCR in a 96-well thermocycler (Roche Applied Science LightCycler 480) using SYBR Green (Vazyme, http://www.vazyme.com) and set the cycling conditions as follows: firstly, denaturation (95ºC, 5 min); Secondly, amplification and quantification program-40 cycles (95ºC for 10 s, 60ºC for 30 s, 72ºC for 60 s) with a single fluorescence measurement; thirdly, the melting curve (60ºC–95ºC) with a heating rate of 0.1ºC per second and continuous fluorescence measurement); and finally, cooling step (40ºC). We performed the three independent replicates. The primer sequences of qRT-PCR are shown in Table S10. We calculated the relative gene expression of the target gene using the equation: $Exp=2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{18S rRNA}}$.

4.5 Generation of *OsFLA2* transgenic plants

The full-length genomic DNA of *Os02g0142875* was amplified by PCR from A7444 rice and cloned into the pBWA(V)HII vector (Table S10). This construct (pBWA(V)HII-*OsFLA2*) was then transformed into Nipponbare by *Agrobacterium* EHA105. And the corresponding empty vector transformed into Nipponbare was used as a control. Thirty-two independent T_1 seedlings obtained were grown to maturity under natural conditions. In the next rice growing season (May to October), the T_2 seeds harvested from T_1 plants at the maturity stage were grown in the paddy field. At the tillering stage, the three allele genotypes (TT, TC, CC) on the *Os02g0142875* locus were determined using the primers listed in Table S10 and the FLA were measured in the $Os02g0142875^{TT}$ and $Os02g0142875^{CC}$ plants at full heading stage.

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Authors' contributions

J.J. and X.D. designed the experiments and managed the project. C.H., L.X., Y.Z. and D.W. conducted field planting and phenotypic identification. X.D. and C.H. prepared RNA samples, qRT-PCR and transformation analysis. X.D. and L.X. performed the data analysis. X.D. and J.J. wrote the manuscript draft, which was revised by J.J.

Competing financial interests

The authors declare no competing financial interests.

Supporting information

Figure S1. Manhattan plots and quantile-quantile plots depicting GWAS results for the FLA using a mixed line model in the 353 cultivated rice accessions in each environment.

Table S1. A list of QTLs controlling flag leaf angle published so far.

Table S2. The results of joint analysis of variance for flag leaf angle.

Table S3. The distribution of the significant association SNP loci with flag leaf angle detected in the rice population composed of 353 accessions in the six environments.

Table S4. The SNP information in the 30.70–30.92 Mb candidate region for flag leaf angle.

Table S5. The SNP information in the 29.63–30.14 Mb candidate region for flag leaf angle.

Table S6. Candidate gene annotation in the LD region 29.63–30.14 Mb associated with flag leaf angle.

Table S7. The SNP information in the 2.16–2.50 Mb candidate region for flag leaf angle.

Table S8. Candidate gene annotation in the LD region 2.16–2.50 Mb associated with flag leaf angle.

Table S9. The results of significantly associated SNP loci detected in this study overlapped with the QTLs/genes of rice flag leaf angle reported previously.

Table S10. Primers used in this study.

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Figure legends

Figure 1. Phenotypic characteristics of FLA in *indica* subgroup, *japonica* subgroup and six environments, respectively. (a) Phenotypic value distributions of FLA in *indica* subgroup and *japonica* subgroup. The number of varieties within each population was 172 and 181, respectively. (b) Phenotypic value distributions of FLA

in six environments. (c) Phenotypic statistics of FLA in six environments. The box edges represent the upper and lower quantile, with the median value shown by the black line in the middle of the box. Vertical lines represent the data from the lowest quantile to the top quantile. Individuals falling outside the range of the whiskers are shown as asterisks.

Figure 2. GWAS for FLA and identification of the candidate gene *OsFLA6* (*Os06g0704300*). (a) Manhattan plots for FLA on chromosome 6. Arrowheads indicate the associated loci containing the candidate gene *Os06g0704300*. Horizontal lines indicate the significance threshold ($-\log_{10} P = 7.0$). (b) Local Manhattan plot (top) and LD heatmap (bottom). The arrow indicates the position of nucleotide variation in *Os06g0704300*. The candidate region lies between the red solided lines. (c) SNPs in *OsFLA6* cDNA between HapA and HapB. (d) Boxplots for FLA based on the two alleles (n=232 versus 116). Center line, median; box limits, upper and lower quartiles; whiskers, 1.5× the interquartile range; dots, outliers. Differences between the alleles were statistically analyzed based on Welch's *t*-test (** $P< 0.01$). (e) Relative expression of *Os06g0704300* in flag leaf and flag leaf lamina joint at development stages 6–8 from the three accessions (Ludao, Haomake and A7444) with a large FLA and the three accessions (Nipponbare, Kendao 13 and Chenwan 3hao) with a small FLA, determined by qRT-PCR (**$P<0.01$, *$P<0.05$, two-tailed Welch's *t*-test). Data are presented as means ± s.e.; n=3 independent biological replicates. FLA, flag leaf angle.

Figure 3. GWAS for FLA and identification the causal gene *OsFLA2* (*Os02g0142875*). (a) Manhattan plots for FLA. Arrowheads indicate the position of strong peaks. The red lines represent significance thresholds ($-\log_{10} P=7.0$). (b) Local Manhattan plot (top) and LD heatmap (bottom). The arrow indicates the position of nucleotide variation in *Os02g0142875*. The candidate region lies between the red solided lines. (c) SNPs in *OsFLA2* cDNA between HapA and HapB. (d) Boxplots for FLA based on the two alleles (n=286 versus 24). Center line, median; box limits, upper and lower quartiles; whiskers, 1.5× the interquartile range; dots, outliers. Differences between the alleles were statistically analyzed based on Welch's *t*-test (** $P< 0.01$). (e) Relative expression of *Os02g0142875* in flag leaf and flag leaf lamina joint at development stages 6–8 from the three accessions (Ludao, Haomake and A7444) with a large FLA and the three accessions (Nipponbare, Kendao 13 and Chenwan 3hao) with a small FLA, determined by qRT-PCR (**$P < 0.01$, two-tailed Welch's *t*-test). Data are presented as means ± s.e. ; n=3 independent biological replicates. (f) Images of FLA of transgenic plants transformed with the empty vector (VEC), C allele, and T allele. Scale bar = 3 cm. (g) FLA of transgenic plants. Data are presented as means ± s.e. (n=20).

Figure 4. The gene allele frequency differences at the causal polymorphisms of *OsFLA2* and *OsFLA6* in five geographic groups. The type of reference allele is indicated in blue, and the alternative is indicated in red. Indo, Indonesia; SC, southern China; CC, central China; EC, eastern China; NEC, northeastern China. The

accessions from SC were mainly wild rice and *indica* subspecies. The accessions from Indo were mainly tropical *japonica* subspecies. The accessions from CC were mainly *indica* subspecies. The accessions from EC and NEC were mainly temperate *japonica* subspecies.

Table 1 The summary of SNP significantly associated with flag leaf angle.

Chromosome	SNP location	Local LD	Allele	Range -$\log_{10}(P)$	Range R^2(%)	Environment
1	31510189	31259625-31511021	T/C	7.11-7.60	1.80-3.27	E1-E3, E5
1	31557795	31310573-31780397	T/G	7.02-7.23	1.53-2.16	E1-E3, E5
1	31557823	31412710-31653328	A/G	7.02-7.16	1.53-1.95	E1-E6
1	31558572	31412710-31653328	G/A	7.04-7.65	1.59-3.42	E1-E6
2	2205168	2174604-2449915	A/C	7.04-7.62	1.59-3.33	E2-E5

2	2372437	2174604-2449915	C/T	7.85-9.59	4.02-9.27	E1-E6
2	9979855	9979855-9979855	G/T	7.31-8.36	2.40-5.55	E1-E6
2	22948971	22948971-22948971	C/T	7.16-7.90	1.95-4.17	E1-3, E5
4	4651577	4558421-4816199	A/C	7.12-7.61	1.83-3.30	E2-E4, E6
4	4694627	4694627-4732062	A/G	7.08-7.58	1.71-3.21	E1-E3, E6
4	4694847	4694627-4732062	T/C	7.06-7.14	1.65-1.89	E1-E6
4	4694932	4694627-4732062	A/T	7.07-7.48	1.68-2.91	E1-E3, E6
4	4716577	4694627-4732062	T/C	7.17-7.45	1.98-2.82	E1-E3, E6
4	4722888	4722888-4874394	T/C	7.04-7.50	1.59-2.97	E1-E6
4	4865528	4722888-5062959	C/T	7.29-7.61	2.34-3.30	E1-E4, E6
4	5010851	4870317-5062370	T/A	7.04-7.18	1.59-2.01	E1-E6
4	5012383	4794812-5062959	T/C	7.07-7.34	1.71-2.49	E1-E6
4	5034015	4816199-5062959	A/G	7.04-7.29	1.59-2.34	E1-E6
4	5060780	4865528-5062959	T/C	7.25-7.52	2.20-3.03	E1-E3, E6
5	4715861	4680302-4718467	T/A	7.01-9.08	1.50-7.71	E1-E6
5	22333371	22327857-22376878	G/C	7.04-7.55	1.59-3.12	E1-E6
5	22360006	22327857-22376878	G/A	7.11-7.50	1.80-2.97	E1-E6
5	22376878	22327857-22376878	C/T	7.14-7.58	1.89-3.21	E1-E6
6	4712788	4684367-4766915	C/T	7.05-7.94	1.62-4.29	E1-E3, E5, E6
6	24430896	24393060-24660723	T/C	7.06-7.92	1.65-4.23	E2-E5
6	26595688	26586772-26597162	A/G	7.03-8.44	1.56-5.79	E1, E2, E4, E5
6	29740496	29739306-30896016	A/G	7.29-8.15	2.34-4.92	E2-E6
6	30938389	30938389-30947929	A/T	8.08-8.84	4.71-6.99	E1-E6
6	30938760	30938389-30947929	G/T	7.02-7.07	1.53-1.68	E2-E5
7	26706376	26641024-26726197	C/G	7.26-7.67	2.25-3.48	E2, E4-E6
7	26715083	26641024-26726197	C/A	7.20-7.40	2.07-2.67	E2-E6
8	8508983	8471294-8606630	T/C	7.06-7.15	1.65-1.92	E2-E5
8	8637090	8471294-8704450	A/G	7.05-7.34	1.62-2.49	E1-E6
9	13036365	13005216-13154929	T/C	7.24-7.92	2.19-4.23	E1-E6
9	14928244	14928244-14928244	T/C	7.04-7.63	1.59-3.36	E1-E6
9	15322414	15271847-15337382	G/C	7.07-7.67	1.68-3.48	E1, E3-E6
10	1447463	1427948-1473753	A/G	7.47-7.95	2.88-4.32	E1-E6

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