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ARTICLE

Meta-Analysis of Flowering-Related Traits and Mining of Candidate Genes in Maize

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ABSTRACT

Maize flowering is an important agronomic character, which is controlled by quantitative trait loci (QTL). Over the years, a large number of flowering-related QTL have been found in maize and exist in public databases. However, combining these data, re-analyzing and mining candidate loci and fine mapping of flowering-related traits to reduce confidence intervals has become a hot issue in maize. In this study, the QTL of 6 important agronomic traits of maize flowering were collected from 15 published articles, including flowering period (DA), Days to tasseling (DTT), Days to silking (DS), Days to pollen shedding (DTP), anthesis-silking interval (ASI) and the photosensitive (PS). Through meta-analysis, 622 QTL were integrated into 26 meta-QTLs (MQTL). Finally, the candidate genes related to maize flowering (Gene IDs: ZM00001D005791, ZM00001D019045, ZM00001D050697, ZM00001D011139) were identified by Gene Ontology (GO) enrichment and hierarchical cluster analysis of expression profile. Based on the results of this study, the genetic characteristics of maize flowering traits will be further analyzed, which is of great significance to guide the improvement of important agronomic characters and improve the efficiency of breeding.

KEYWORDS

Maize; flowering-related traits; meta-analysis; MQTL; candidate gene

1 Introduction

Maize (*Zea mays* L.) originated in the American continent and spread to Europe at the end of the 15th century, and then gradually spread to the whole world along the Mediterranean Sea. Corn is one of the most important sources of food, feed and industrial raw materials in the world. Flowering is an important agronomic character of maize, which is affected by complex factors such as light, temperature, latitude, agronomic measurement and stress [1]. Therefore, a better understanding of the genetic mechanism of maize flowering is helpful to improve the adaptability of different dimensions of maize, improve agronomic characters, and enhance stress resistance and yield.

At present, there have been a large number of reports on quantitative trait loci mapping of related agronomic, physiological and biochemical traits in maize. However, it is still difficult to apply these QTL directly to breeding practice [2]. The main reasons are as follows: (1) The primary population is often

used for QTL analysis; (2) The mapping population is small, and the population type and genetic background are different; (3) Little is known about the relationship between QTL which controls different traits in different materials; (4) The accuracy of the map is low, and the interval span of the located QTL is large, so it is difficult to find closely linked molecular markers related to QTL. By integrating the QTL information of different mapping populations, the QTL meta-maps of related traits were constructed, which made it possible for these QTL information to be used in molecular marker-assisted selection breeding [3].

Meta-analysis was originally proposed and named by Glass (1976). Since then, meta-analysis has become a research method widely used in many disciplines. QTL meta-analysis is an effective method for generating meta-QTLs (MQTL). By integrating information from different mapping populations, it can more accurately identify the genomic intervals of controlled traits and reduce the confidence intervals of these QTL [4]. In recent years, the application of meta-analysis in maize has also been reported. Wang et al. [5] Integrated 637 QTL of 6 agronomic traits in maize, including yield, plant height, ear position, leaf angle, stay-green and maize disease. These authors reported that the *GA3ox2* genes controlling plant height, *lg1* and *lg4* regulating leaf angle, and *zfl1* and *zfl2* genes controlling the flowering stage of maize were screened from 113 MQTL formed by bioinformatics technology. Chen et al. [6] formed 76 MQTL by integrating 999 QTL of maize yield-related traits, and screened candidate genes related to maize kernel size and weight by regional association mapping. Arfang et al. [7] formed 102 MQTL by integrating 388 QTL of maize insect resistance related traits, including leaf insect resistance, stem insect resistance and kernel insect resistance. They found the co-localization of fiber and hydroxycinnamate components in MQTL, and confirmed the new idea of mechanical insect resistance. Salvi et al. [8] integrated 313 QTL on Chromosomes 8 and 10, identified 62 MQTL related to flowering, and finally successfully cloned the *Vgt1* gene in bin 8.05 by association mapping analysis. These successful cases prove that meta-analysis is an effective method to predict candidate genes and integrate molecular markers for complex traits. It also provides a new way for comparative mapping of genes with different traits. This provides a theoretical basis for our meta-analysis of flowering characters of maize.

Table 1: Sources of QTL used in this study

Reference	Parent	QTL number	Cross type	Population size	Trait
Wang et al. [9]	Huangzao4, CML288	13	RIL	207	DTT, PS
Wang et al. [10]	Huangzao4, CML288	29	RIL	201	DA, DTP, PS
Steinhoff et al. [11]	ParentA, ParentB, ParentC, ParentD	10	F_3	684	DA, DS
Almeida et al. [12]	CML444, MALAWI	21	RIL	234	ASI
Chardon et al. [13]	F7p, F_2	13	F_3	200	DA, DTP
Jiao et al. [14]	Qi319, Chang7-2	14	DH	119	DTP
Li et al. [15]	B73, B97	101	RIL	500	ASI, DA, DS, DTP
Vanous et al. [16]	PH251, PHB47	26	DH	252	ASI, DA
Journal et al. [17]	Natural group	13	Natural group	72	DS, DTT
Chen et al. [18]	W22, TIL01	14	BC_1S_4	1257	ASI, DTP, DS

(Continued)

Table 1 (continued)

Reference	Parent	QTL number	Cross type	Population size	Trait
Matteo et al. [19]	A632, B73, B96, F7, H99, HP301, Mo17, W153R	29	MAGIC	1636	DS
Liu et al. [20]	B73, Mo17	69	RIL	244	DTP
Negro et al. [21]	DROPS, UH007	221	F_3	247	DA
Cui et al. [22]	Y915, Z58	10	F_3	192	DTT
Ren et al. [23]	X178, 9782	39	RIL	262	DS, DTP, DTT

In this study, six QTLs for flowering agronomic traits in maize were integrated using meta-analysis, and the integrated MQTL was mapped onto the physical map of B73. This work provides a reference for consistent QTL analysis, fine mapping, candidate gene cloning and molecular breeding. The meta-QTL and candidate genes reported in this paper deserve further study based on metabonomics and molecular biology methods.

2 Materials and Methods

2.1 QTL Data Collection and Data Generation

We collected QTL information for flowering-related traits in maize from 15 studies published between 2005 and 2020 (Table 1). We surveyed published QTL experiments on maize: throughout the flowering period (DA), Days to tasseling (DTT), Days to silking (DS), Days to pollen shedding (DTP), anthesis-silking interval (ASI), and the photosensitive (PS). QTL information on flowering-related traits of maize was collected from different databases retrieved by PubMed in NCBI (http://www.ncbi.nlm.nih.gov/pubmed). Additionally, MaizeGDB (http://www.maizeGDB.org) web resources were surveyed to collect more QTL information. When QTL information is not given in the article, its attachment can be downloaded to obtain QTL confidence interval, LOD value, R^2 and other information. When only the location of QTL is given in the article, you can find the left and right makers in the IBM2 2008 reference map for the next experiment. When entering QTL information, the parameters included QTL name, trait, logarithm of the odds (LOD) value, phenotypic contribution rate (R^2) and the most likely location of QTL and the beginning and end of its confidence interval. When some articles did not provide LOD values or phenotypic contribution rates (R^2), R^2 were estimated using the individual LOD value of QTL, and the formula was $R^2 = 1 - 10^{(-2LOD/N)}$, where N is the population size. When selecting map, if the article used the single nucleotide polymorphism (SNP) tag to locate the QTL, we usually downloaded the generic map IBM2 2008 Neighbors at MaizeGDB (http://www.maizegdb.org). Also, if the article used the SSR molecular marker to locate the QTL, we used the map in the article to generate them. When marker coordinates were not available, we used the AdobeReader distance measurement tool (https://helpx.adobe.com) to measure the interval of different markers for each chromosome on the map. Then, we used the scale to convert the distance to centimeters (cM).

2.2 Meta-Analysis

First, all the QTLs collected were projected, followed by a meta-analysis. All QTLs were superimposed on the reference map IBM2 2008 to generate a consensus map. IBM2 2008 Neighbors is a high-density genetic linkage map formed by integrating all kinds of maize molecular markers, which often share some common markers with the original mapping map of QTL. Through the Maps-compilations mapping function of BioMercator4.2 software, the distance of common markers is calculated by homogeneous

function, and the maximum possible position and confidence interval of the initial QTL of different mapping groups are proportionally integrated into the IBM2 2008 Neighbors reference map. According to the integration results, the meta-analysis of ten chromosomes was carried out, and the lowest QTL value of the model was selected as the most suitable model. In addition, the most likely position and confidence interval of consistent QTL were determined by including 2 or more populations and 3 or more Akaike information criterion (AIC) as screening conditions.

2.3 GO Annotation of Candidate Genes

The physical intervals of these MQTL were identified by MaizeGDB. The physical location of the left and right markers of these MQTL was confirmed on the IBM2 2008 neighbor graph. According to the annotated version of maize B73 genome AGPv3, all the annotated transcripts in the MQTL region were mined in the maizeGDB (http://curation.maizegdb.org/). The annotated version of AGPv3 was then converted to AGPv4 through Assembly Converter in Gramene. Visualize the results of GO comments through the WEGO2.0 website.

2.4 Expression Profile Analysis of Maize Candidate Genes

To determine the expression patterns of candidate genes in maize tissues, a comprehensive expression analysis based on genome-wide coverage was first performed based on published microarray data, which are publicly available on MaizeGDB (http://www.maizegdb.org). According to the expression information in MaizeGDB, 33 genes enriched by GO were screened for the first time, and the genes with no expression or very low expression were removed, and finally 26 genes were obtained. Maize plant material B73 is provided by plant Biotechnology Center of Jilin Agricultural University. The environment is in an artificial weather room, with temperature controlled at 25°C and humidity controlled at 60%RH. Proper irrigation ensures normal flowering and powder scattering. Root, Stem, Leaf and shoot apical meristem (SAM) of plant materials are taken at the tasseling stage, pollen and tassel are taken at the pollen shedding, and embryo, endosperm and seed are taken at the filling stage. Weigh 10 g of each sample and store in a refrigerator at −80°C. Total RNA was isolated from the collected samples using Trizol reagent (Invitrogen, USA) and then treated with DNase I to remove any genomic DNA contamination. The first cDNA strand was synthesized from 1 μg total RNA using the catenary ect Rev. Transcription Kit. The qRT-PCR primers were designed and commercially synthesized by Comate Bioscience Co., Ltd., (China). The qRT-PCR machine was set to 40 cycles and the annealing temperature was 60°C. At least three biological replicates were performed on each cDNA sample. The relative mRNA level of each gene was calculated by the $2^{-\Delta\Delta CT}$ method to calculate the multiple changes of the expression level of related genes. Microarray analysis was accomplished to identify expression patterns in representative tissues, including seed, root, pollen, stem, embryo, leaf, tassel, SAM (shoot apical meristem) and endosperm. By using China's online mapping software bioinformatics (http://www.bioinformatics.com.cn) we carried out the hierarchical clustering analysis based on the average linkage Pearson coefficient: the corresponding gene expression in several different tissues was analyzed by cluster analysis.

3 Results

3.1 Collected QTL Distribution

A total of 622 QTL for maize throughout the flowering period (DA), Days to tasseling (DTT), Days to silking (DS), Days to pollen shedding (DTP), anthesis-silking interval (ASI), and the photosensitive (PS) were collected in this study. The mapping population includes seven recombinant inbred lines (RIL), five F_3 populations, two double haploid (DH) populations, one Multiparent Advanced Generation Inter-Cross (MAGIC) population and one natural population. Among them, the size of QTL mapping population was between 72–1636. The number of QTL for each trait was 10–221 (Table 1). The 622 QTLs collected

were unevenly distributed on ten chromosomes (Fig. 1A). The maximum number of QTL distributed on Chromosome 1 was 115, while Chromosome 10 exhibited the fewest, with 34 (Fig. 1A). In the statistics of collected QTL traits, it was found that a total of 80.23% of $R^2 < 10\%$, and that the phenotypic contribution rate of QTL explanation greater than 10% was relatively small (Fig. 1B). The results showed that the flowering and related traits of maize were mainly controlled by many small genetic loci and showed complex heredity.

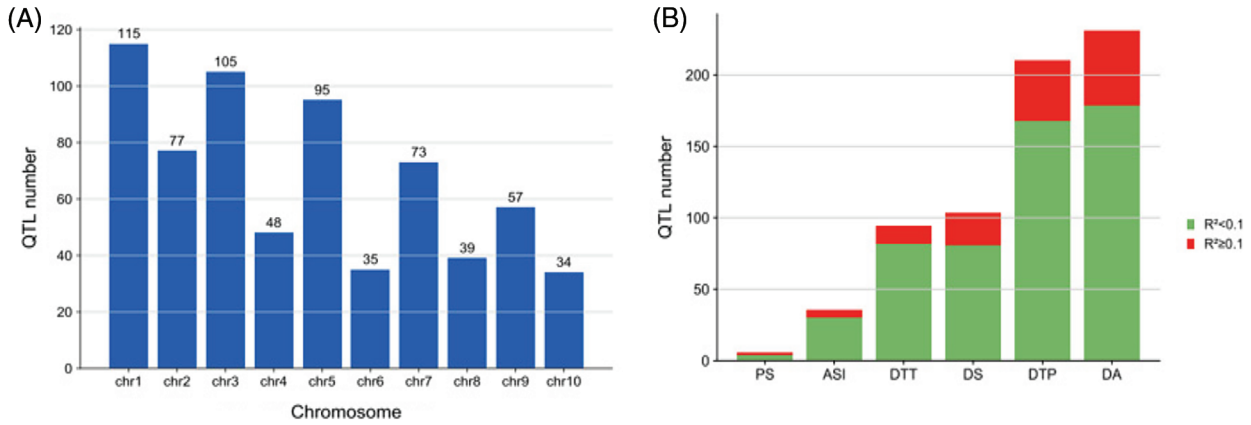

Figure 1: The number of QTL and related R^2 values are distributed throughout the genome. (A) The number of QTL on each chromosome. (B) R^2 of different characters in this study

3.2 Meta-QTL Identification

Through meta-analysis, the collected QTL information of maize flowering was mapped to the target map IBM2 2008, and the MQTL was mined to narrow the QTL confidence interval. According to the model with the lowest AIC value, a total of 26 MQTL were identified (Table 2, Fig. 2). All MQTL were unevenly distributed on ten chromosomes: there was one MQTL on Chromosome 5; two MQTL on Chromosomes 1, 3 and 6, and three MQTL on Chromosomes 2, 4, 8, 9 and 10. Among them, the number of MQTL on Chromosome 7 of maize was the highest. These MQTL are sequenced from MQTL1 to MQTL26 according to their chromosomal position (Table 2).

Table 2: QTL meta-analysis results

MetaQTL name	Chr.	MetaQTL position	Left marker	Right marker	MetaQTL interval (cM)	Physical distance (Kb)	MetaQTL interval (Kb)
MQTL1	1	439.24	hb127	bzip45	439.24–439.28	139,125–140,632	1507
MQTL2	1	454	umc1076	glk9	440–468	141,636–163,233	21597
MQTL3	2	341.17	umc1454	hagtf12	339.3–343.04	69,935–75,862	5927
MQTL4	2	347.16	lug6	pco111783	346.72–347.6	104,705–106,286	1581
MQTL5	2	383.32	wrky55	myb111	383.04–383.6	181,753–183,333	1580
MQTL6	3	235.6	thx41	caf2	235.2–236	58,035–58,422	387
MQTL7	3	251.08	cl16458_1	ks4	249.76–252.4	99,433–100,981	1548
MQTL8	4	297.72	zhd19	hb90	297.68–297.76	86,930–87,735	805
MQTL9	4	298.4	sap5	e2f16	298.24–298.56	106,651–107,858	1207
MQTL10	4	298.64	e2f16	invan3	298.56–298.72	111,078–113,895	2817

(Continued)

Table 2 (continued)

MetaQTL name	Chr.	MetaQTL position	Left marker	Right marker	MetaQTL interval (cM)	Physical distance (Kb)	MetaQTL interval (Kb)
MQTL11	5	294.5	bzip14	nlp15	294–295	74,803–75,892	1089
MQTL12	6	88.6	sin1	ereb72	88–89.2	46,523–47,087	564
MQTL13	6	91.2	limtf11	abi37	90.4–92	53,854–54,982	1128
MQTL14	7	138.62	his1a	rps1	125.2–152.04	11,490–16,498	5008
MQTL15	7	183.2	hak18	cncr2	183.08–183.32	45,664–47,726	2062
MQTL16	7	183.8	uaz187	rif1	183.7–184	54,502–55,386	884
MQTL17	7	184.4	ereb189	brl1	184–184.8	61,278–65,403	4125
MQTL18	8	255.4	mlkt2	pro1	255.2–255.6	94,636–95,222	586
MQTL19	8	265.37	umc1427	phi100175	255.84–274.9	96,268–100,867	4599
MQTL20	8	370	cl17205_1	umc2650	357.2–382.8	129,794–138,584	8790
MQTL21	9	224.4	phd27	TIDP3766	224.2–224.6	40,233–40,755	522
MQTL22	9	226.7	phs1	IDP375	226.6–226.8	57,737–58,259	522
MQTL23	9	254.9	AW257883	bnl5.04	253.7–256.1	101,627–103,455	1828
MQTL24	10	189.58	gpt1	glx1	187.16–192	59,075–60,076	1001
MQTL25	10	204.1	pza01677	propep4	204–204.2	71,090–71,841	751
MQTL26	10	250.06	acc1	GRMZM2G157313	250–250.12	98,374–99,125	751

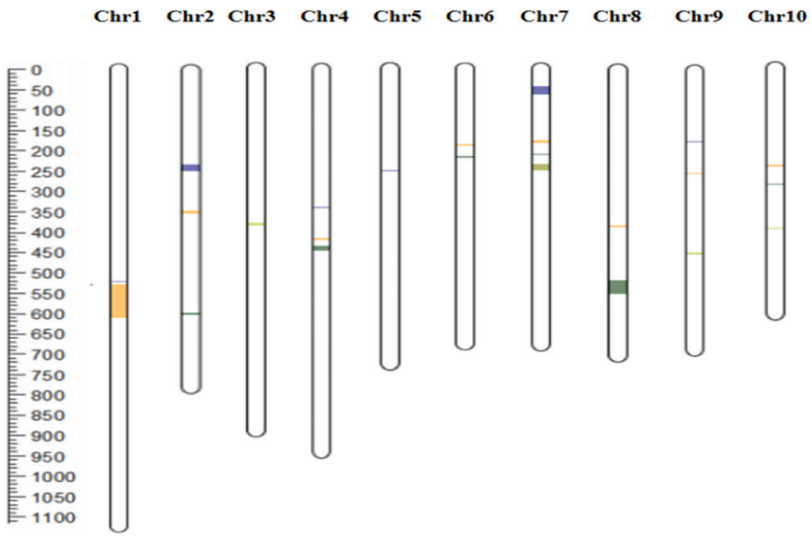

Figure 2: Identification of the location and distribution of MQTL on ten chromosomes. The y-scale measures chromosome length and QTL relative location

3.3 GO Annotation in Meta QTL Region Genes

The annotated transcripts of a total of 26 MQTL were analyzed by gene (GO). There are 331 genes in 26 MQTL regions (Table S1). According to WEGO classification, 33 genes were annotated, which were divided into three categories (cellular composition, molecular function and biological pathway) (Fig. 3).

We found that 47 common genes related to flowering-related traits were enriched in GO term including internal protein amino acid acetylation, internal peptidyl-lysine acetylation, histone acetylation, histone H4 acetylation, histone H4-K16 acetylation, chromatin organization, histone H4-K5 acetylation, histone H4-K8 acetylation, protein acetylation, peptidyl-lysine modification (Fig. 3). We were pleasantly surprised to find that the enriched genes were related to histone acetylation. Histones block the process by which chromosomes convert chromatin through methylation, acetylation, and phosphorylation. By changing the degree of relaxation in the double helix structure of chromosomes, the expression of *FT (FLOWERING LOCUS T)* and other related flowering genes can be prevented, so as to inhibit or promote the flowering of plants.

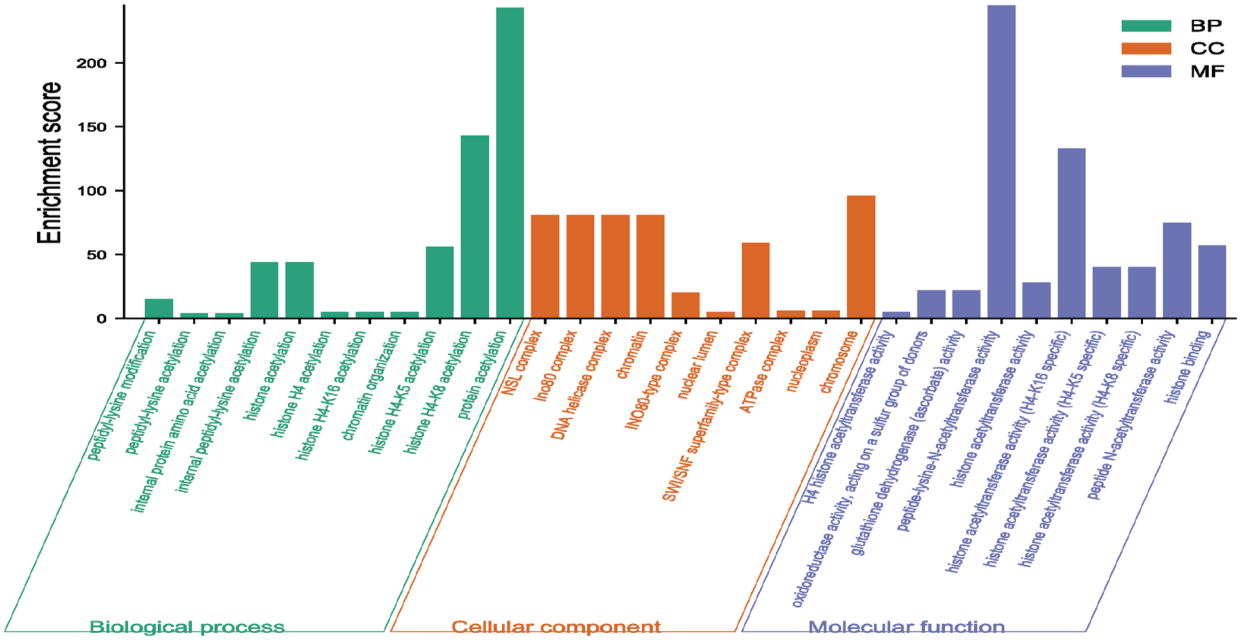

Figure 3: GO analysis of genes located in MQTL region

3.4 Expression Analysis of Maize Flowering Genes

The results of heat maps of different tissues of 26 candidate genes showed that Zm00001D035869, Zm00001D011092, Zm00001D011139, Zm00001D019045, Zm00001D005791, Zm00001D050697 have the highest expression levels in roots, stems, seeds, embryos, SAM and tassel (Fig. 4). We found that the expression of all candidate genes in pollen was not high, and it is possible that candidate genes are not involved in the formation of mature pollen. We found that candidate genes related to flowering were highly expressed in roots, which may be related to the collected ASI traits. The ASI is an important Index of drought resistance in maize. Plants can blossom ahead of time, improve survival, and ensure heredity and yield of offspring. We chose the candidate genes with high expression of seeds, embryos, SAM and tassel because flowering-related candidate genes are actively expressed in these maize tissues and are closely related to flowering.

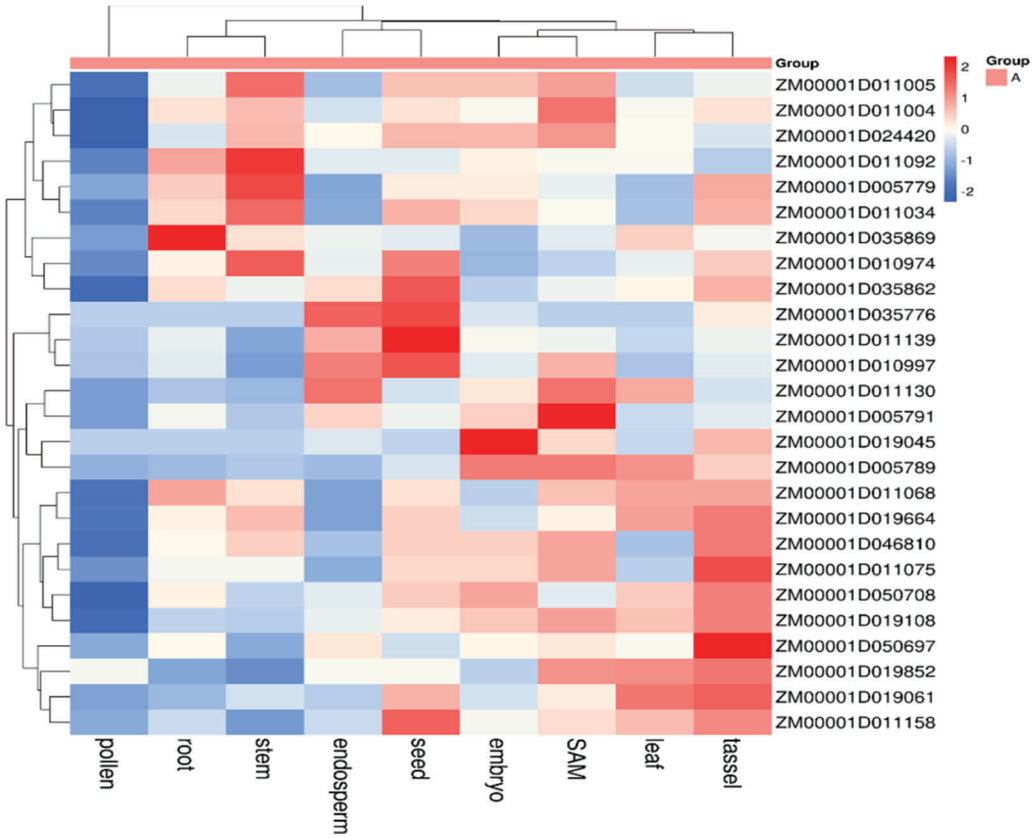

Figure 4: Cluster analysis of 26 gene expression profiles in 9 tissues. The color represents the proportion of the $\log^2$ signal value. Blue indicates a lower level and red indicates a higher level of transcript. Below are the different tissues in corn, and on the right are the genes annotated by GO analysis

4 Discussion

Flowering time is not only an important agronomic character in maize breeding, but also a core problem of other plants. In out-pollinated maize, flowering time is controlled by many tiny genes to form a complex network of contacts. In this study, we collected QTL of maize flowering-related traits from different sources. The QTL of different molecular marker localization was mapped to IBM2 2008 Neighbors reference map. Meta-analysis methods were used to analyze, and MQTLs were obtained, respectively. QTLs for flowering-related traits located by SNP marker technology were effectively used. By further searching for common genes in MQTLs of the same related traits, we can mine maize flowering candidate genes, which reduces the screening of candidate genes. Compared with non-common genes, common genes are more likely to participate in the establishment of maize and flowering traits.

Functional annotation of the genes could better understand the metabolic pathways involved in them, and provide a reference for the next step to mine candidate flowering genes in maize. In this study, two main pathways (histone acetylation, chromatin organization) were obtained by GO enrichment in the common gene set of flower-related traits (Fig. 3). Histone modification enzymes exist in different organisms and regulate chromatin conversion into chromosomes by changing the structure or location of nucleosomes [24]. Histone modifying enzymes covalently alter nucleosome histones by acetylation, methylation, or phosphorylation. These histone modification enzymes regulate other proteins by modifying nucleosomes in a complex network, and also regulate chromatin state in conjunction with

ATP-dependent chromatin remodelers. In *Arabidopsis thaliana*, the regular arrangement of nucleosomes in the genome requires ISWI chromatin remodeling agents CHROMATIN REMODELING 11 (CHR11) and CHR17, thereby inhibiting the expression of the inflorescence transfer genes *FT* and *SEP3* (*SEPALLATA 3*) [25]. They also interact with MADS-box transcription factors and are responsible for the morphogenesis and formation of floral organs.

Through the heat map of the expression levels of 26 candidate genes in different tissues, it was found that the expression levels of 4 candidate genes were higher in seeds, embryos, SAM and tassel, respectively (Fig. 4). In maize, ear genes can regulate the normal development of corn flowers, in which ZmMADS1 and ZmMADS3 genes affect the growth of corn ears [26]. ZmMADS47 gene can affect the storage activity of endosperm through the interaction with Opaque2 [27]. Overexpression of other MADS-like genes can lead to changes in the structure and content of starch in seeds and embryos, and changes in sugar content. SAM is positively associated with flowering. Through literature review, We found that when SAM transforms into male inflorescences, called tassels, the apex of the plant ends [28]. In SAM, *FT* interacts with 14-3-3 receptor protein and bZIP transcription factor *FD (FLOWERING LOCUS D*) to form a fluorescein activation complex *(FAC)*. *FAC* activates the transcription of *AP1 (APETELA 1)*-like MADS box genes, which marks the beginning of reproductive development. The genetic module of *FT-FD* is conserved in maize. The *DLF1 (DELAYED FLOWERING1*) gene encodes a *bZIP* transcription factor like FD, which mediates the floral signal in the shoot tip [29]. The extended gene family like *FT* in maize is called *Zea ZCN* (*CENTRORADIALIS*), reflecting their diversified functions. *ZCN8* gene has the function of promoting flowering, and it has recently been proved to be helpful to adapt to flowering at different latitudes. So we chose four respectively in seeds, embryos, SAM and tassel expressed in a high percentage of candidate genes, which contains a complex of genes and gene networks, providing a theoretical basis for further understanding the genetic characteristics. Based on the above results, four candidate genes related to maize flowering were screened, which provided a reference for the excavation of maize flowering candidate genes.

5 Conclusion

Flowering and its related traits are complex quantitative traits controlled by many less affected QTL in maize. The integration and meta-analysis of a large number of QTL provide valuable information for the fine mapping of QTL and the cloning of key genes. A total of 622 QTLS related to maize flowering were collected in this study, and 26 MQTLS were identified in the maize genome by meta-analysis. The candidate genes were obtained by MQTL mapping on the reference map of B73 maize. Firstly, GO enrichment analysis was performed to determine the histone pathway and its effect on chromatin structure through methylation, acetylation and phosphorylation to inhibit or promote flowering. After that, 33 genes obtained by GO enrichment were preliminarily screened by the open expression level on MaizeGDB, and genes with no expression or very low expression level were eliminated. Subsequently, fluorescence quantitative PCR was performed on the 26 genes obtained in seed, root, pollen, stem, embryo, leaf, tassel, SAM and endosperm respectively, and we could clearly see the expression changes of different genes in different tissues. And finally obtained 4 candidate genes closely related to flowering. The results laid a foundation for elucidating the genetic basis of maize agronomic characters. In addition, the information of meta-QTL and candidate genes reported in this study is helpful to different functional markers and fine mapping of genes in maize breeding projects.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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