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Genome-wide association study identifies elite alleles of *FLA2* and *FLA9* controlling flag leaf angle in rice

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Abstract

Background In hybrid rice seed production, rice varieties with a small flag leaf angle (FLA) experience obstacles to cross-pollination at the early heading stage, and farmers usually need to remove flag leaves to achieve artificial pollination. Therefore, the cultivation of rice varieties with large FLAs can not only save a substantial amount of labour in the leaf-cutting process during artificial pollination but also accelerate the mechanization of hybrid rice seed production.

Results In this study, 431 rice accessions were included in a genome-wide association study (GWAS) to identify quantitative trait loci (QTLs) and the superior haplotypes for rice FLA in 2022 and 2023. The aim of the study was to identify new QTLs and provide germplasm resources for the genetic improvement of rice FLA. The population exhibited rich phenotypic variation in FLA in both years. The FLA GWAS was performed with more than 3 million single-nucleotide polymorphisms (SNPs), and eight QTLs associated with FLA were detected; of these, six QTLs located on rice chromosomes 1, 2, 8 and 9 were novel and detected in both years. In addition, these QTLs were analysed by haplotype analysis and functional annotation, and *FLA2* and *FLA9*, which encode xyloglucan fucosyltransferase and cytokinin-O-glucosyltransferase 2, respectively, were identified as candidate genes for FLA regulation in rice. Quantitative real-time polymerase chain reaction (qRT–PCR) results validated *FLA2* and *FLA9* as candidate genes. The results of this study showed that the elite alleles of *FLA2* and *FLA9* can increase FLA in rice. Excellent parents for FLA improvement were predicted through pyramiding breeding.

Conclusions A total of six new QTLs and two candidate genes (*FLA2* and *FLA9*) were identified by a GWAS of 431 rice accessions over two years. The elite alleles and excellent parents predicted in our study can provide important information for the functional analysis of rice FLA-related genes and improvement through pyramiding breeding.

Keywords Rice, Flag leaf angle, Genome-wide association study, Candidate genes, Favourable haplotypes

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Introduction

Rice is a staple food used to meet the energy needs of more than half of the world's population [1]. Hybrid breeding is a key method for improving rice yield, which is highly important for overcoming food shortages worldwide [2]. The practice of crop improvement shows that increasing yield through heterosis is an effective way to breed rice [3]. This process requires ensuring the stable cross-pollination of rice to avoid trait segregation in F_1 hybrid seeds because of the inability to fix heterosis [4, 5]. However, the upright leaves produced by the small flag leaf angle (FLA) can block normal pollination of the rice flowers. To eliminate cross-pollination obstacles in the early heading stage of rice, farmers usually remove one-third or one-half of the top of the flag leaf during hybrid rice seed production, which requires not only more labour but also more careful operations to avoid cutting young panicles. Moreover, the wounds caused by leaf cutting can also adversely affect the rice grain-filling process [6]. Therefore, the FLA is an important trait affecting the mechanized production of hybrid rice. A large FLA can solve the problems associated with the breeding of sterile lines. Selecting and regulating quantitative trait loci (QTLs) and genes related to FLA is highly important for hybrid rice seed production and rice yield improvement [7].

Many studies have shown that FLA is a complex quantitative trait [8]. A total of 77 QTLs for FLA have been identified via linkage analysis, with 13, 7, 5, 3, 5, 7, 8, 8, 7, 1, 8 and 5 QTLs on the 12 chromosomes [9–21]. Through association analysis, more than 100 QTLs have been detected [4, 22–24]. Although many QTLs related to FLA have been mapped, only one cloned gene (*OsFLA2*) has been reported to be directly related to FLA [4]; the other cloned genes have focused mainly on the angle of other leaves. Therefore, in rice hybrid seed production, more favourable FLA alleles need to be identified to improve the outcrossing rate.

Previous studies have shown that plant hormones play important roles in controlling rice leaf angle [25]. Genes encoding the key enzymes involved in brassinosteroid (BR) biosynthesis play crucial roles in the regulation of rice leaf bending. The overexpression of BR-related genes, including *BRD2*, *OsDWARF4*, *D11*, *OsBRI1*, *OsBAK1*, *OsBZR1* and *OsOFP8*, was reported to positively regulate BR biosynthesis, which increases the leaf angle [26–32], whereas the overexpression of *brd1* and *OsLIC1* decreases the leaf angle [33–34]. Genes such as *FIB*, *LC1*, *OsIAA1*, *OsTIR1*, *OsAFB2*, *OsmiR393*, *OsARF19*, *LC3* and *OsSPY* indirectly regulate leaf angle by regulating auxin levels [35–41]. Among them, *OsmiR393* may mediate the interactions of *OsAFB2* with *OsTIR1* and *OsIAA1* to alter the auxin response, changing the leaf angle [38]. For gibberellin (GA) signal transduction pathway, the studies showed that the gene *OsSPY* involved in GA-GID1-DELLA pathway, while the genes *D1*/*RGA1* and *OsGSR1* involved in G-protein pathway. These three genes affected the angle of rice leaves by interacting with genes associated with BRs biosynthesis [42–43]. In addition to these genes involved in hormone biosynthesis and signal transduction to control rice leaf angle, some other genes, such as *LPA1*, *LAZY1* (*LA1*) and *ILA1*, can affect the geotropism of rice plants and the mechanical tissue strength of the pulvinus, which affect the size of the leaf angle [25, 44, 45, 46]. *LPA1* encodes a plant-specific domain-uncertain transcriptional repressor that regulates leaf angle by controlling cell growth on the adaxial surface of the occipital node [44]. The *LA1* gene regulates the geotropism of rice stems. A loss of function of this gene significantly increases the polar transport of auxin, which in turn leads to a decrease in the geotropism of the aboveground part of the plant and an increase in the angle between rice leaves [45]. *ILA1* affects the leaf angle by regulating the formation of pulvinar mechanical tissue and abnormalities in cell wall composition, and its mutation increases rice leaf angle [46].

The genes discussed above positively or negatively regulate rice leaf angle through various pathways, but only one cloned gene directly related to FLA has been reported. Therefore, further study of the molecular regulatory mechanism of FLA is needed. In this study, a genome-wide association study (GWAS) was used to identify multiple QTLs and elite alleles of candidate genes. These results provide a reference for cloning and improvement of FLA-related genes. Favourable haplotypes and excellent parents can be used to improve FLA through pyramiding breeding.

Materials and methods

Material source and field planting

A total of 431 rice accessions were selected from the 3000 Rice Genome Project (3KRGP). The natural population was divided into four subgroups: *Xian* (269), *Geng* (137), *admix* (17) and *Bas* (8) (Table S1). The accessions were planted at the Dayang Experimental Station of Anhui Agricultural University (31.93 N, 117.39 E) in 2022 and planted at the Lujiang Experimental Station of Hefei City (31.25 N, 117.48 E) in 2023. Each plot consisted of three rows with nine hills per row, with the hills spaced at 17 cm×33 cm. The field trials were arranged in a completely randomized block design, with three replications.

Phenotypic investigation

At 5 to 10 days after heading, 10 plants with the same growth were randomly selected, and the FLA, defined as the angle between the stem and the base of the flag leaf, was measured with a digital display protractor (Fig. 1a and b). To minimize experimental errors, 10 replicates

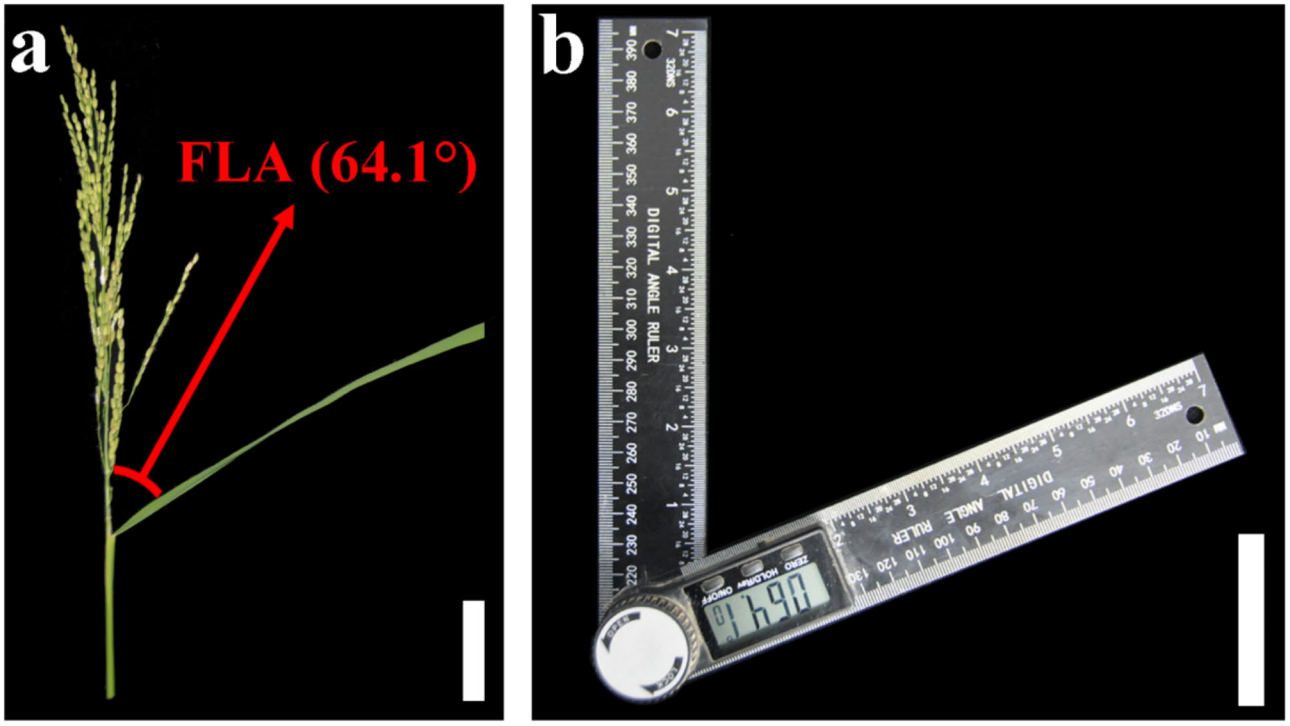

Fig. 1 FLA measurement methods and tools. Scale bar = 5 cm. **(a)** FLA measurement example. **(b)** Digital display goniometer

were measured, and the mean was taken as the phenotypic value. The FLA measurements were carried out in the experimental field.

Statistical analysis of phenotypic data

Phenotypic FLA data collected over two years were statistically analysed. The frequency distributions of the traits were calculated using Excel 2018 (Microsoft, Redmond, WA, USA), SPSS 2022 (IBM, Armonk, NY, USA) and Origin software 2022 (Northampton, MA, USA).

Genotype data acquisition and annotation

The resequencing data of the 431 rice accessions have been published in NCBI (https://www.ncbi.nlm.nih.gov/), and the variant locus information is available through the SNP-Seek database (http://snp-seek.irri.org/) [47].

We used PLINK (version 1.9, BGI Cognitive Genomics, Shenzhen, China) [48] software to perform secondary allele frequency calculation and site integrity and multiallelic site filtering on genotype data and screened 3,203,141 (MAF > 0.05) single-nucleotide polymorphisms (SNPs) with the criteria of a deletion rate greater than 20% and a secondary allele frequency less than 5%. The Nipponbare genome sequence was downloaded from the International Rice Genome Sequencing Project (http://rice.plantbiology.msu.edu) [49], and all paired terminal sequence readings were compared using Bowtie 2 software. The reads for SNP calls needed to have a unique location in the Nipponbare genome; reads with a location score of more than 60.95% were mapped to the Nipponbare genome, and 3% of reads were not mapped to any location or mapped to multiple locations and were deleted.

The SNPs of the Nipponbare genome sequence were annotated using ANNOVAR software [50]. The annotation results were divided into exons, introns, untranslated regions (UTRs), regions between genes, and upstream and downstream regions. SNPs in exons of the coding region were divided into two types: synonymous and non-synonymous [51].

Population structure analysis

The filtered SNPs were used to construct a distance matrix using VCF2Dis (https://github.com/BGI-shenzhen/VCF2Dis), and a phylogenetic tree was constructed with iTol (https://itol.embl.de/) [52]. Principal componen t analysis (PCA) was performed with PLINK using GCTA software [53], and the PCA diagram was drawn in R. We used PLINK to filter SNPs according to linkage disequilibrium (LD), retain unlinked SNP loci, and convert these loci into a structural format for population structure analysis to predict the optimal number of subgroups. The genetic relationships were analysed with TASSEL software, and a heatmap was drawn in R [54].

Linkage disequilibrium analysis

In our study, the r^2 value [55] was used to measure the degree of LD between loci in the whole rice genome. The

LD analysis of the 431 rice accessions was carried out via PLINK software. The default parameters were used to transform the genotype file format into .ped and .map formats. The script decay_chrom.pl was used to sort and summarize the results. Using the output file, the LD diagram was drawn using the LDheatmap package in R statistical software [56].

Genome-wide association study

After quality control, the SNPs were further processed by TASSEL software [54], and GWAS was performed via a mixed linear model (MLM) and a general linear model (GLM) [57]. Moreover, to ensure the accuracy of the results, the Bonferroni correction [51] was used to correct for multiple tests in this study. Combined with the actual results of the Manhattan plot, the significance threshold was set as $P \geq 1e\text{-}10^{-6}$. We selected the rmvp package in R for visual analysis of the GWAS results. The position of SNPs on the chromosome was used as the X-axis coordinate, and the significance $-\log_{10}(P)$ value was used as the Y-axis coordinate of the Manhattan diagram of the GWAS. The actual significance *P* value of each SNP was subsequently used as the Y-axis, and the theoretical *P* value was used as the X-axis to draw the quantile–quantile plot (Q–Q plot) of the GWAS results. The Manhattan diagram was used to determine whether SNPs were significantly associated with the phenotype, and the Q–Q plot was used to verify the reliability of the GWAS results and reduce the number of false-negative or false-positive results caused by statistical tests.

In this study, the genome bast linear unbiased prediction (BLUP) [58] method was used to verify the accuracy of cross-environment and cross-year GWAS results. The BLUP method can integrate multi-environmental data, eliminate environmental impacts, and obtain stable individual genetic phenotypes [59]. BLUP is a common method for phenotypic processing, and the lmer function in lme4 in R package is a common method for BLUP analysis. We use the R package "CMplot" to construct the Manhattan diagram. The threshold of the MLM model is $P \geq 1e\text{-}10^{-5}$, which is the same as the threshold setting of the multi-environment GWAS method.

Identification of candidate genes and haplotype analysis.

Based on the results of the GWAS analysis and LD decay distance, the candidate gene regions on chromosomes were determined. The functions of the candidate genes were searched in the National Rice Database Center (https://www.ricedata.cn/). We referred to the Nipponbare genome sequence to analyse the SNP types in the candidate regions, focusing on non-synonymous SNPs in exons that cause amino acid changes [51, 60]. Combining the functions of FLA-related genes reported in previous studies with the functions of candidate region genes and non-synonymous SNPs further narrowed the range of candidate genes. Haplotype analysis was performed on non-synonymous SNPs in exons. Differences in FLA were tested for significance via Tukey's test [60].

Detection of *FLA2* and *FLA9* expression levels via qRT–PCR

The candidate genes *FLA2* and *FLA9* were validated via qRT–PCR in 5 large FLA accessions and 5 small FLA accessions. The seedlings of 10 rice accessions were collected for RNA extraction. A 1 μg aliquot of total RNA was reverse transcribed into single-stranded cDNA using a Prime Script RT Reagent Kit (TaKaRa, Japan). Real-time quantitative RT–PCR was performed in a total volume of 20 μL, which included 2 μL of template cDNA, 10 μL of 2×ChamQ Universal SYBR qPCR Master Mix (Vazyme), 0.5 μL of the forwards and reverse gene-specific primers, and 7 μL of ddH_2O. Gene expression was normalized to that of the internal control 18 S rRNA. The amplification reaction was performed in a 96-well thermocycler (Roche Applied Science LightCycler 480) using an AceQ qPCR Kit (Vazyme). The cycling program consisted of 5 min at 95 °C, followed by 40 amplification cycles (95 °C for 10 s and 60 °C for 30 s). The primers used are listed in Table S6. Relative quantification of the transcript levels was performed using the comparative Ct method (Livak and Schmittgen 2001).

Prediction of excellent parents

The average positive (negative) haplotype effect (AHE) within a gene locus was calculated as follows:

$$AHE = \sum h_c/n_c$$

where h_c represents the phenotypic value of the cth haplotype with a positive (negative) effect and n_c represents the number of haplotypes with positive (negative) effects within the gene locus [61].

The rice accessions with the greatest positive haplotype effects on all FLA gene loci were predicted to be the most promising parents for FLA improvement in rice breeding.

Results

Phenotypic variation of the FLA in the natural population

In 2022 and 2023, there was a significant difference in FLA among the varieties; coefficients of variation ranged from 52.20 to 58.69%, and the mean values of FLA ranged from 14.26° to 20.28° (Table 1). To show the rich phenotypic variation, ten rice varieties were selected to represent the phenotypic differences in FLA, including PsBRC28, with the smallest FLA (6.65°), and SE122 (129.39°), with the largest FLA (Fig. 2a). In addition, the average broad-sense heritability (H_B^2) of FLA in the two environments was 66.7% (Table 1). The frequency

Table 1 Descriptive statistics of FLA in 431 rice accessions

Trait	Location	Year	Mean ± SD (°)	Max (°)	Min (°)	CV (%)	H_B^2(%)
FLA	DY	2022	20.28 ± 11.90	129.40	6.62	58.69%	66.67
FLA	LJ	2023	14.26 ± 7.50	82.88	5.70	52.20%	

SD: standard deviation, CV: coefficient of variation, H_B^2: broad heritability, DY: Dayang Experimental Station, LJ: Lujiang Experimental Station

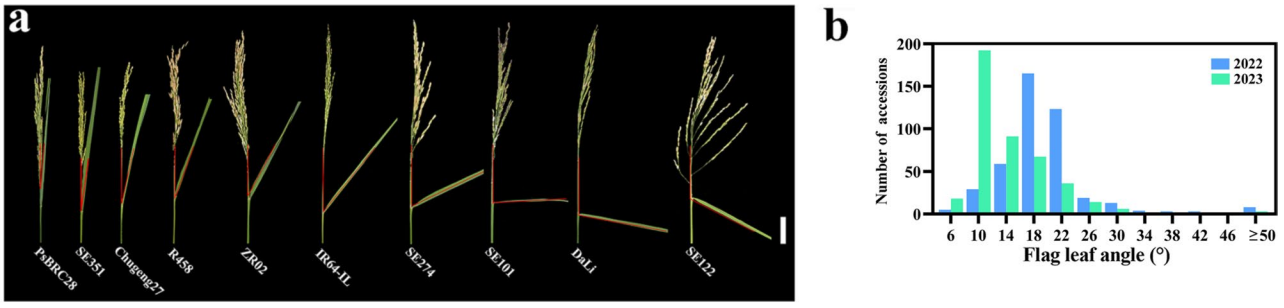

Fig. 2 **(a)** Phenotypes of FLA in 10 rice accessions. Scale bar = 5 cm. **(b)** Histogram of the phenotypic frequency distribution of FLA in 431 rice accessions

histogram of the FLA phenotype revealed that the two-year FLA traits were essentially normally distributed among the 431 rice accessions (Fig. 2b). These results indicate that the FLA is a quantitative trait controlled by multiple genes and that there is abundant phenotypic variation among varieties.

Population structure and LD decay analysis

The phylogenetic tree revealed that the 431 rice accessions could be divided into four subgroups: *Xian*, *Geng*, *admix* and *Bas* (Fig. 3a). Similar results were obtained with PCA (Fig. 3b). The results of the genetic relationship analysis revealed that most of the varieties had no obvious genetic relationships, and a small number of accessions had close genetic relationships (Fig. 3c), indicating that the population used in this study was suitable for association analysis. When r^2 was reduced to half of the maximum value, the corresponding decay distance was 395 kb (Fig. 3d).

GWAS of FLA traits

The GWAS of the 431 rice accessions was analysed with an MLM. A total of 11 QTLs, which were located on chromosomes 1, 2, 7, 8 and 9, were identified in both 2022 and 2023 (Table 2). After eliminating the effect of environment on phenotypic data using the genomic BLUP method, we conducted a combined GWAS for all environments across years. GWAS results showed that a total of eight QTLs (*qFLA1.2*, *qFLA1.4*, *qFLA2*, *qFLA7.2*, *qFLA8.1*, *qFLA8.2*, *qFLA9.1*, *qFLA9.2*) were identified, which was included in the QTLs of the single-environment GWAS (Fig. 4c; Table 3). Among them, *qFLA7.2* and *qFLA8.2* were close to the physical locations of *qFLA7e* and *qFLA8f* [7], respectively (Fig. 4a and b). Therefore, the stability of QTLs identified by the multi-environment and the multi-year GWAS is reliable. The remaining six QTLs (*qFLA1.2*, *qFLA1.4*, *qFLA2*, *qFLA8.1*, *qFLA9.1*, and *qFLA9.2*) were novel QTLs detected in this study, which were selected as the main QTLs for further study.

Identification of two candidate genes associated with FLA

The candidate gene analysis was performed on all identified novel regions. Through gene functional annotation (http://rice.plantbiology.msu.edu) [49], non-homologous SNPs and haplotype analysis, only two regions on chromosome 2 and 9 were focused on candidate genes selection. After removing the genes encoding hypothetical proteins, retrotransposons, and transposon proteins, 37 genes associated with significant SNP sites in the 31.8–32.2 Mb region of chromosome 2 were identified; 11 of the 37 genes had non-synonymous mutations (Fig. 5a and Table S2). Among these 11 genes, the gene *LOC_Os02g52590* encodes xyloglucan fucosyltransferase which was revealed that plays an important role in the mechanism and control of plant cell expansion, differentiation, maturation and wall repair [73]. The haplotype analysis showed that the FLA of HapA and HapB has significant difference (Fig. 5c). Therefore, the gene *LOC_Os02g52590* on chromosome 2 was selected as the candidate gene. Similarly, the gene *LOC_Os09g03140* was identified in the 1.37–1.77 Mb region of chromosome 9 that harboured a non-synonymous mutation, which encodes cytokinin-O-glucosyltransferase 2 (Table S3). Previous studies revealed that cytokinin-O-glucosyltransferase 2 promotes cell division and increases cell expansion during the proliferation and expansion stages of leaf cell development [80]. The haplotype analysis also showed significantly difference among three haplotypes (Fig. 6c). Therefore, the genes *LOC_Os02g52590* (*FLA2*) and *LOC_Os09g03140* (*FLA9*) were predicted to be candidate genes controlling the FLA in rice.

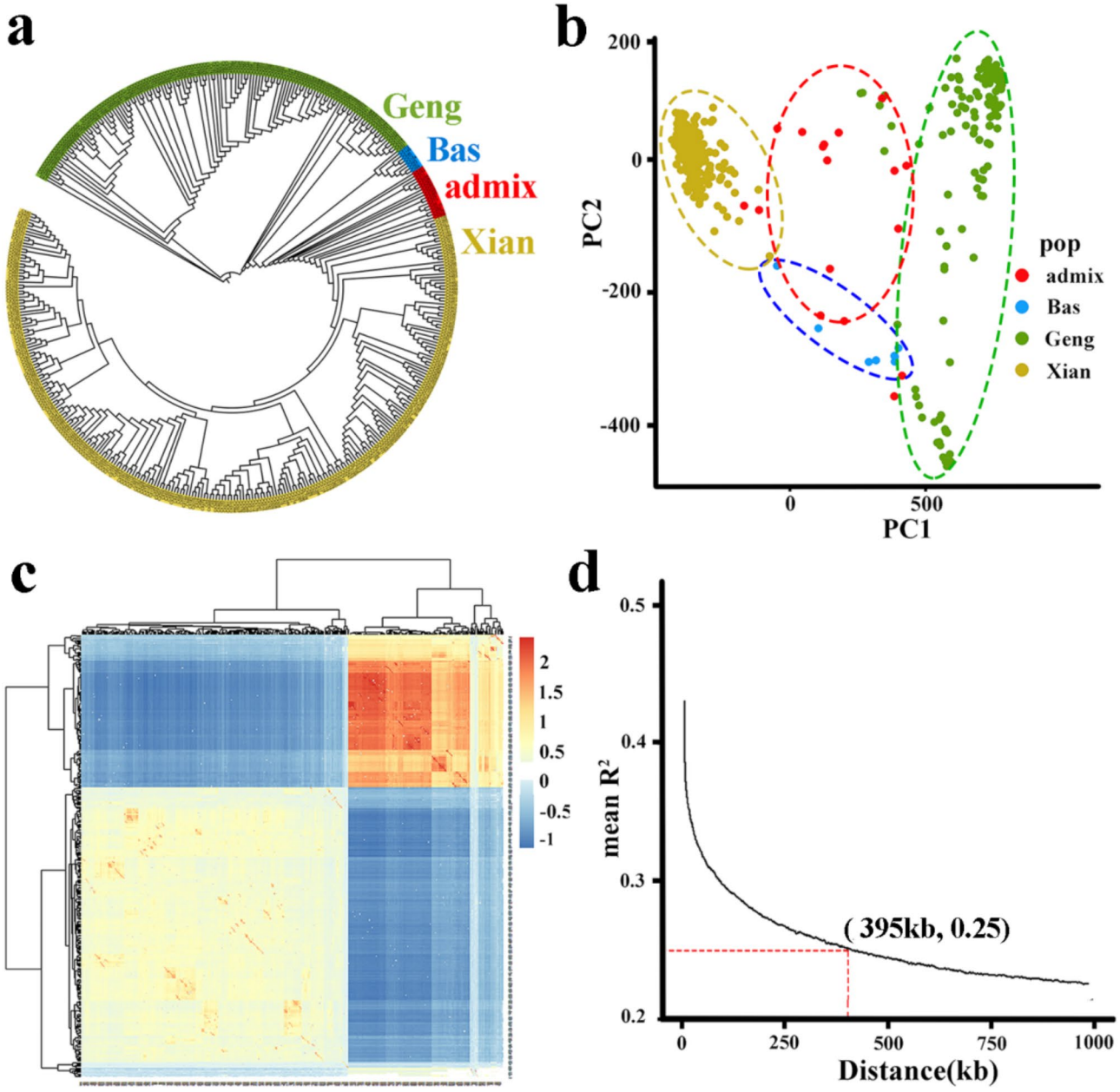

Fig. 3 Genetic structure analysis of the natural population constructed from 431 rice accessions. **(a)** Phylogenetic tree: Each branch represents a rice variety. **(b)** Principal component analysis was performed on 3.20 million SNPs from 431 rice varieties. PC1 and PC2 refer to the first and second principal components, respectively. The points in the figure represent the 431 rice varieties. The shorter the distance between the points is, the closer the relationship is. **(c)** Heatmap of kinship generated with the R package pheatmap. **(d)** LD decay analysis of the whole genome of natural rice populations

Validation by real-time PCR analysis

Real-time PCR analyses were conducted to verify the candidate genes of *FLA2* and *FLA9*. The results of qRT–PCR revealed that the expression of *FLA2* in 4 large FLA accessions (IRAT140, SE282, SE369, SE111) was significantly greater than that in the small FLA accessions (Fig. 7a and b). Compared with that in the small FLA accessions, *FLA9* expression was significantly greater in 4 accessions (IRAT140, SE282, SE369, SE87), whereas the other 1 accession (SE111) presented no significant differences (Fig. 7a and c). These results indicate that high expression of *FLA2* and *FLA9* can improve FLA, which confirms that *FLA2* and *FLA9* are candidate genes controlling the FLA in rice.

Elite haplotype analysis

A non-synonymous SNP was detected in the exon of the *FLA2* gene, which encodes xyloglucan fucosyltransferase in the glycoside hydrolase family. All germplasms of the *FLA2* gene could be divided into two haplotypes

Table 2 Genome-wide significant association of rice FLA traits

QTLs	Chromosome	Position	Allele	$Log_{10}(P)$[a]	Environment	MAF[b]	Known Genes/QTLs
qFLA1.1	1	12,713,277	G/C	7.43	2022, DY	0.35	
	1	12,908,893	T/C	7.04	2023, LJ	0.34	
qFLA1.2	1	13,332,762	G/A	6.31	2022, DY	0.38	
	1	13,158,926	T/G	8.24	2023, LJ	0.34	
qFLA1.3	1	16,203,835	A/C	6.67	2022, DY	0.40	
	1	16,418,160	A/G	6.37	2023, LJ	0.38	
qFLA1.4	1	24,305,157	T/C	6.29	2022, DY	0.38	
	1	24,514,119	T/C	8.61	2023, LJ	0.37	
qFLA2	2	32,057,055	C/T	6.57	2022, DY	0.36	
	2	32,035,468	G/A	7.15	2023, LJ	0.37	
qFLA7.1	7	19,733,783	A/T	6.65	2022, DY	0.34	
	7	19,528,068	A/T	6.38	2023, LJ	0.05	
qFLA7.2	7	29,419,260	C/T	6.36	2022, DY	0.06	*qFLA7e* [7]
	7	29,641,042	A/G	6.29	2023, LJ	0.26	
qFLA8.1	8	15,708,371	A/G	7.75	2022, DY	0.11	
	8	15,704,229	C/T	6.04	2023, LJ	0.67	
qFLA8.2	8	21,508,180	A/G	8.89	2022, DY	0.32	*qFLA8f* [7]
	8	21,599,529	C/T	6.37	2023, LJ	0.32	
qFLA9.1	9	1,568,461	C/T	6.54	2022, DY	0.32	
	9	1,403,199	G/A	6.57	2023, LJ	0.06	
qFLA9.2	9	4,404,631	C/T	6.42	2022, DY	0.06	
	9	4,217,431	A/G	6.36	2023, LJ	0.06	

[a]The $-log_{10}(P)$ value obtained by GWAS in different environments are described [58]

[b]Minimum allele frequency

according to the SNPs in the cDNA (Fig. 5b). The mean FLA of HapA was 17.48 ± 0.38°, whereas that of HapB was 15.64 ± 0.52°. Haplotype analysis of the whole population revealed that there was a significant difference between the FLAs of HapA and HapB (Fig. 5c). The larger FLA was selected as the elite haplotype. The elite haplotype of *FLA2* was HapA, which was composed mainly of the *Xian* (234), *Geng* (137), *admix* (16) and *Bas* (8) subgroups (Fig. 8a). At the non-synonymous SNP site at 32,178,088 bp, a G-T substitution occurred in the allele. A comparison of the amino acid sequences revealed that amino acid 174 of HapA and HapB is aspartic acid (D) and tyrosine (Y), respectively, which might be the reason for the significant difference in FLA between the two haplotypes (Fig. 5d).

Similarly, five non-synonymous SNPs were found in the exons of *FLA9*, which encodes cytokinin-O-glucosyltransferase 2. All the germplasms were divided into three haplotypes according to the SNPs in the cDNA of *FLA9* (Fig. 6b). The average FLA of HapA was 17.04 ± 0.34°, that of HapB was 19.94 ± 1.88°, and that of HapC was 14.23 ± 1.56°. Haplotype analysis of *FLA9* revealed that the FLA of HapB was significantly greater than that of the other two haplotypes and significantly differed from that of HapC (Fig. 6c). The elite haplotype HapB with the largest FLA was mainly composed of the *Geng* (22), *Bas* (5) and *Xian* (3) subgroups (Fig. 8b). There were two differences (58D/G, 358E/G) in the protein sequence among the three haplotypes, and these alterations might cause the large FLA observed in HapB (Fig. 6d).

As shown on the haplotype geographic distribution map (Fig. 8c), the elite haplotypes of *FLA2* are widely distributed, concentrated in the middle and low latitudes of Southeast Asia (China) and in the *Xian* subgroup, with a small proportion distributed in the high latitudes (Brazil) (Fig. 8a and c); the elite haplotype distribution of *FLA9* is concentrated in the low latitudes (Philippines) and in the *Geng* subgroup (Fig. 8b and c).

Excellent parental combinations predicted for FLA

According to AHE analysis, among the two haplotypes of *FLA2*, only HapA has an AHE of 0.16° and is a favourable haplotype of *FLA2*. AHE analysis of the *FLA9* gene revealed that HapB was a favourable haplotype, with an AHE of 2.62° (Table S4). We identified 30 parents containing both *FLA2* HapA and *FLA9* HapB in 431 rice accessions (Table S5). Among them, we selected 11 rice accessions with high seed-setting rates as excellent parents (Table 4). All the predicted elite alleles and excellent parents can increase the FLA through pyramid breeding to improve the outcrossing rate and yield of hybrid rice varieties.

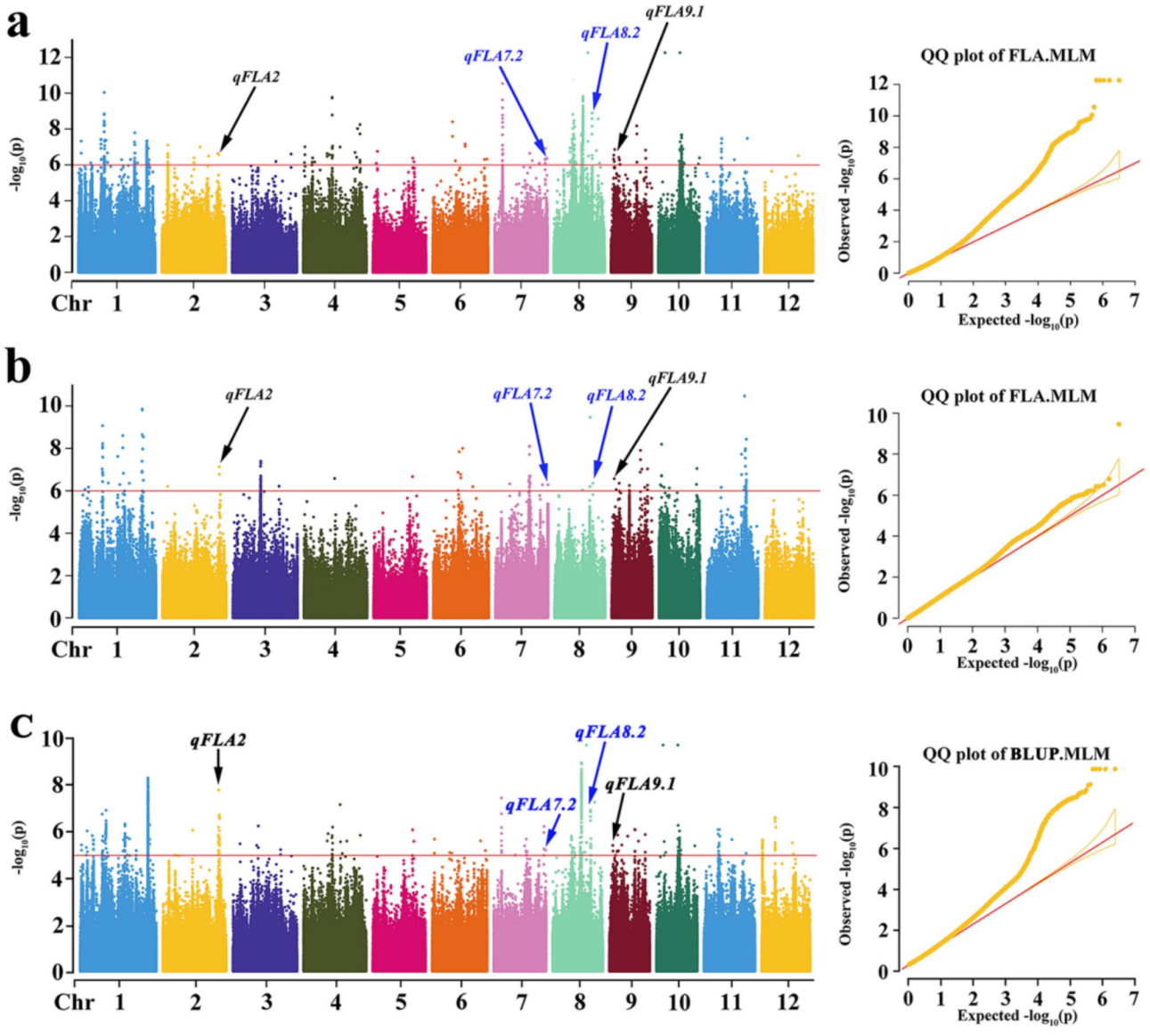

Fig. 4 The black arrow represents the QTLs detected by an MLM; the blue arrow represents a QTL colocalized with previous studies. **(a)** Manhattan diagram and Q–Q diagram of the 2022 FLA GWAS. **(b)** Manhattan diagram and Q–Q diagram of the 2023 FLA GWAS. **(c)** Manhattan diagram and Q–Q diagram of the BLUP GWAS

Table 3 Genome-wide significant association of rice FLA traits (BLUP)

QTLs	Chromosome	Position	Allele	$Log_{10}(P)$[a]	MAF[b]	Known Genes/QTLs
qFLA1.2	1	13,332,762	G/A	5.21–5.83	0.38	
qFLA1.4	1	24,305,157	T/C	5.19–5.68	0.38	
qFLA2	2	32,057,055	C/T	5.11–6.75	0.36	
qFLA7.2	7	29,419,260	C/T	5.22	0.06	*qFLA7e* [7]
qFLA8.1	8	15,708,371	A/G	5.08–5.77	0.11	
qFLA8.2	8	21,508,180	A/G	5.01–6.96	0.32	*qFLA8f* [7]
qFLA9.1	9	1,907,184	C/T	5.19	0.07	
qFLA9.2	9	4,404,631	C/T	5.16–5.88	0.06	

[a]The $-log_{10}(P)$ value and $-log_{10}(P)$ value range obtained by GWAS in single environments (BLUP) are described [58]

[b]Minimum allele frequency

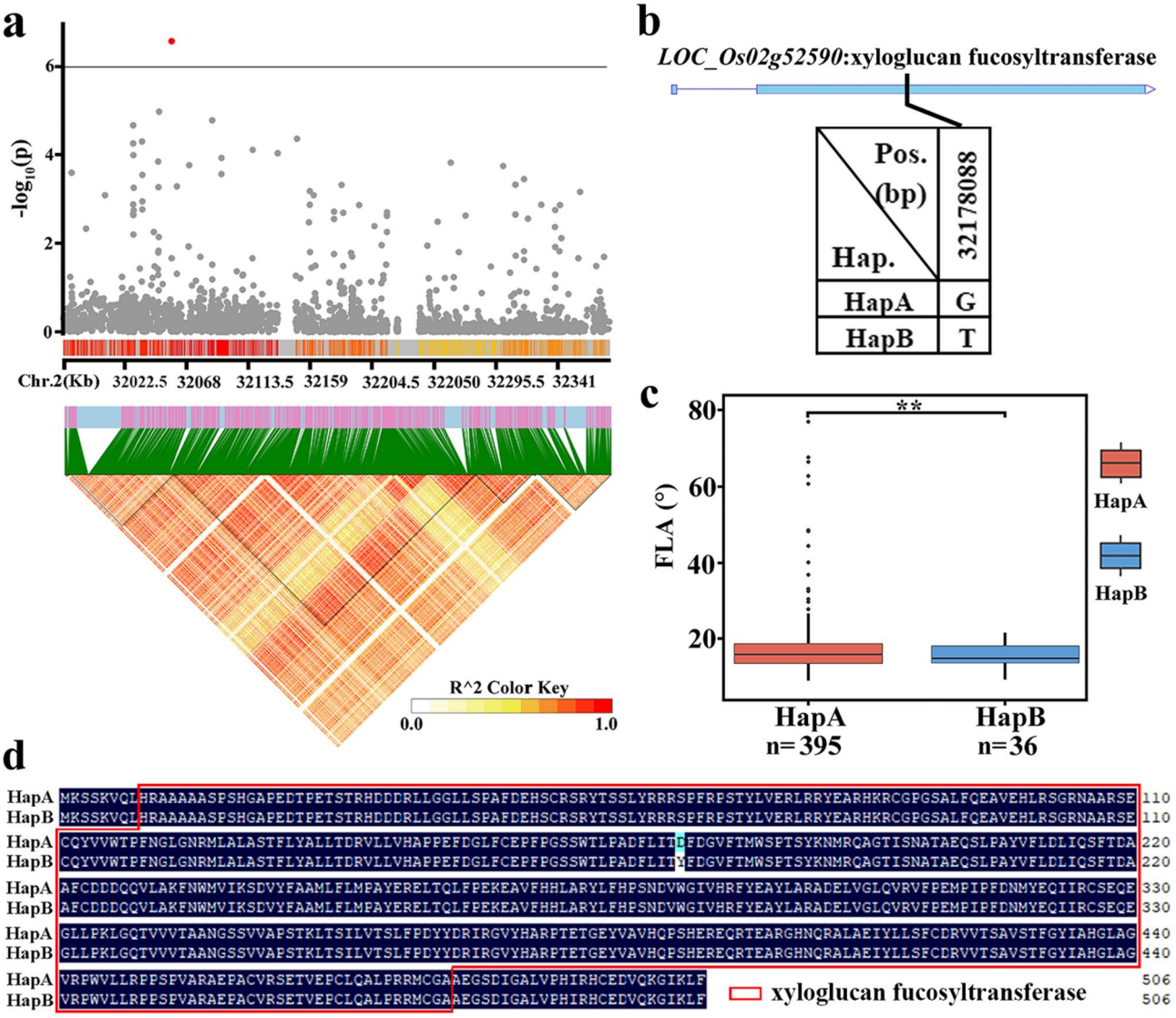

Fig. 5 Identification of the *qFLA2* candidate gene. **(a)** Local Manhattan graph based on a single polymorphism and LD heatmap of the candidate gene *FLA2*. **(b)** Two haplotypes of *FLA2* were identified based on 1 SNP in all the evaluated rice materials. In the gene structure map of *FLA2* (http://rice.plantbiology.msu.edu), the promoter is indicated by the white box; exons are represented by blue boxes; introns and intergenic regions are marked with blue lines. The thin black line represents the genomic location of each SNP. **(c)** Tukey's test was used to analyse the differences between haplotypes. ** indicates significance at the $p < 0.01$ level. **(d)** The amino acid sequences of HapA and HapB were compared, and the red line indicates the amino acid length of the protein encoded by the *FLA2* gene

Discussion

In this study, the FLA phenotypic data of 431 rice varieties were investigated for two consecutive years, and the results revealed extensive phenotypic variation. The coefficient of variation of FLA was between 52.20% and 58.69% (Table 1), which was similar to the coefficient of variation reported in other studies. In the study by Jiang et al. [4], the coefficient of variation of 353 FLAs measured in six different environments in Hefei and Nanjing over three years ranged from 55.55 to 58.48%. Gui et al. [62] used 221 microcore germplasm resource populations planted in Liuyang, Hunan Province, in 2018 and 2019 as research materials, and the FLA variation coefficient ranged from 59.14 to 64.90%. These phenotypic variations are all moderate variations [63] and may be related to high genetic diversity [64]. The average generalized heritability of FLA over two years was 66.7% (Table 1), indicating that FLA was strongly affected by environmental factors but that heredity was stable. There was a significant interaction among FLA, genotype and the environment, indicating that FLA has a complex genetic regulatory mechanism. In addition, the average, maximum and minimum values of FLA in 2022 were greater than those in 2023. We speculate that this difference is caused by extremely high-temperature weather in 2022,

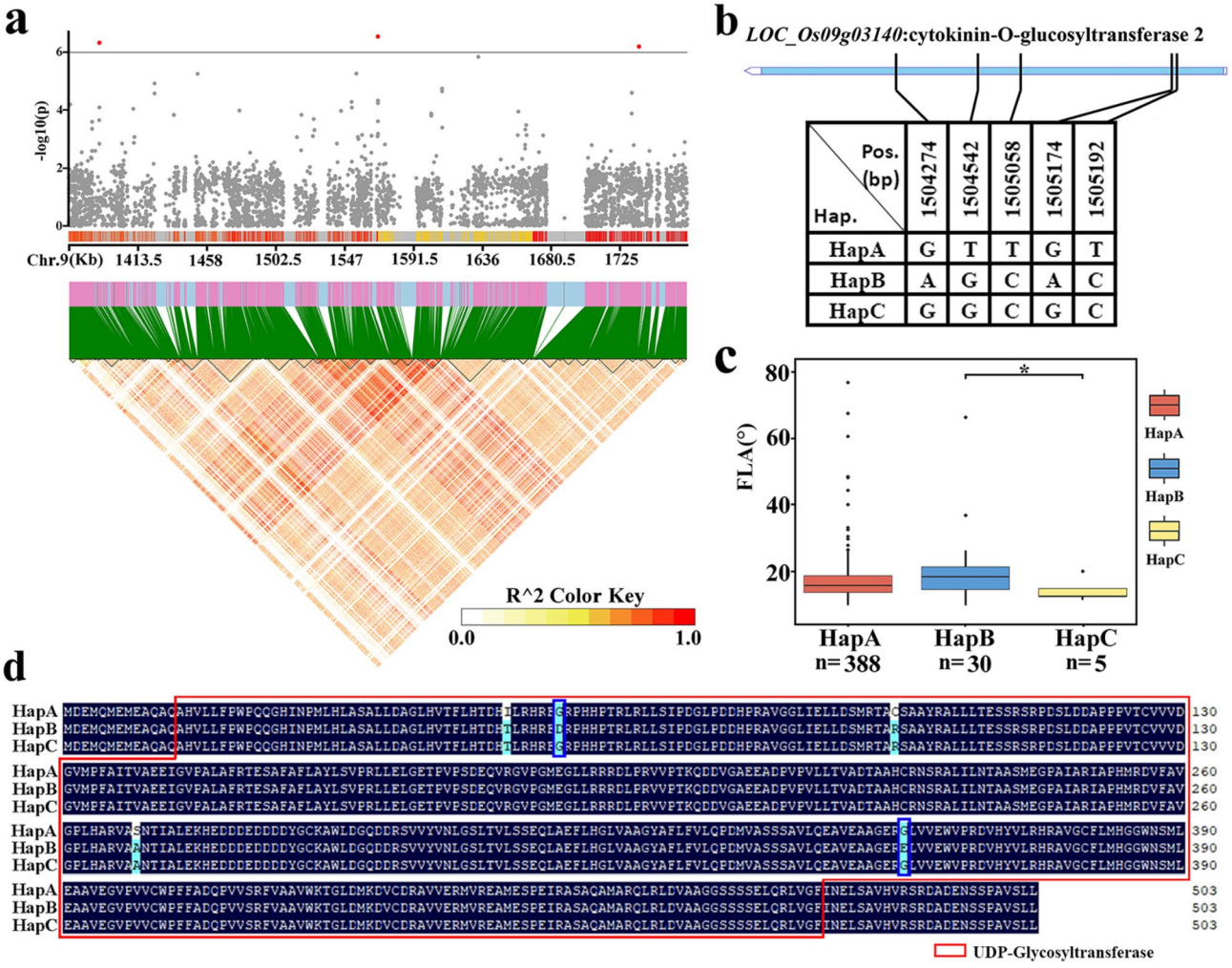

Fig. 6 Identification of the *qFLA9.1* candidate gene. **(a)** Local Manhattan graph based on a single polymorphism and LD heatmap of the candidate gene *FLA9*. **(b)** Three *FLA9* haplotypes were identified based on 5 SNPs in all the evaluated rice materials. In the gene structure map of *FLA9* (http://rice.plantbiology.msu.edu), the promoter is indicated by the white box; exons are represented by blue boxes; introns and intergenic regions are marked with blue lines. The thin black line represents the genomic location of each SNP. **(c)** Tukey's test was used to analyse the differences between haplotypes. * indicates significance at the $p < 0.05$ level. **(d)** The amino acid sequences of HapA, HapB and HapC were compared, and the red line indicates the amino acid length of the protein encoded by the *FLA9* gene

indicating that the size of the rice FLA is determined by both genetic and environmental factors [25].

In GWASs, population structure information is usually incorporated to avoid incorrect marker–trait associations [65]. Genetic relationships and PCA are helpful for inferring population structure from genotype data in GWASs [66]. Genetic relationship analysis and PCA were used to divide the FLA genotype into four subgroups (Fig. 3b and c), which was consistent with the phylogenetic tree obtained by iTol. The genotypes contained in the four subgroups were substantially different. The *Xian* subgroup contained 269 genotypes, with an average FLA of 16.42°; the *Geng* subgroup contained 137 genotypes, with an average FLA of 18.7°; the *admix* subgroup contained 17 genotypes, with an average FLA of 17.5°; and the *Bas* subgroup contained 8 genotypes, with an average FLA of 23.28°. This finding indicates that the *Geng* and *Bas* subgroups contain mainly large FLA genotypes, whereas the *Xian* and *admix* subgroups contain most of the small FLA and medium FLA genotypes. In addition, the *FLA2* favourable haplotype was mainly found in the *Xian* subgroup, and the *FLA9* favourable haplotype was mainly found in the *Geng* subgroup.

A GWAS is a genome-wide system tool based on LD used to study the associations between population traits and SNPs. This method has been widely used to identify QTLs and genes associated with important traits in many crop species [67–69]. Regression models are often constructed to test whether there is a correlation between markers and phenotypes. GLMs are often used to assess genetic markers. However, a GLM leads to a serious overestimation of the site effect value and produces

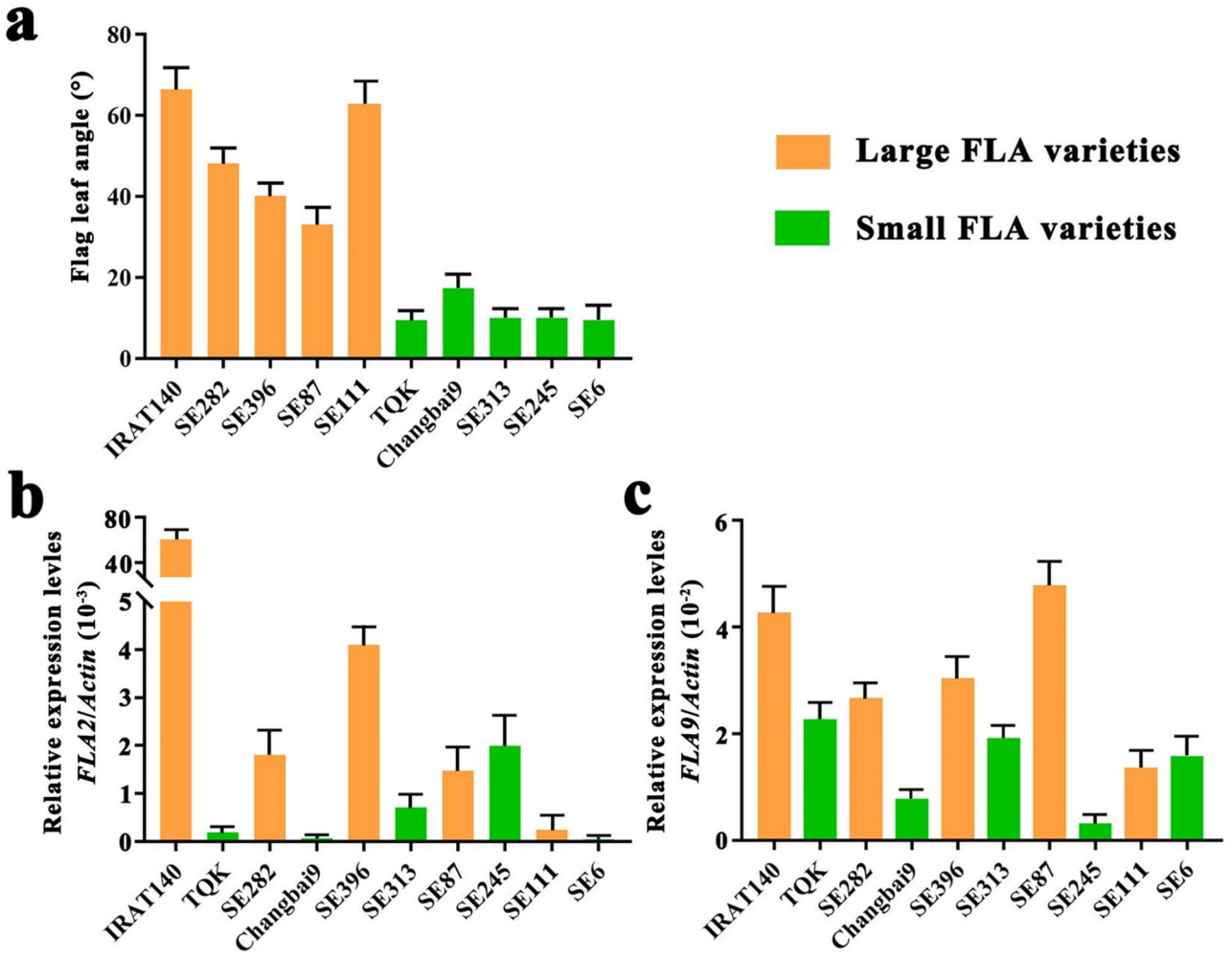

Fig. 7 qRT–PCR analysis of *FLA2* and *FLA9*. **(a)** The phenotypic data of five large FLA and five small FLA materials for qRT-PCR analysis were expressed as mean ± s.d. **(b)** qRT–PCR analysis of *FLA2*. **(c)** qRT–PCR analysis of *FLA9*

false-positive results. An MLM can effectively adjust for the population structure and complex genetic relationships within the population and better control false positives [51, 70]. Therefore, we used an MLM to ensure the accuracy of the results.

We compared the QTLs for FLA identified in this study with previously reported QTLs (Fig. 9). Among the eight QTLs, the locations of *qFLA7.2* (29,419,260 ~ 29,641,042 bp) and *qFLA8.2* (21,508,180 ~ 21,599,529 bp) are close to those of the previously reported QTLs *qFLA7e* (28,893,769 ~ 29,673,928 bp) and *qFLA8f* (20,968,463 ~ 21,468,267 bp) [7] and are considered the same loci. According to gene function and haplotype analysis, two candidate genes, *FLA2* and *FLA9*, were ultimately preselected. *FLA2* is annotated as xyloglucan fucosyltransferase, which is a complex plant polysaccharide in the glycoside hydrolase family and has high intrinsic affinity for cellulose [71]. *FLA2* encodes the catalytic enzymes xyloglucan endotransglucosylase (XET) and xyloglucan endotransglucosylase/hydrolase (XTH) [72, 73], which are necessary for controlling cell wall extension and remodelling, especially the recombination of cellulose microfibers with cross-linking [74, 75]. This process promoted the deposition of hemicellulose and cellulose, increased the accumulation of starch in flag leaves, and thickened the cell wall of flag leaf mesophyll cells, thus affecting the FLA (Fig. 10). In addition, in Arabidopsis, XTH participates in shade avoidance response to adapt to the decrease of low-red/far-red light ratio, which is mainly manifested by the elongation of stems and petioles [76]. The petiole elongation rate of the gene knockout mutant under green shade and low-red/far-red light was lower than that of the wild type. Whether it has the same performance in rice needs further exploration [77]. Another candidate gene *FLA9* was annotated as cytokinin-o-glycosyltransferase 2, which is a key enzyme in plant regulation of cytokinin (CTK) level and function [78]. Cytokinin glycosylation of glycosyltransferases fine-tunes the synthesis, metabolism and function

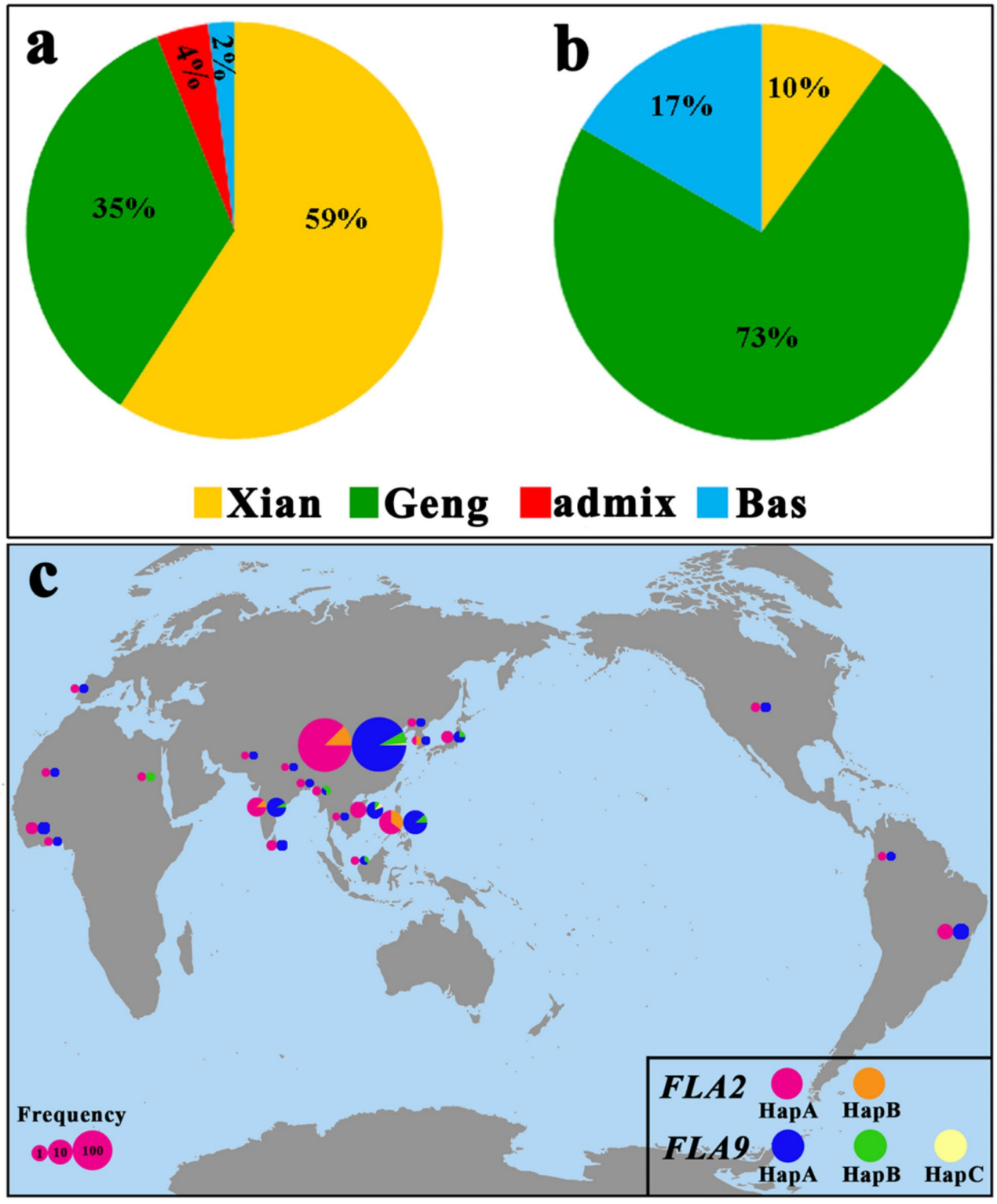

Fig. 8 Population information and geographical distribution of favorable haplotypes. **(a)** Population composition of favourable haplotypes of *FLA2*.**(b)** Population composition of favourable haplotypes of *FLA9*.**(c)** Geographic information distribution map of 431 haplotypes

Table 4 Excellent parents predicted according to FLA for hybrid breeding

Best Predicted Parents	Subgroup	Predicted FLA Improvement (°)		Total Predicted FLA Improvement (°)	Seed-setting Rate
		FLA2	FLA9		
SE352	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.71
SE349	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.71
SE65	*Bas*	HapA (0.16)	HapB (2.62)	2.78	0.71
IRAT140	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.72
SE131	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.75
SE351	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.76
SE368	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.78
SE204	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.84
Handao297	*Geng*	HapA (0.16)	HapB (2.62)	2.78	0.85
SE119	*Xian*	HapA (0.16)	HapB (2.62)	2.78	0.90
SE71	*Bas*	HapA (0.16)	HapB (2.62)	2.78	0.91

of cell division peptides, thereby affecting the transport and distribution of cell division proteins in cells and tissues, related signal transduction processes and upstream regulatory factors, as well as the normal growth and development of plants [79]. It is mainly manifested in maintaining the growth potential (pluripotency) of shoot apical meristem, providing stem cells for the formation of leaf primordia at the initial stage of leaf formation, and determine the phyllotaxis pattern by interacting with auxin (IAA) (Fig. 10) [80]. However, whether cytokinins have a direct regulatory effect on FLA remains to be explored.

In the rice production of pure lines, in order to maximize the planting density, crop lines with erect flag leaves are preferred, which can withstand higher planting rates, and erect flag leaves can significantly improve photosynthetic capacity, increase grain filling rate, and increase yield [81]. However, for hybrid rice production, upright leaves will hinder cross-pollination and reduce cross pollination rate. Large FLA can be pollinated normally without artificial leaf cutting. So the large FLA can increase the pollination rate and improve the seed setting rate during hybrid rice production [4]. Therefore, both small FLA and large FLA are benefit for rice yield improvement during different types of rice breeding. If the elite alleles of *FLA2* and *FLA*9 are used for hybrid rice seed production, the pollen of restorer or maintainer lines can be avoided from being occlusion by the flag leaves of sterile lines, and the artificial cutting of flag leaves can be avoided, so that more pollen of the sterile line falls on the stigma to complete pollination. (Fig. 10). Increasing the yield of hybrid seeds saves labour, greatly improves the outcrossing rate, and promotes the mechanization of hybrid rice. Considering that small FLA can increase the yield of pure line rice production, we have also predicted the excellent parents with alleles of small FLA for pure line rice breeding (Table S7).

The elite alleles identified in this study can improve FLA in rice, considering that the seed-setting rate is an important factor in hybrid rice. We ultimately identified 11 rice varieties with both excellent alleles and better seed-setting rates as predicted excellent parents. One of the eleven predicted excellent parents belonged to the *Xian* group, two belonged to the *Bas* group, and eight belonged to the *Geng* group. The FLA of *Geng* rice is theoretically greater than that of *Xian* rice (Table 4), indicating that *Geng* rice performed better. Moreover, we speculate that the large difference in the seed-setting rates in this study is due to the high-temperature weather during the flowering period. Under high-temperature stress, the flowering period of rice is shortened, the daily flowering time is prolonged, the peak value is reduced, anther dehiscence is blocked, and pollen viability and stigma viability are decreased, resulting in a decrease in the seed-setting rate [82]. The performance of all the predicted excellent parents in this study needs to be verified in the production environment.

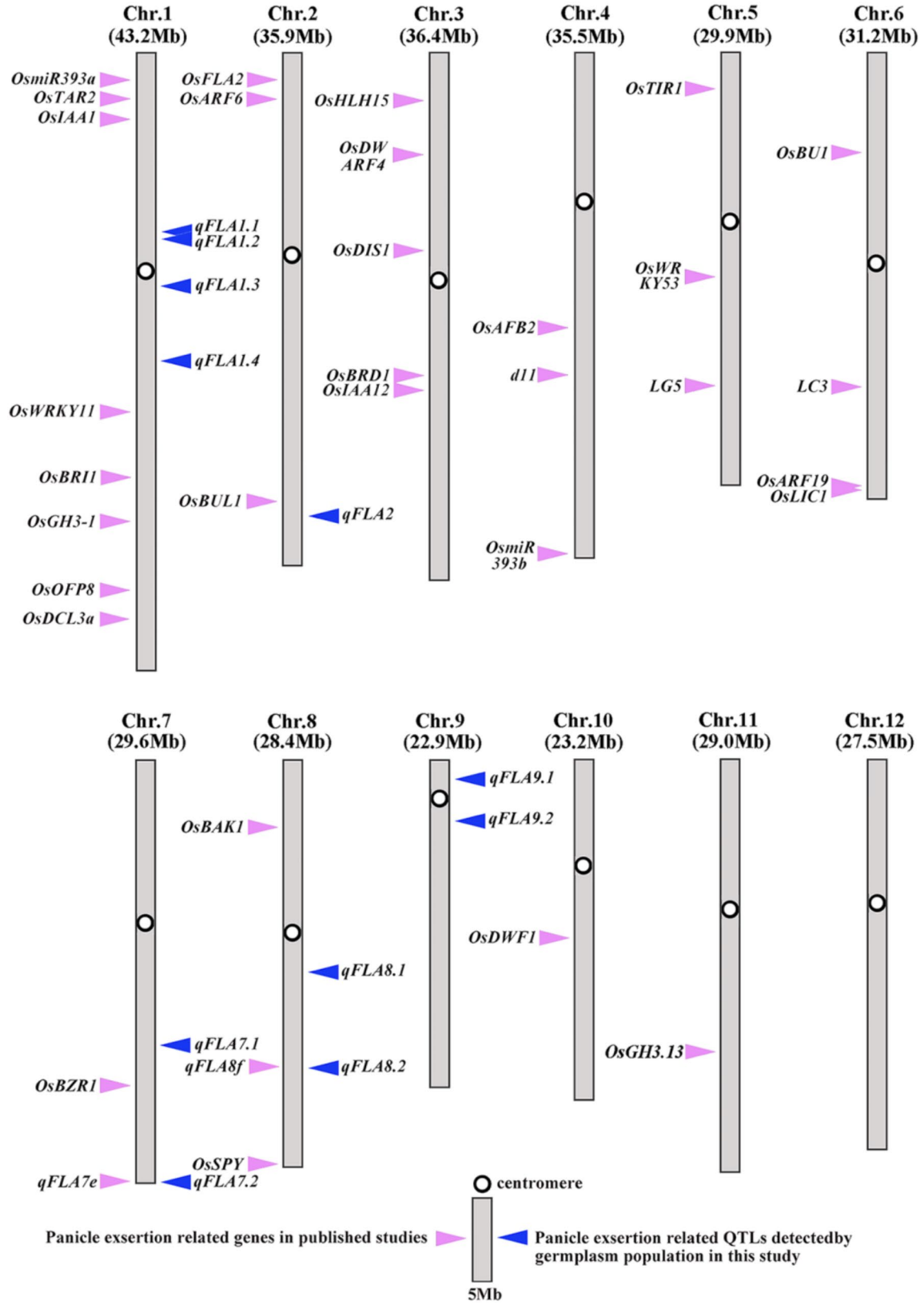

Fig. 9 Pink represents previously reported genes, and blue represents the QTLs mapped in this study

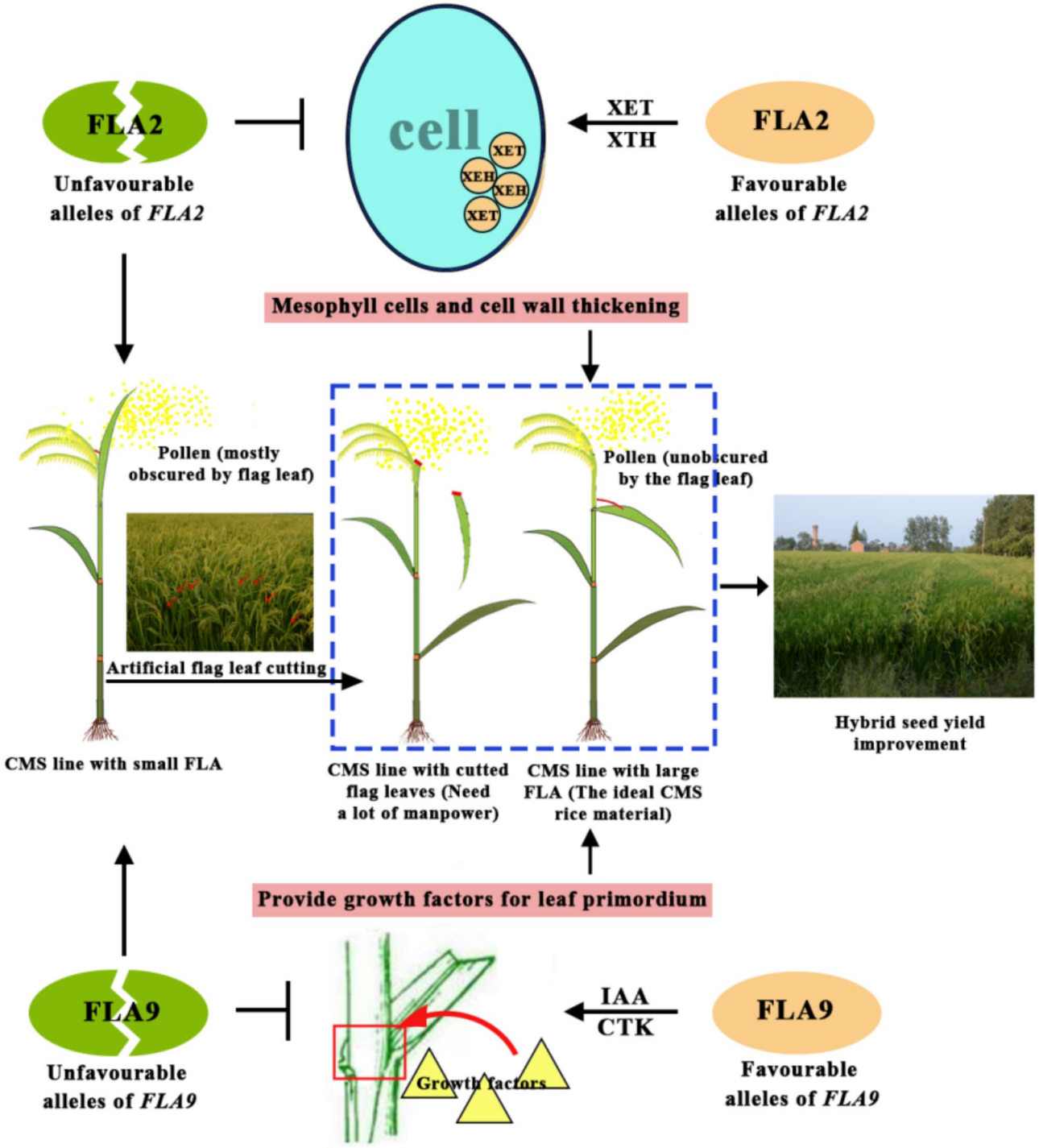

Fig. 10 Hypothetical model of the functions of *FLA2* and *FLA9* in terms of the FLA. XET: Xyloglucan endotransglucosylase, XTH: Xyloglucan endotransglucosylase/hydrolase, CMS: Cytoplasmic male sterility, FLA: Flag leaf angle, IAA: Auxin, CTK: Cytokinin

Conclusions

A total of six new QTLs associated with FLA were detected by a GWAS of 431 rice accessions in 2022 and 2023. *FLA2* (*LOC_Os02g52590*) and *FLA9* (*LOC_Os09g03140*), which encode xyloglucan fucosyltransferase and cytokinin-O-glucosyltransferase 2, respectively, were identified as candidate genes for FLA. In addition, all the elite alleles and excellent parents predicted in this study can provide a molecular basis for improving the FLA in hybrid rice breeding.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11487-z.

Supplementary Material 1: Table S1. List of all the rice accessions used for SNP genotyping. Rice accessions are labelled with their geographical location, sequence number, subpopulation, material name, source, and phenotypic data for 2022 and 2023.

Supplementary Material 2: Table S2. Candidate gene annotation in the 31.8–32.2 Mb LD region associated with FLA.

Supplementary Material 3: Table S3. Candidate gene annotation in the 1.37–1.77 Mb LD region associated with FLA.

Supplementary Material 4: Table S4. Gene haplotype distribution of 431 accessions.

Supplementary Material 5: Table S5. Thirty parents containing ***FLA2*** (HapA) and ***FLA9*** (HapB) and their seed-setting rate information.

Supplementary Material 6: Table S6. The primers used for qRT-PCR in this study.

Supplementary Material 7: Table S7. The parents with small FLA predicted for pure line breeding.

Author contributions

Y-N Z, Z-B L, H S, Y-P C and J-H S conducted the experiments and collected the data. X-P C, M-Y D and Y-Y Z carried out data collation and statistical analyses. T-H L and Z Y constructed the graphics. T-H L wrote the paper. E-B L designed the experiments and reviewed the paper. All the authors have read and agreed to the published version of the manuscript.

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Data availability

The data supporting this article are included within the article and its Supplementary Material. The resequencing data of the rice experimental materials used in this study have been published in the NCBI database (https://www.ncbi.nlm.nih.gov/), and the variant locus information is available through the SNP-Seek database (http://snp-seek.irri.org/). The Nipponbare genome sequence and protein sequence were downloaded from the International Rice Genome Sequencing Project (http://rice.plantbiology.msu.edu). The gene function of the candidate region was obtained from the National Rice Database Center (https://www.ricedata.cn/). The map resources used in the haplotype geographic information distribution map can be downloaded from the standard map service system (http://211.159.153.75/).

Declarations

Ethics approval and consent to participate

The research reported here did not involve experimentation with human participants or animals. Therefore, there was no need to obtain consent for participation.

Consent for publication

The research does not contain any individual person's data in any form, and all the authors have provided consent for publication. There were no human participants, so there was no need to obtain consent for publication.

Competing interests

The authors declare no competing interests.

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