BMC Plant Biology

RESEARCH Open Access

Molecular mechanisms of flowering time differentiation revealed by transcriptomic sequencing and *de novo* analysis in Chinese invasive populations of *Ambrosia artemisiifolia*

Xiao-Feng Yang[1,4], Xiao-Meng Li[2,4*], Pär K. Ingvarsson[3], Chao Xi[4] and Wan-Jin Liao[1,4*]

Abstract

Background *Ambrosia artemisiifolia* is a highly invasive herb with deleterious effects on public health and agricultural systems. Flowering time in this species has been reported to vary along a latitudinal gradient, which may contribute to local adaptation and invasion success in China. However, the molecular basis for the flowering time differentiation remains unclear.

Results A common garden experiment confirmed a latitudinal gradient in flowering time among seven Chinese populations. Differentially expressed genes (DEGs) across sampling times and flowering time groups were identified through transcriptome sequencing and analyses of DGE and WGCNA, and were partially annotated to circadian rhythm, light response and hormone response through GO enrichment. By annotating to Flowering Interactive Database (FLOR-ID) and protein-protein interaction (PPI) databases, 53 candidate genes for flowering time differentiation were identified, with 23 of these genes linked to the photoperiod pathway. Additionally, 43 of 53 candidate genes exhibited expression correlated with latitude. Six genes, including *FKF1*, *FT*, *FUL*, *MAF2*, *WNK4* and *WNK5*, were inferred to promote flowering, while 5 genes, *FBH3*, *FLK*, *NCL(1)*, *POL2A*, and *ZHD4*, likely repress flowering, based on their expression patterns in relation to latitude and sampling times. Notably, *NCL(1)*, *FBH3*, *MAF2*, and *FLK* may function differently in *A. artemisiifolia* compared to *Arabidopsis thaliana*.

Conclusions This study identified key candidate genes related to the differentiation of flowering time in Chinese ragweed populations, providing valuable insights into molecular mechanisms of phenological adaptation and invasive success of ragweed.

Keywords Differentiation of flowering time, Flowering regulatory pathway, Invasive plants, Transcriptome

*Correspondence:
Xiao-Meng Li
xiaomengli@bnu.edu.cn
Wan-Jin Liao
liaowj@bnu.edu.cn
[1]Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Beijing Normal University, Beijing 100875, China
[2]National Demonstration Center for Experimental Life Sciences and Biotechnology Education, Beijing Normal University, Beijing 100875, China
[3]Department of Plant Biology, Uppsala BioCenter, Swedish University of Agricultural Sciences, PO Box 7080, Uppsala 750 07, Sweden
[4]College of Life Sciences, Beijing Normal University, Beijing 100875, China

Background

Plant adaptations to their habitats are crucial for their growth, survival and reproduction [1]. Invasive plant populations generally experience novel environments from their native habitats and this may lead to the rapid evolution of local adaptation to their introduced abiotic or biotic conditions [1, 2], which may result in them eventually outperforming native populations [3]. Numerous studies have confirmed that local adaptation plays an important role in the successful establishment and spread of invasive species [4–6]. One clear manifestation of local adaptation is clinal variation in characteristics related to adaptation [1]. Flowering time, a key trait in the transition from vegetative to reproductive development, is known to be highly susceptible to local selection [7]. It has been reported that latitudinal clinal variation of flowering time plays crucial roles in the successful invasion of several species, such as *Lythrum salicaria* [4], *Medicago polymorpha* [8], and *Ambrosia artemisiifolia* [6]. Many studies have demonstrated that genes associated with flowering time and plant growth are linked to the success of invasive species [2, 9]. These findings suggest that the ability to adapt to different environments through genetic variation in key developmental processes, such as flowering time, can promote the successful invasion of introduced plants. Hence, unraveling the molecular mechanisms underlying the differentiation of flowering time is imperative for understanding factors that contribute to successful invasion of introduced species.

Flowering time is thought to be a quantitative trait and is influenced by both genetic and environmental factors [10]. Environmental factors, including light, temperature, water availability, and humidity, as well as endogenous factors, such as hormones and age, collectively contribute to the intricate regulation of flowering in plants [11–13]. Extensive research in model species, such as *Arabidopsis thaliana*, has identified numerous genes and pathways involved in regulating flowering time [14]. In *A. thaliana*, the dynamic process of flowering is primarily induced by seven pathways, including the photoperiod, vernalization, gibberellin, temperature, autonomous, age, and sugar pathways [15, 16]. These exogenous and endogenous signals function as upstream signals and transmit information to floral integrators, such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). These integrators subsequently integrate and relay diverse signals to downstream genes that ultimately trigger the process of flowering [16]. Genetic mechanisms regulating flowering time have also been elucidated in many other species, revealing both conservation and divergence in flowering time regulation across taxa [17, 18]. For example, key elements of the genetic network that regulate flowering time, including homologous genes such as photoreceptors (*PHYTOCHROMEs*, *PHYs*), circadian clock genes (*GIGANTEA*, *GI*), and integration factors (*FTs*), are conserved across species such as *A. thaliana*, rice (*Oryza sativa*), and many temperate cereals [17]. However, functional disparities in primary flowering-related genes have been observed among species. The *BOLTING TIME CONTROL 1* (*BTC1*) has been shown to regulate flowering time in sugar beet [19] and *E11* in soybean [20], but not in *A. thaliana*. In addition, genetic factors regulating flowering time differ among species or geographical regions within species. For example, allelic variation at the *FLOWERING LOCUS C* (*FLC*) locus accounts for variation in flowering time in *Capsella rubella* [21]. Genes such as *Pseudo-Response Regulator 37* (*PRR37*), *FRI-LIKE3a* (*FRL3a*), and *GIGANTEA 1* (*GI1*) have been identified as significant contributors to the variation in flowering time in *Zea mays* [22]. Additionally, the effect of *FLOWERING LOCUS T-like 9* (*PhFTL9*) on flowering time has been observed in *Panicum hallii* [23]. These findings highlight the complexity and diversity of the genetic mechanisms controlling flowering time across species, emphasizing both the conservation of key regulatory elements and the emergence of species-specific adaptations.

Common ragweed (*Ambrosia artemisiifolia*) is a wind-pollinated annual herb native to North America and Mexico [24]. This species has been unintentionally introduced into multiple regions around the world, including Asia, South America, Oceania, and Europe. Invasive populations of this species commonly have high genetic diversity probably due to multiple introductions and genetic admixture from diverse origins [25–28]. The detrimental impact of *A. artemisiifolia* on the economy and public health is increasing in both native and invasive areas because of its highly allergenic pollen and the formidable challenge it poses in disturbed habitats [29–33]. Investigating the underlying mechanisms behind its extensive spread will facilitate the effective management of this species in invasive ranges. Latitudinal variation in flowering time of *A. artemisiifolia* has been documented in both its native and invasive ranges [5, 6]. The genetic differentiation of flowering time and other phenotypic traits between different Chinese populations has been observed in common garden experiments, and flowering time is considered to play an important role in the local adaptation of invasive populations through its direct and indirect effects on fitness [6]. Therefore, elucidating the genetic factors underlying variation in flowering time would greatly contribute to unraveling the intricate mechanisms facilitating its successful spread. Although several flowering-related genes have been quantitatively analyzed via real-time quantitative PCR and have been shown to be associated with differentiation of flowering time in Chinese populations [34], a more thorough investigation is needed to obtain more

comprehensive information on the genetic mechanisms of this differentiation.

In this study, we investigated the variation in flowering time and the genetic basis underlying this variation among seven populations of *A. artemisiifolia* from different latitudes across China. We observed a latitudinal cline in flowering time in a common garden experiment [6]. To explore candidate genes controlling flowering time differentiation, RNA-Seq analyses were conducted on these seven populations at three sampling times covering different developmental stages of the populations. Analysis of differential gene expression (DGE) was conducted to examine variation in gene expression across populations with different flowering times at each sampling time and across sampling times for each flowering time group. In addition, weighted gene co-expression network analysis (WGCNA) was also performed on the seven populations, using flowering time and population as phenotypes, to identify critical modules and pivotal genes responsible for the differentiation of flowering time between populations in *A. artemisiifolia*. Through these analytical methods, we aim to determine (1) the molecular mechanism for flowering time differentiation in Chinese invasive populations of *A. artemisiifolia*; (2) the mechanisms of local adaptation for Chinese populations of *A. artemisiifolia* and (3) the crucial regulatory networks and pathways that govern flowering time. The present study provides novel insights into the genetic and environmental interactions influencing flowering time in *A. artemisiifolia*, paving the way for more effective strategies in management of invasive plants.

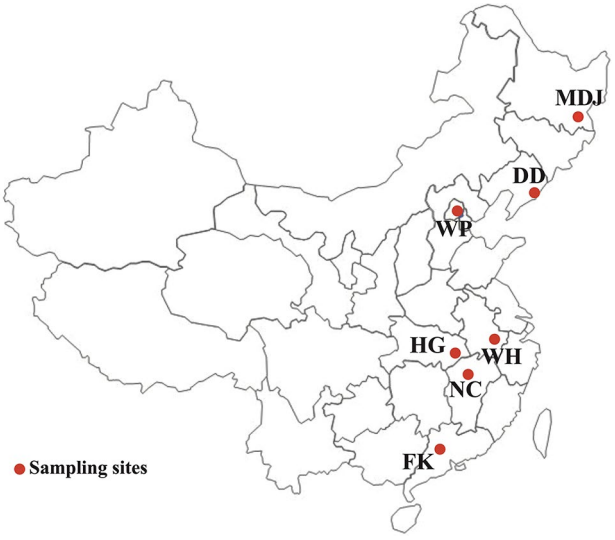

Fig. 1 Populations of *Ambrosia artemisiifolia* included in this study. The red dots indicate the sampling sites. The population codes used in this study are as follows: MDJ for Mudanjiang, DD for Dandong, WP for Wangping, WH for Wuhu, HG for Huanggang, NC for Nanchang and FK for Fengkai

Methods

Common garden experiment

The common garden experiment was conducted in the field garden at Beijing Normal University (39.96°N, 116.36°E). In 2019, seeds of *A. artemisiifolia* (voucher specimens were identified by Prof. Wan-Jin Liao and deposited in the herbarium of Beijing Normal University) were collected from seven geographically separated populations in China along a latitudinal cline, including Mudanjiang (MDJ, 44.60°N, 129.68°E), Dandong (DD, 40.18°N, 124.32°E), Wangping (WP, 39.98°N, 116.01°E), Wuhu (WH, 31.22°N, 118.37°E), Huanggang (HG, 30.46°N, 114.93°E), Nanchang (NC, 28.68°N, 115.83°E), and Fengkai (FK, 23.45°N, 111.50°E) populations, with each population consisting of seeds obtained from 30 maternal families (Fig. 1). On April 18, 2020, ten seeds were randomly chosen from each of 10 families and sown in Petri dishes filled with moist vermiculite, resulting in a total of 100 seeds sown per population. The Petri dishes were placed at 4 °C for cold stratification for two weeks and then transferred to ambient temperature to facilitate germination. Time to germination was recorded for each sample. After the development of the first set of true leaves, 25 seedlings for each population were randomly chosen and transplanted into 9 cm pots filled with a blend of vermiculite and potting soil at a 1:1 ratio. Finally, a total of 96 individuals with similar growth performance, were monitored throughout the growing season, each population with 5–22 plants. Flowering time was recorded as the duration in days from seed germination to the initial appearance of male inflorescences, taking into consideration the potential protandry of this species [35]. Difference in flowering time among populations was tested by carrying out the Kruskal-Wallis test in R (v 4.1.1), followed by Dunn's Test for pairwise comparisons with Benjamini-Hochberg adjusted *p* values.

On July 18th, August 21st, and September 27th, 2020, leaf tissues were collected from three biological replicates for each population at approximately 12 p.m. Samples were cut into pieces and immediately stored in RNA*later* solution (Life Technologies, CA, USA) at -20 °C until RNA extraction. The three sampling times covered almost all the developmental stages of all seven populations (Additional file 1 Table S1). Leaf tissue was used for transcriptome analysis to elucidate the genetic underpinnings of interpopulation variation in flowering time, as genes associated with flowering may be expressed in leaves and regulate the development of floral organs and the timing of flower emergence [36].

RNA extraction, library construction and Illumina sequencing

Total RNA from each sample was extracted via the TRIzol® Plus RNA Purification Kit (Thermo Fisher

Scientific, Inc.). The quality of the RNA was assessed via agarose gel electrophoresis and a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and the concentrations were evaluated via a Qubit® RNA Assay Kit on a Qubit® 2.0 fluorometer (Life Technologies, CA, USA).

Libraries for each sample were constructed using approximately 3 μg of RNA as input material. The sequencing libraries were prepared via the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's guidelines, and index codes were added to assign sequences to each respective sample. The index-coded samples were subjected to clustering on a cBot Cluster Generation System via the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina NovaSeq 6000 platform using 150 bp paired-end reads at a depth of 6 Gb per sample.

De novo transcriptome assembly

Reads were cleaned using Trimmomatic (version 0.39) [37] with default settings. This process involved trimming adapter sequences, removing reads containing poly-N regions and deleting reads with quality less than 3. Additionally, reads shorter than 36 bases were excluded from the analysis. A *de novo* transcriptome assembly was conducted to assemble the contigs into a nonredundant set of unigenes via Trinity (v2.1.1), setting 300 as the minimal length of assembled contigs. Sequence redundancy was reduced using CD-HIT (v4.8.1) by removing sequences with over 97% identity. The longest transcripts were considered as nonredundant unigenes. The effectiveness of the assembly was evaluated through N50 calculation in Trinity (v2.1.1) and examination of gene mapping to OrthoDB database in benchmarking universal single-copy ortholog (BUSCO) (v3.0.2). Potential protein coding domain sequences (CDS) for unigenes were predicted using TransDecoder (v5.5.0). We explored multiple assembly strategies, including assembling all individuals together and assembling samples from different populations at specific time points, and selected the final assembly based on N50 values and completeness evaluations using BUSCO. After comparing various assemblies, the July sample from the DD population provided the highest N50 and BUSCO scores and was selected as the reference.

Differential gene expression analysis of samples among sampling times and among flowering time groups

For all high-quality reads after Trimmomatic filtering, read counts were obtained by mapping to the reference of DD population in July. Then, the Fragments Per Kilobase Million (FPKM) values for each sample were estimated using the RSEM package (v1.3.3) and employed as the indicator of gene expression level [38, 39]. Genes with FPKM-value greater than 1 in at least one individual in each population were retained and their FPKM-values were normalized into Trimmed Mean of M-values (TMM) for subsequent study.

To conduct DGE analysis, we categorized the seven populations into three groups according to the flowering stages of the populations at sampling times (Additional file 1 Table S1): the early-flowering (EF) group, which included the MDJ population; the late-flowering (LF) group, which included the FK population; and the group with moderate flowering time (MF), which included the DD, WP, WH, HG, and NC populations. The DGE analysis was conducted on samples from different sampling times (July, August and September) and samples from the different flowering time groups (LF, MF and LF) using DESeq2 (version 1.24.0) based on a model using the negative binomial distribution [40]. The comparisons with log2 (fold change) value greater than 1 or smaller than −1 and adjusted p-value less than 0.05 were considered to be significant and the genes were considered to be differentially expressed genes (DEGs). The DEGs were enriched to the Gene Ontology (GO) database via the clusterProfiler package (version 3.12.0), using enricher function with BH (FDR correction with Benjamini-Hochberg) method. The enriched terms for the DEGs were identified to be significant when adjusted p-value ≤ 0.05. GO terms that had annotations of "photosynthesis" and "photosystem" were considered as photosynthesis related functions, terms of "water deprivation", "stress", "cold" and "heat" were flagged as functions of response to stress, term of "biosynthetic process" was regarded as functions of biosynthetic process and terms of "hormone", "salicylic acid", "abscisic acid", "ethylene" and "jasmonic" were deemed as hormone related functions. The DEGs were subsequently subjected to a search for orthologous genes regulating flowering in the curated Flowering-Interactive Database (FLOR-ID) [41], which is an interactive database of flowering-time gene networks in *Arabidopsis thaliana*. Additionally, via protein–protein interaction (PPI) analysis, the DEGs were annotated to the STRING database [42], and genes with functions related to flowering regulation were considered as flowering-related genes. Flowering-related DEGs among sampling times and among flowering time groups are considered to play crucial roles in the differentiation of flowering time among populations of *Ambrosia artemisiifolia*. The correlations between the expression levels of these candidate genes and origin latitude were analyzed using Spearman correlation analyses in R software (v 4.1.1), to further assess correlations between candidate gene expression and the latitudinal cline in flowering time.

Weighted gene co-expression network analysis (WGCNA)

To investigate the genetic basis underlying the differentiation of flowering time, we constructed a co-expression network on the basis of gene expression data obtained from samples collected at each sampling time. The WGCNA package (v 1.72-5) in R (v 4.1.1) was utilized to build the expression network [43]. The similarity matrix between each pair of genes across all samples was calculated based on their Pearson's correlation values, and the genes were clustered into different modules using "dynamic tree cut" method in WGCNA. The power threshold parameter was set to 5. The type of topological overlap matrix (TOM) was set to unsigned, and the minimal number of genes in the modules was set to 50. We performed two WGCNA analyses using flowering time and population as the phenotype, respectively. The Pearson correlation coefficients between the phenotypes and gene modules were calculated using the cor and corPvalueStudent functions and visualized within a heatmap plot using labeledHeatmap function. The gene modules significantly related to flowering time differentiation were identified. Subsequent annotation of all genes within these pivotal modules was conducted using the GO database via the R package clusterProfiler (version 3.12.0) to ascertain their functions. Moreover, to further delineate critical genes specifically involved in the regulation of flowering time, all genes within the pivotal modules were cross-referenced with the Flowering-Interactive Database [41] and the PPI protein STRING database [42]. The candidate genes identified in these WGCNA were considered to be related to the differentiation of flowering time if they differ in expression among sampling time in the DGE analyses simultaneously. The correlations between the expression levels of these genes and the origin latitudes of populations were evaluated using Spearman correlation analyses in R (v 4.1.1) to elucidate the candidate flowering-related genes responsible for the latitudinal clinal pattern of flowering time.

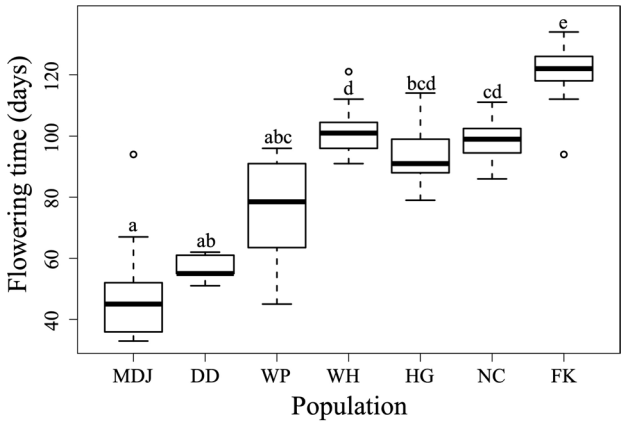

Fig. 2 Box plot showing the flowering times of different populations of *Ambrosia artemisiifolia* in China. The central line in the box represents the median value. Lowercase letters indicate differences between populations ($p < 0.05$)

Results

Variation in flowering time among populations

Flowering time showed a notable disparity among populations ($W = 0.923$, $p < 0.001$), with populations originating from lower latitudes exhibiting delayed flowering compared with those from higher latitudes (Fig. 2). The flowering time, days from germination to flowering, was 48.3±5.99 (mean±SE) for plants from the northern MDJ population, 122.5±1.13 for plants from the southern FK population. Flowering time varied from 56.8±2.06 to 101.2±1.95 for the moderate five populations.

De novo transcriptome assembly

About 1230 million clean reads were obtained, with an average of 21 million per library. After Trimmomatic filtering, 1212 million high-quality reads remained (Additional file 1 Table S2). The high-quality reads were assembled, leading to the identification of 79,600 transcripts. The assembly of the DD July sample provided the highest N50 value and BUSCO completeness score. Specifically, the N50 was 958 bp, with a GC content of 40.44%. BUSCO analysis revealed that approximately 77.5% of the 1440 core embryophyte genes were complete, indicating comprehensive mapping of the sequences. Therefore, the July sample from the DD population was employed as the reference for subsequent analyses.

Identification of DEGs among sampling times

A total of 26,628 genes were identified to differ among sampling times through DGE analysis, with 11,897 upregulated and 14,731 downregulated (Fig. 3A). For each flowering group (EF, MF, and LF), the lowest number of DEGs of July vs. August was observed, compared to the July vs. September and August vs. September contrasts (Fig. 3A). Specifically, the EF group had 37 upregulated and 31 downregulated genes, the MF group had 371 upregulated and 592 downregulated genes, and the LF group had 71 upregulated and 54 downregulated genes in the July vs. August contrast.

GO enrichment analysis revealed DEGs were partially annotated to photosynthesis, response to stress, biosynthetic processes and response to phytohormones functions (Additional file 1 Table S3-1 – S3-9). Among the genes upregulated in July and August compared to September in the EF group, 200 and 48 genes were related to photosynthesis, respectively. Similarly, in the MF group, 252 genes in July and 254 genes in August that showed higher expression levels than September were related to photosynthesis. In the EF and MF groups, 48 and 485 genes that were upregulated in September compared to

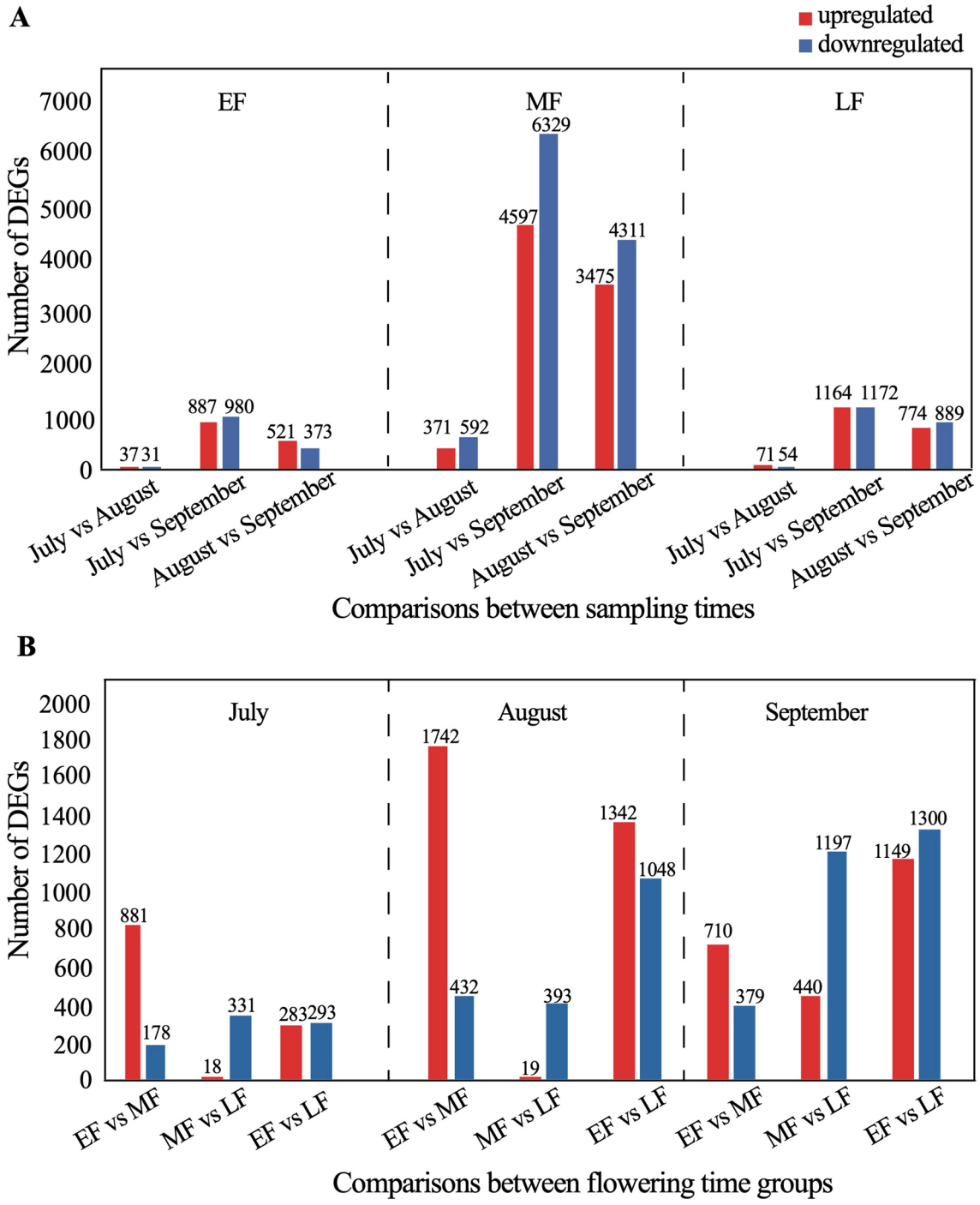

Fig. 3 Number of up- and downregulated DEGs between sampling times **(A)** and between flowering time groups **(B)** for the Chinese invasive populations of *Ambrosia artemisiifolia*. EF, MF and LF represent the population groups with early, moderate and late flowering times, respectively

July and August were associated with stress response, respectively. In the LF group, there were 110 upregulated genes involved in biosynthetic processes in July and August compared to September, respectively. Additionally, 209 upregulated genes in September were associated with the response to phytohormones in the LF group. The results of annotation to the FLOR-ID and PPI STRING databases indicated that 27, 126, and 39 flowering-related genes were differentially expressed across sampling times in the EF, MF and LF groups, respectively (Additional file 1 Table S4).

Candidate genes involved in flowering time differentiation identified through DGE analysis

A total of 12,135 genes were identified to differ among flowering time groups through DGE analysis, with 6584 upregulated and 5551 downregulated (Fig. 3B). Some DEGs were enriched in terms related to hormone regulation, sugar metabolism, and floral development (Additional file 1 Table S3-10 – S3-18). The annotation to the FLOR-ID and PPI STRING databases indicated that 21, 35 and 48 flowering-related DEGs between flowering time groups were identified in July, August and September, respectively (Additional file 1 Table S5).

There were 41 genes identified to be flowering-related and differed in expression among sampling times and among flowering time groups through DGE analysis (Fig. 4). These genes are involved mainly in pathways regulating transition to flowering, including the photoperiod, temperature, age, autonomous, sugar and hormone pathways and the integrator and floral meristem identities controlling flower development (Fig. 4). Notably, 16 of 41 genes were involved in photoperiod pathway in regulating flowering. 34 of 41 genes showed significant latitude-correlated expression (Table 1). Specifically, 5, 9 and 9 genes were positively correlated with latitude in July, August and September, respectively (Table 1). While 8, 9 and 9 genes were negatively correlated with latitude in July, August and September, respectively (Table 1). Among the genes whose expression levels were positively correlated with latitude, *FLAVIN-BINDING, KELCH REPEAT, F BOX 1(1) (FKF1(1))*, *FKF1(2)*, *FT*,

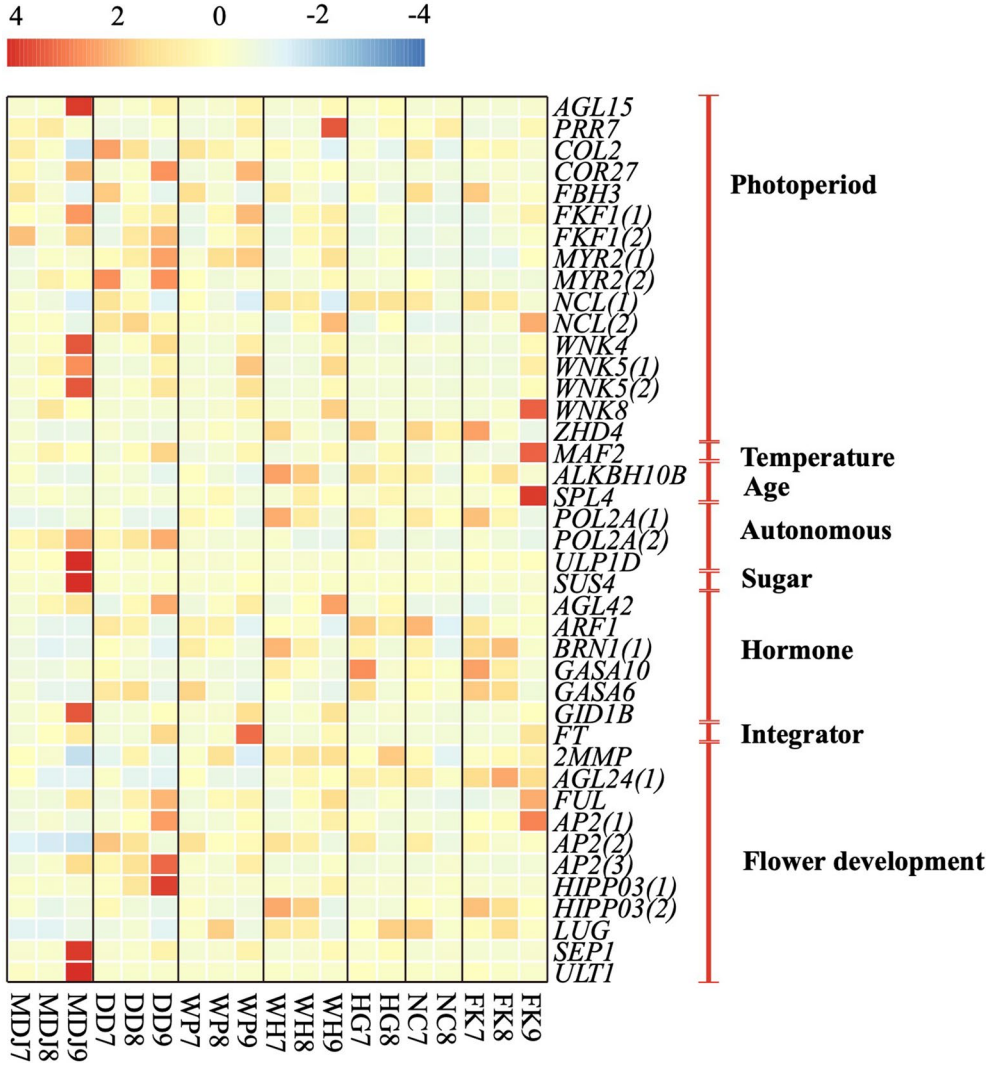

Fig. 4 Heatmap of expression levels of flowering-related genes that exhibited different expression levels among sampling times and among flowering time groups through DGE analysis, with blue rectangles indicating low expression and red rectangles denoting high expression. The rows and columns are not clustered. Each column represents the expression levels of candidate genes in samples from different populations and different times (with letters representing different populations and numbers representing different sampling times). Each row represents the expression level of a specific candidate gene. The flowering regulatory pathways in which these genes are involved, are labeled on the right

Table 1 Spearman correlations between the expression levels of the candidate genes which were identified through the DGE analysis and WGCNA and origin latitude of Chinese invasive *Ambrosia artemisiifolia* populations at each sampling time

Gene	Sampling time			Method	Gene	Sampling time			Method
	July	August	September			July	August	September	
2MMP	-0.22	-0.11	**-0.82*** **	DGE	***GASA10***	-0.38	**-0.51***	0.16	DGE
ADF1	**0.52***	0.24	0.07	WGCNA	***HIPP03(1)***	**0.54***	**0.67*** **	0.11	DGE
AGL15	0.29	0.21	**0.80*** **	DGE	***HIPP03(2)***	-0.35	**-0.57****	-0.34	DGE
AGL24(1)	**-0.50***	**-0.87*** **	**-0.88*** **	DGE	***LUG***	**-0.47***	**-0.48***	-0.28	DGE
AGL24(2)	-0.13	**-0.51***	**-0.80*** **	WGCNA	***MAF2***	**0.74*** **	**0.58****	-0.34	DGE
AGL42	0.29	**0.48***	0.31	DGE/WGCNA	***MYR2(1)***	0.37	**0.59****	0.07	DGE
ALKBH10B	-0.39	**-0.78*** **	**-0.61***	DGE/WGCNA	***MYR2(2)***	0.12	**0.48***	0.47	DGE
AP2(1)	**-0.51***	-0.16	-0.50	DGE	***NCL(1)***	**-0.46***	**-0.57****	**-0.61***	DGE
AP2(2)	-0.12	-0.12	**-0.53***	DGE	***NCL(2)***	**0.450***	0.46	**-0.77*** **	DGE/WGCNA
AP2(3)	0.15	0.37	**0.70****	DGE	***POL2A(1)***	**-0.66****	**-0.67*** **	0.06	DGE
ARF1	**-0.44***	-0.26	0.24	DGE/WGCNA	***RFI2***	**-0.62****	**-0.60****	0.11	WGCNA
BRN1(1)	**-0.54***	**-0.66****	-0.46	DGE	***SEP1***	0.29	0.21	**0.80*** **	DGE
BRN1(2)	**-0.66****	**-0.70*** **	**-0.79*** **	WGCNA	***SPL4***	-0.32	-0.21	**-0.81*** **	DGE
CIB1	-0.08	0.04	**-0.58***	WGCNA	***SUS4***	-0.25	-0.32	**0.62***	DGE/WGCNA
COR27	-0.21	-0.05	**0.75****	DGE	***TPL***	**-0.70*** **	**-0.472***	0.14	WGCNA
FBH3	-0.05	0.36	**-0.59***	DGE/WGCNA	***ULP1D***	0.14	0.17	**0.58***	DGE
FKF1(1)	0.28	0.24	**0.57***	DGE	***WNK11***	**0.44***	0.34	0.08	WGCNA
FKF1(2)	0.19	0.43	**0.82*** **	DGE	***WNK4***	0.37	**0.44***	0.46	DGE
FLK	**-0.51***	**-0.54***	**-0.59***	WGCNA	***WNK5(2)***	0.19	**0.65*** **	**0.76*** **	DGE
FT	**0.54***	**0.75*** **	0.01	DGE	***WNK8***	0.21	**0.59****	**-0.59***	DGE
FUL	**0.45***	0.19	-0.26	DGE	***ZHD4***	**-0.83*** **	**-0.60****	-0.25	DGE
FVE	**-0.60****	**-0.70****	**-0.61***	WGCNA					

The values indicate the correlation coefficients

Bold values indicate significant correlations. Statistical significance is denoted as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Candidate genes that showed no correlation with latitude at any of the sampling times were not listed

FRUITFULL (FUL) and *MADS AFFECTING FLOWERING 2 (MAF2)* had higher expression in August compared to July in the MF group which was in vegetative stage in July and flowering stage in August (Additional file 1 Table S1, 6). Meanwhile, *FKF1(1)*, *FKF1(2)*, *FT*, *FUL*, *MAF2*, *WNK lysine deficient protein kinase 4 (WNK4)* and *WNK5(2)* showed higher expression in September compared to July or August in the LF group which was in vegetative stage in July or August and flowering stage in September (Additional file 1 Table S1, 6). For the genes with expression levels negatively associated with latitude, *FBH3* displayed a higher expression level in July compared to August in the MF group, and *Neuronal Calcium sensor-1-Like (NCL)*, *polymerase epsilon catalytic subunit A (POL2A)(1)* and *Zinc-finger homeodomain protein 4 (ZHD4)* exhibited a lower expression level in September compared to July or August in the LF group (Additional file 1 Table S6).

Candidate genes associated with flowering time differentiation identified through WGCNA

There were 25, 46, and 37 co-expression modules identified for samples collected in July, August and September, respectively. The green and pink modules were significantly correlated with both flowering time and the earliest-flowering MDJ population in July (Additional file 2 Fig. S1A). In August, 8 modules (magenta, steel blue, light cyan1, red, salmon, dark green, green, and blue modules) showed significant correlation either with flowering time and flowering populations (DD, WP, WH, HG, or NC), or with flowering time and the population that had not yet flowered (FK) but not with the DD, WP, WH, HG and NC populations (Additional file 2 Fig. S1B). In September, 3 modules (magenta, midnightblue and lightgreen) were significantly correlated with flowering time and the FK population, which initiated flowering latest in September (Additional file 2 Fig. S1C). These gene modules were considered to be the most relevant modules for flowering time differentiation and were subjected to subsequent analyses.

Through GO enrichment of the identified modules, we found that 6 and 7 genes were annotated to "pollen germination" and "pollen tube growth", respectively (Additional file 1 Table S7) and these were considered to be relevant for flower development. In addition, 4 genes were annotated to "response to red light" which may be involved in flowering regulation, and 4 genes were annotated to "regulation of jasmonic acid-mediated signaling pathway" in hormone regulation (Additional file 1 Table S7). Furthermore, 79 genes from the candidate

modules were identified to be related to flowering regulation through annotation to the FLOR-ID and the PPI STRING databases (Additional file 1 Table S8). Among these genes, 18 genes displayed differential expression levels among sampling times in our DGE analysis (Fig. 5) and are thus considered to be candidate genes for flowering time differentiation across populations. These genes were mainly involved in the photoperiod, age, autonomous, sugar, hormone and flower development pathways in flowering regulation, with 9 of 18 genes in the photoperiod pathway.

The correlation analysis between candidate gene expression levels and origin latitude showed that 5 genes, including *Actin-depolymerizing factor 1 (ADF1)*, *NCL(2)* and *WNK11* in July, *AGAMOUS-LIKE 42 (AGL42)* in August, and *SUCROSE SYNTHASE 4 (SUS4)* in September, were positively correlated with origin latitude (Table 1). On the other hand, 6, 7 and 8 genes were negatively correlated with latitude in July, August and September, respectively (Table 1). Among the genes that were negatively correlated with latitude, *FLOWERING BHLH 3 (FBH3)* showed lower expression level in August compared to July in MF group which was in vegetative stage in July and flowering stage in August (Additional file 1 Table S6). *FLOWERING LOCUS KH DOMAIN (FLK)* had lower expression level in September compared to July in LF group which was in vegetative stage in July and flowering stage in September (Additional file 1 Table S6).

Discussion

Latitudinal cline of flowering time in Chinese ragweed populations

Multiple studies have shown that *Ambrosia artemisiifolia* has a remarkable capacity for local adaptation, enabling it to thrive and reproduce effectively in novel environments [5, 6, 34, 44]. Flowering time, a pivotal trait, has been observed to be negatively correlated with latitude of origin in *A. artemisiifolia* and is thought to play an important role in adaptation to the local environment [5, 6]. Given the sensitivity of fruit development to frost in *A. artemisiifolia*, only early-flowering plants can produce viable seeds at high latitudes [44]. In addition, Li et al. conducted a reciprocal transplant experiment on *A. artemisiifolia* and revealed that the northern population exhibited earlier flowering and greater production of female flowers and seeds than their southern counterparts did, suggesting local adaptation through enhanced female fitness in the northern population [6]. Based on the current common garden planting, the MDJ and DD populations, which originate from the northernmost regions, presented the earliest onset of flowering, whereas the FK population, from the southernmost site, presented the latest flowering time, approximately sixty days later than that observed in the northernmost populations (Fig. 2). These results confirmed the latitudinal flowering time clines in China (Fig. 2). We have investigated the genetic structure of Chinese invasive populations based on microsatellite markers and found

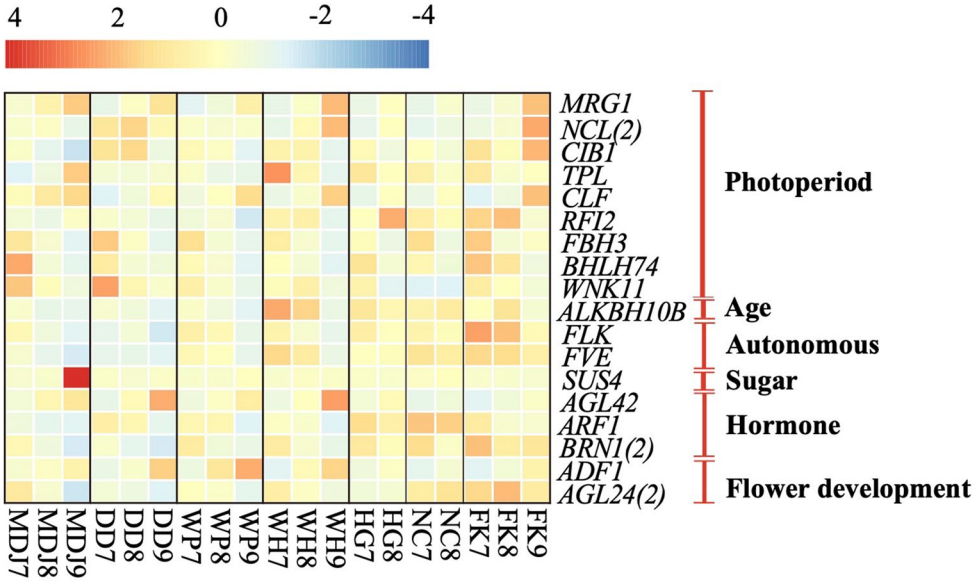

Fig. 5 Heatmap of expression levels of flowering-related genes that exhibited different expression levels among sampling times through DGE analysis and among flowering time groups through WGCNA, with blue rectangles indicating low expression and red denoting high expression. The rows and columns are not clustered. Each column represents the expression levels of candidate genes in samples from different populations and different times, with the names of the samples listed below the image (with letters representing different populations and numbers representing different sampling times). Each row represents the expression level of a specific candidate gene. The flowering regulatory pathways in which these genes are involved, are labeled on the right

that each population is comprised of two shared genetic clusters (Li et al. unpublished). Therefore, the latitudinal cline of flowering time in Chinese populations may result from local adaptation to the abiotic conditions rather than from genetic origin of the populations.

Candidate genes for the flowering time differentiation

The DGE analysis and WGCNA, based on the transcriptome sequences, identified 53 candidate genes underlying the differentiation of flowering time among populations (Figs. 4 and 5). Through our DGE analysis, we identified 41 genes with differing expression across both sampling times and flowering time groups (Fig. 4). By combining DGE analysis among sampling times with WGCNA among populations, we found that 18 genes exhibited expression differences across both sampling times and populations (Fig. 5). Given 6 genes were identified in both analyses, a total of 53 genes demonstrated differential expression across sampling times and flowering time groups, indicating their association with flowering time differentiation (Figs. 4 and 5). These genes were mainly involved in the pathways of photoperiod, temperature, age, autonomous, sugar, and hormone, integrator and floral meristem identity in the flowering regulation pathways (Figs. 4 and 5). Notably, some of our candidate genes have also been identified to be related to flowering time differentiation in other studies. For instance, *FT*, *MAF2*, *FKF1* and *PSEUDO-RESPONSE REGULATOR (PRR)* were identified to be responsible for flowering time adaptation in *A. artemisiifolia* in the introduced European range [9]. In *Lycoris radiata*, *AP2*, *AGL*, and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* were found to play an important role in the differentiation of flowering time [45]. In *Mikania micrantha*, *SPLs*, *FLK*, and *FT* were identified to be related to flowering differentiation at different altitudes [46]. Additionally, *FVE*, *PRRs* and *FT* were detected to be critical in the genetic regulation of flowering time in *Brassica napus* [47].

Environmental factors driving flowering time adaptation

Through GO enrichment and annotation with Flowering-Interactive and PPI protein STRING databases, we found that 23 of 53 candidate genes were annotated to the photoperiod pathway, greater than all other pathways (Figs. 4 and 5), highlighting the significant role of photoperiodic factors in the differentiation of flowering time among *A. artemisiifolia* populations in China. The evolution of photoperiodic regulation of flowering mainly encompasses the diversity of photoperiod response types, variations in the magnitude of photoperiodic response and changes in daylength thresholds [48]. For example, in an annual ecotype of *Mimulus guttatus*, higher elevation plants had greater critical photoperiod for flowering to ensure that floral initiation awaits the start of the growing season [49]. Photoperiodic regulation of phenology traits has also been reported in numerous woody species [50, 51]. Moreover, temperature plays a significant role in flowering regulation and plant adaptation [48]. A strong effect of warming on first flowering has been reported through a meta-analysis of manipulative experiments across the globe [52, 53]. In this study, *MAF2* is related to the temperature pathway and was identified to regulate the flowering time in *A. artemisiifolia* (Fig. 4). In addition, genes of age, autonomous, sugar and hormone pathways were also identified to be involved in the differentiation of flowering time (Figs. 4 and 5). The overrepresentation of photoperiod pathway genes in this study suggest that photoperiod was the major cue driving the divergence of flowering time, leading to local adaptations in *A. artemisiifolia.* Controlled experiments could be performed to verify this photoperiodic adaptation of flowering time. Responses of phenology to photoperiod and temperature could be used to predict the potentially range expansions of *A. artemisiifolia* populations, given their effects on plant development and flowering [54].

Roles of candidate genes in regulating flowering time

There were 43 of 53 candidate genes exhibiting latitudinal correlated expressions (Table 1), suggesting their strong associations with the differentiation of flowering time along latitude. There were 7, 9 and 9 genes positively correlated with latitude in July, August and September, respectively (Table 1). These results suggest their positive effects on flowering because plants from higher latitude flowered earlier (Fig. 2). Conversely, 13, 15 and 14 genes were negatively correlated (Table 1) and may negatively affect flowering. Combined with the results of comparisons among sampling times (Additional file 1 Table S6), we further inferred that *FKF1(1)*, *FKF1(2)*, *FT*, *FUL*, *MAF2*, *WNK4* and *WNK5(2)* may positively affect flowering, because these genes were upregulated in the early-flowering populations from higher latitude and showed increased expression after flowering compared to the vegetative stage. Similarly, *FBH3*, *FLK*, *NCL(1)*, *POL2A(1)* and *ZHD4* were inferred to have negative effects on flowering, as they were downregulated in the early-flowering population from higher latitudes and also showed reduced expression after flowering compared to the vegetative stage (Additional file 1 Table S6). *FKF1*, *WNK4*, *WNK5*, *FBH3*, *NCL* and *ZHD4* have been reported to be involved in photoperiod pathway [55–59]. *FKF1*, *WNK* gene family, and *NCL* are regulated by circadian rhythms and facilitate flowering in *Arabidopsis thaliana* [56–59]. *FBH3*, as a *CONSTANS (CO)* transcriptional activator, caused early flowering when overexpressed [58]. *ZHD4* increases expression in the meristem adjacent to floral primordia after exposure to long days which can induce flowering in *A. thaliana* [60]. *MAF2* is associated with

the temperature pathway, acting as a floral repressor in *A. thaliana* [61]. *FLK* and *POL2A* are involved in the autonomous pathway [62, 63]. *FLK* functions as a repressor of *FLOWERING LOCUS C (FLC)* expression and promotes flowering in *A. thaliana*, whereas *POL2A* may increase the expression of *FLC* and delay flowering [62, 63]. The upstream signals from the photoperiod, vernalization, gibberellin, temperature, autonomous, age, and sugar pathways act on the integrators *FT* and *SOC1*, activating downstream genes involved in the initiation of flowering, such as *APETALA 1 (AP1)*, *CAULIFLOWER (CAL)*, *FUL* and *LEAFY (LFY)* [64].

In this study, the roles of *NCL(1)*, *FBH3*, *MAF2*, and *FLK* in flowering regulation, as indicated by their expression patterns, appear to differ from those observed in *Arabidopsis thaliana.* We suspect that the difference in the functions of homologous flowering genes may be partially attributed to the difference in day length threshold for flowering, because *Ambrosia artemisiifolia* is a short-day plant [65] while *Arabidopsis thaliana* is a long-day plant. For example, in the short-day *Oryza sativa*, *Hd1* follows circadian rhythm, homologous to *CO* in *A. thaliana*, but regulates the *FT* ortholog *Hd3a* in a opposite way to the long-day *A. thaliana* [66]. Furthermore, as the samples in this study were collected from July to September, significant changes in genes that function during early life stage could not have been detected [13], potentially biasing our conclusions. It has been reported that some flowering time regulators, especially the circadian rhythms genes, were expressed at a very early stage [36, 67]. Continuous sampling from the seedling phase to the flowering phase would allow us to avoid this problem in future studies.

Conclusions

In this research, we confirmed the latitudinal cline in flowering time across Chinese populations of invasive *Ambrosia artemisiifolia* and identified 53 candidate genes for the flowering time differentiation based on transcriptome sequence. After annotation, photoperiod was inferred to be the main environmental factor driving this differentiation as 43% (23 out of 53) candidate genes were annotated to the photoperiod pathway. Among the candidate genes displaying latitudinal correlated expressions, 7 genes were inferred to promote flowering, whereas 5 genes likely exert a repressive effect on flowering. Interestingly, 4 genes appear to function differently in *A. artemisiifolia* compared to their well-characterized roles in *Arabidopsis thaliana*, indicating diversity in regulating flowering time across plant taxa. Our findings revealed the molecular mechanisms of flowering time differentiation in *A. artemisiifolia*, providing insights into its local adaptation to invasive environments and range expansions in China.

Abbreviations

FT	Flowering locus T
SOC1	Suppressor of overexpression of CO 1
PHYs	Phytochromes
GI	Gigantea
BTC1	Bolting time control 1
FLC	Flowering locus C
PRR37	Pseudo-response regulator 37
FRL3a	Fri-like3a
GI1	Gigantea 1
FTL9	Flowering locus T-like 9
DGE	Differential gene expression analysis
WGCNA	Weighted gene co-expression network analysis
BUSCO	Benchmarking universal single-copy orthologs
CDS	Coding domain sequences
FPKM	Fragments per kilobase million
TMM	Trimmed mean of M-values
DEGs	Differentially expressed genes
GO	Gene ontology
FLOR-ID	Flowering interactive database
PPI	Protein-protein interaction
TOM	Topological overlap matrix
FKF1	Flavin-binding, kelch repeat, F BOX 1
FUL	Fruitfull
MAF2	Mads affecting flowering 2
WNK	WNK lysine deficient protein kinase
NCL	Neuronal calcium sensor-1-Like
POL2A	Polymerase epsilon catalytic subunit A
ZHD4	Zinc-finger homeodomain protein 4
AGL15	Agamous-like 15
ULP1D	Ubiquitin-like-specific protease 1D
GID1B	GA insensitive dwarf 1B
SEP1	Sepallata 1
AP2	Apetala 2
HIPP03	Heavy metal-associated isoprenylated plant protein 3
ADF1	Actin-depolymerizing factor 1
AGL42	Agamous-like 42
SUS4	Sucrose synthase 4
FBH3	Flowering bhlh 3
FLK	Flowering locus kh domain
PRR	Pseudo-response regulator
SPL	Squamosa promoter binding protein-like
CO	Constans
FLC	Flowering locus C
AP1	Apetala 1
CAL	Cauliflower
LFY	Leafy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05830-x.

Supplementary Material 1: Table S1-Developmental stages of Chinese *Ambrosia artemisiifolia* populations at each sampling time for transcriptome analysis in the common garden. Table S2-Data quality of RNA-seq for leaf samples of *Ambrosia artemisiifolia*. Table S3-Significant Biological Process terms in the GO enrichment analysis for DEGs identified through DGE analysis across sampling times and among flowering time groups in *Ambrosia artemisiifolia*. Table S4-Differentially expressed flowering-related genes across sampling times for each flowering time group according to the DGE analysis and the FLOR-ID and PPI annotation in *Ambrosia artemisiifolia*. Table S5-Flowering-related genes differentially expressed among flowering time groups at each sampling time from the results of DGE analysis and the FLOR-ID and PPI annotation in *Ambrosia artemisiifolia*. Table S6-The expression levels (mean ± SE) of candidate genes at each sampling time for each flowering time group in *Ambrosia artemisiifolia*. Candidate genes that showed no correlation with latitude at any of the sampling times were not listed. Table S7-Significant Biological Process terms from the GO enrichment analysis for critical modules genes identified by WGCNA in *Ambrosia artemisiifolia*. Table S8-Flowering-related genes differentially expressed among flowering time groups at each

sampling time according to the results of WGCNA and the FLOR-ID and PPI annotation in *Ambrosia artemisiifolia*.

Supplementary Material 2: Heatmap of the module-trait relationships with flowering time and populations considered as traits in the analysis of *Ambrosia artemisiifolia* samples in July (A), August (B) and September (C). The values in each box indicate the correlation coefficient between modules and the trait, with values in bold present significant correlations. Statistical significance is denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Acknowledgements
We express our gratitude to Bo Li from Jiangxi Agricultural University, Nian-Xun Xi from Sun Yat-sen University and Dan-Ni Xing for helping us with the collection of *Ambrosia artemisiifolia* seeds, and we extend our appreciation to Quan-Bao Zhang for assisting in the data analysis of the transcriptome.

Author contributions
YXF, LXM and LWJ designed the research. LXM, XC and LWJ performed the common garden experiment, and YXF performed transcriptome analysis and wrote the article draft. LXM, LWJ and PKI provided editorial input on the writing. All the authors have read and approved the manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (32371582 and 31700326).

Data availability
The datasets used and analyzed during the current study have been successfully stored in the SRA database of NCBI; the RNA-Seq accession number is available from accession number SAMN40142471.

Declaration

Ethics approval and consent to participate
No specific permission was needed to collect *Ambrosia artemisiifolia* materials for research.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 September 2024 / Accepted: 14 November 2024
Published online: 21 November 2024

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