

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

Xiao-Feng Yang

Beijing Normal University

Xiao-Meng Li

xiaomengli@bnu.edu.cn

Beijing Normal University

Pär K. Ingvarsson

Swedish University of Agricultural Sciences

Chao Xi

Beijing Normal University

Wan-Jin Liao

Beijing Normal University

Research Article

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

Posted Date: March 8th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4015147/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background *Ambrosia artemisiifolia* is a highly invasive species with a notorious reputation for its deleterious effects on both public health and agricultural systems. Previous research has demonstrated that flowering time varies latitudinally along a cline and this may have significant implications for local adaptation and the successful invasion of *A. artemisiifolia* in China. However, the molecular mechanisms underlying variation in flowering time in this species remains poorly understood. In this study, a common garden experiment and transcriptome analysis were performed using different populations of *A. artemisiifolia* that originated from diverse locations across a latitudinal gradient in China.

Results Our study confirmed the presence of a latitudinal cline in the flowering time of *A. artemisiifolia*. Transcriptome comparisons identified substantial variation in gene expression among populations exhibiting different flowering time, with a greater abundance of differentially expressed genes (DEGs) observed between populations spanning wider latitudinal ranges. The functional annotation results revealed the involvement of light, sugar, vernalization and autonomous pathways in the modulation of flowering time variation among populations. Furthermore, a comprehensive set of DEGs encompassed various transcription factors (TFs) associated with flowering time regulation, including WRKY, AP2/ERF, bZIP, C2H2, bHLH, NAC and MYB-related TFs. Several candidate genes associated with variation in flowering time were identified. *CO*, *GAI*, *FES1* and *FD* function as promoters of flowering induction, while *LFY*, *FVE*, *FLK* and *SVP* act as inhibitors of flowering initiation in *A. artemisiifolia*. Several previously uncharacterized unigenes, involved in the regulation of flowering time in *A. artemisiifolia*, were also been identified.

Conclusions The findings in this study suggest that the differentiation of flowering time in *A. artemisiifolia* is collectively influenced by numerous genes involved in the photoperiodic, vernalization, gibberellin, temperature, sugar and autonomous pathways. The elucidation of the molecular mechanisms underlying the differentiation of flowering time will significantly advance our understanding of the factors facilitating the successful invasion of *A. artemisiifolia* in China.

Background

The ability of plants to adapt to their environment is crucial for their growth, reproduction and survival. Invasive plants that undergo distinct selective pressures from their local environment may lead to the evolution of local adaptation [1], which may result in them outperforming native populations [2]. Numerous studies have confirmed that local adaption play an important role in the successful establishment and spread of invasive species [3, 4, 5]. 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 [6]. It has been reported that latitudinal clines in flowering time have played crucial roles in the successful invasion of several species, such as *Lythrum salicaria* [3], *Medicago polymorpha* [7] and *Ambrosia*

artemisiifolia [5]. Hence, unraveling the mechanisms underlying the differentiation of flowering time is imperative for comprehending the factors contributing to successful invasion of non-native species.

Flowering time is thought to be a quantitative trait, influenced by both genetic and environmental factors [8]. Environmental factors, including light, temperature, water availability, as well as endogenous factors, such as hormones and age, collectively contribute to the intricate regulation of flowering in plants. Extensive research in model species, such as *Arabidopsis thaliana*, a long-day plant, have identified numerous genes and pathways involved in regulating flowering time [9]. In *A. thaliana*, the dynamic process of flowering is primarily induced by seven pathways, including photoperiod, vernalization, gibberellin, temperature, autonomous, age, and sugar pathways [10, 11]. 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*). Subsequently, these integrators effectively integrate and relay diverse signals to downstream genes that ultimately trigger the process of flowering [11]. Genetic mechanisms regulating flowering time have also been elucidated in numerous species, revealing both conservation and divergence in flowering time regulation across different taxa [12, 13]. For example, key elements of the genetic network that regulates flowering time, including homologous genes like photoreceptors (*PHYTOCHROMES*, *PHYs*), circadian clock genes (*GIGANTEA*, *GI*), and integration factors (*FT*), are conserved among various species such as *A. thaliana*, rice (*Oryza sativa*), and numerous temperate cereals [12]. However, functional disparities in the primary flowering-related genes have been observed across species. Novel genes related to flowering time not previously reported in *A. thaliana* have been discovered in other species, such as *BOLTING TIME CONTROL 1* (*BTC1*) in sugar beet [14] and *E11* in soybean [15]. In addition, the genetic factors governing variation in flowering time across distinct populations exhibit substantial diversity among different species or across different geographical regions within species. For example, allelic variation at the *FLOWERING LOCUS C* (*FLC*) locus have been found to confer variation in flowering time in *Capsella rubella* [16]. Genes like *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* [17]. Additionally, the effect of *FLOWERING LOCUS T-like 9* (*PhFTL9*) in regulating flowering time has been observed in *Panicum hallii* [18].

Ambrosia artemisiifolia is a wind pollinated, annual herbaceous plant native to North America and Mexico [19]. This species has been unintentionally introduced into multiple regions around the world, including Asia, South America, Oceania, and Europe, commonly with high genetic diversity in the invasive populations [20, 21]. The detrimental impact of *A. artemisiifolia* on the economy and on public health is escalating in both native and invasive areas because of its highly allergenic pollen and the formidable challenge it poses in agricultural landscapes as an invasive weed [22, 23, 24, 25, 26]. Investigating the underlying mechanism behind its extensive spread will facilitate enhanced management strategies. Latitudinal variation in flowering time of *A. artemisiifolia* has been documented in both native and invasive ranges and has been considered an important mechanism for its extensive invasive spread in China [4, 5, 7]. Therefore, elucidating the genetic factors underlying variation in flowering time would greatly contribute to unraveling the intricate mechanisms governing its successful colonization.

However, investigations into the genetic mechanisms of this species have been hindered by a dearth of accessible genetic information [27]. Relying solely on information about the regulatory networks governing flowering time in *A. thaliana*, a long-day plant, may prove inadequate for understanding flowering in *A. artemisiifolia* because it is a short-day plant and might therefore have unique regulatory mechanisms. Recently, second-generation sequencing RNA-seq has been employed to explore the molecular mechanisms underlying variation in flowering time across different non-model species, including chickpea [28], strawberry [13] and Adzuki bean [29]. In the case of *A. artemisiifolia*, several candidate genes associated with male or female organ development have been identified [30], providing support for the existence of unique flowering-related genes in this species. However, current knowledge on the genetic regulation of flowering in *A. artemisiifolia* remains limited, impeding our understanding of the underlying mechanisms driving its successful invasion. Further dedicated research is warranted to address this gap in our knowledge.

In this study, we investigated the variation in flowering time among populations of *A. artemisiifolia* derived from seven distinct regions across China and observed a latitudinal gradient in flowering time, consistent with previous study [5]. To gain insights into the molecular basis underlying this variation, an RNA-Seq analysis was conducted on three populations with distinct flowering time, and differentially expressed genes (DEGs) and their functions were identified, to explore candidate genes controlling flowering time differentiation. Furthermore, we identified potential transcription factors (TFs) and other genes that could account for the observed variation in flowering time across different populations. The findings from this study may provide valuable insights into the molecular regulation of flowering time differentiation and adaptation in *A. artemisiifolia*.

Methods

Common garden experiment

The common garden experiment was conducted in a field at Beijing Normal University (39.96°N, 116.36°E). In 2019, seeds of *A. artemisiifolia* (voucher specimens has been identified by Prof. Wan-Jin Liao and deposited in the herbarium of Beijing Normal University) were collected from seven geographically separated populations in China, including Mudanjiang (MDJ), Dandong (DD), Wangping (WP), Wuhu (WH), Huanggang (HG), Nanchang (NC), and Fengkai (FK) 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 family and sown in petri dishes filled with moist vermiculite, resulting in a total of 300 seeds sown per population. The petri dishes were placed at 4°C for a cold stratification process for two weeks and transferred to ambient temperature to facilitate germination. The days to germination were recorded for each sample. After the development of true leaves, the seedlings were transplanted into 9 cm pots filled with a blend of vermiculite and potting soil in a 1:1 ratio. A total of 96 individuals survived and were incorporated into the subsequent experiment. The individuals were adequately irrigated and monitored throughout the growing season. The flowering time was recorded as the duration in days from seed germination to the initial appearance of inflorescences. Variation in

flowering time among populations was assessed using ANOVA (R v4.1.1), employing the "Tukey" method for conducting pairwise comparisons.

On July 18th, August 21st, and September 27th, leaf tissues were collected from each population, with three biological replicates, at approximately 12 p.m., cut into pieces and immediately stored in RNA*later* solution (Life Technologies, CA, USA) at -20°C until RNA extraction and transcriptome analyses. All the sampled individuals were observed to be in the vegetative stage during the initial sampling on July 18th, while they had all transitioned to the flowering stage by the final sampling time on September 27th. During the second sampling period on August 21st, all sampled individuals, except for those from the FK population, had successfully progressed to the flowering stage.

RNA extraction, library construction and Illumina sequencing

Total RNA for each sample was extracted using the TRIzol® Plus RNA Purification Kit (Thermo Fisher Scientific Inc.). The quality of RNA was assessed using agarose gel electrophoresis and a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), and the concentrations were evaluated using the Qubit® RNA Assay Kit on a Qubit®2.0 Fluorometer (Life Technologies, CA, USA).

Libraries for each sample were constructed using approximately 3 µg RNA as the input material. Sequencing libraries were prepared using NEBNext® Ultra™ 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 using 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 125 bp/150 bp paired-end reads at a depth of 6G per sample.

De novo assembly

The raw reads underwent a filtration process, wherein sequences containing adapters, low-quality sequences (with more than 50% of the bases having $Q \leq 5$), and reads with over 10% unknown nucleotides were discarded [31]. A *de novo* transcriptome assembly was conducted to assemble the contigs into a non-redundant set of unigenes using Trinity (v2.1.1) and CD-HIT (v4.8.1) with the July sample from the DD population employed as the reference. The longest transcripts were considered as the non-redundant unigenes. The effectiveness of the assembly and the number of mapped genes was evaluated based on the N50 calculation and Benchmarking Universal Single-Copy Orthologs (BUSCO) (v3.0.2) examination. Potential protein coding domain sequences (CDS) were predicted using TransDecoder (v5.5.0).

Differentially gene expression analysis

Gene expression levels for each sample were estimated using the RSEM package (v1.3.3) and quantified by the FPKM values [32]. Then, a differential expression analysis was conducted using DESeq2 (version

1.24.0) on the populations of DD, HG, and FK which were categorized as early-flowering, intermediate-flowering, and late-flowering groups based on their respective flowering times. Genes exhibiting a log₂ (fold change) value greater than 1 and an adjusted *P*-value below 0.05 were deemed to be differentially expressed. The differentially expressed genes (DGE) were annotated to terms in the Gene Ontology (GO) database using the clusterProfiler package (version 3.12.0). The identification of significantly enriched terms in DEGs was based on a criterion of a Bonferroni-corrected *P*-value ≤ 0.05 . Subsequently, we submitted extracted coding sequences of all DEGs to Mercator [33] for gene annotation, obtained the corresponding mapping file, and performed functional analysis using PageMan in MapMan (version 3.6.0) to investigate the biological regulation and metabolism-related roles of these DEGs [34]. Furthermore, we extracted the protein sequences of DEGs and employed the iTAK pipeline for identification of transcription factors as well as for functional classification [35].

Identification of flowering-related genes in DEGs

The differentially expressed genes (DEGs) were subsequently subjected to further analysis to identify potential candidate genes underlying the observed variations in flowering time across populations. We conducted a search for orthologous genes in the curated FLOR-ID (Flowering-Interactive Database) [36], which is an interactive database of flowering-time gene networks in *A. thaliana*. Additionally, we conducted a search for flowering-related sequences within the mapping file generated by Mercator [33], and used them to annotate the flowering-related functionalities of the DEGs.

Quantitative real-time PCR (qRT-PCR) validation

Relative qRT-PCR was conducted to validate and assess the expression levels of a subset of candidate genes associated with differentiation of flowering time. Total RNA for each sample was reverse transcribed to produce first-strand cDNA using a PrimeScript RT reagent Kit (Takara, Dalian, China), with 20 µl PCR reactions containing 9 µl of RNase Free dH₂O, 4 µl of 5× PrimeScript Buffer, 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of OligodT Primer (50 µM), 1 µl of random hexamers (100 µM) and 4 µl of RNA. The procedure was performed in a Veriti 96-Well thermal cycler (Applied Biosystems) at 37°C for 15 min and 85°C for 5 s. The cDNAs were diluted 10-fold and used in the following qRT-PCR experiment. Gene-specific primers for qRT-PCR were designed using the Primer Tool (Table S1). Relative qRT-PCR was performed using a 7500 Fast Real Time PCR System (Applied Biosystems) with a 20 µl reaction mixture comprising 10 µl of FastStart Universal SYBR Green Master Mix (ROX; Roche, Germany), 8 µl of dH₂O, 0.3 µl of 10mM forward and reverse primers and 1.4 µl of diluted cDNA as the template for target genes. The relative expression level of each target gene was determined using the $2^{-\Delta\Delta CT}$ method, with the *BETA-TUBULIN* (*TUB*) gene serving as a reference gene [37]. Then, the relative expression level of the target genes among different populations were compared using analysis of variance (ANOVA) followed by Dunnett's multiple comparisons using GraphPad Prism (version 6.0.0 for Windows, GraphPad Software, San Diego, California USA). Six genes, including *SOC1*, *GI*, *FKF1*, *GASA5*, *GAI*, *AP2*, were quantified and compared in this experiment.

Results

Differentiation of flowering time

Flowering time showed a notable disparity among populations, with populations originating from lower latitudes exhibiting delayed flowering compared to those from higher latitudes (Fig. 2). The plants from the MDJ and DD populations exhibited the earliest flowering, with a duration of approximately sixty days from germination to the onset of flowering. The FK population exhibited the most prolonged duration until flowering, approximately twice as long as the earliest group. The populations of WH, HG, and NC all demonstrated a duration of approximately one hundred days to reach the flowering stage.

De novo assembly of transcriptome

We performed RNA sequencing on leaf samples collected from DD, HG, and FK populations at three time points, with three biological replicates. Approximately 20 million raw reads were obtained for each library. After removing low-quality and ambiguous reads, the effective rates (the ratio of clean data to raw data) approached 95% across libraries. The Q20 and Q30 values were greater than 97% and 92%, respectively (Additional file 1), indicating an exceptional quality of the clean reads.

For the specific sample of DD population collected on July 18th, contigs were pooled together and further assembled into a non-redundant unigene set with Trinity and CD-HIT. The raw read counts amounted to 6,509,633, and subsequent transcriptome analysis led to the identification of 79,600 transcripts following rigorous filtering procedures. The N50 of the selected sequences was 958bp, with a GC content exceeding 40%. Moreover, a BUSCO analysis revealed that about 77.5% of the 1440 core embryophyte genes were found to be complete, indicating comprehensive mapping of the selected sequences. Overall, these results demonstrated the high quality of the identified sequences and their suitability as reference materials for subsequent analyses.

Identification of differentially expressed genes (DEGs)

Differentially expressed genes were identified for different populations at different time points (Fig. 3). Due to the mortality of the sampled plants from the HG population in September, it was not feasible to make any comparisons involving the HG population at the final time point. The findings unveiled substantial divergence in gene expression across the three populations, notably in the comparisons between the DD and FK populations, as well as between the DD and HG populations. There were 2985, 2464, and 2749 DEGs between the DD and FK populations at the three sample times. Of these DEGs, 521, 276, and 568 genes exhibited upregulated expression, while 2464, 2166, and 2181 genes displayed downregulated expression. Furthermore, we identified 1368 and 2185 DEGs between the DD and HG populations at two sample times. Among these DEGs, 37 and 141 genes were upregulated, while 1331 and 2044 genes were downregulated. Additionally, we identified a total of 519 and 575 DEGs in the HG and FK populations in July and August. Among these DEGs, there were 202 and 272 genes with upregulated expression, while 317 and 303 genes exhibited downregulated expression.

GO annotation of DEGs

The upregulated and downregulated DEGs for each comparison were enriched in the GO databases, and were divided into three main categories of GO classification, including biological process (BP), cellular component (CC), and molecular function (MF). The results showed distinct GO term profiles for each comparison.

For samples collected in July, the up-regulated DEGs in the comparison of DD vs. FK, DD vs. HG, and HG vs. FK were enriched in DNA replication, methylation, cell activity and biosynthesis process, such as cinnamic acid biosynthetic process, photosystem I reaction center, and phenylalanine ammonia-lyase activity (Fig. 4). The down-regulated DEGs in the comparison of DD vs. FK, DD vs. HG, and HG vs. FK were all enriched in photosynthesis, response to Karrikin, photosystem II and the light system including "photosystem I reaction center" and "ADP binding" (Fig. 4). For samples collected in August, the up-regulated DEGs in the comparison of DD vs. FK, DD vs. HG, and HG vs. FK were enriched in development processes, biosynthesis, and metabolism. The up-regulated DEGs in the comparison of DD vs. FK, DD vs. HG, and HG vs. FK were enriched in photosystem II and epsilon DNA polymerase complex. For samples collected in September, the up-regulated DEGs in the comparison of DD vs. FK exhibited enrichment in photosynthesis, the light system, catabolic processes, metabolic processes, and sugar transport, while the down-regulated DEGs were found to be enriched in response to external stimuli and ribosome activity.

Bioregulation analysis and transcription factor identification of DEGs

The DEGs of different populations in *A. artemisiifolia* were analyzed using MapMan to elucidate their functional roles in biological regulation and metabolism. The results revealed that genes related to hormone regulation, such as indole-3-acetic acid (IAA), abscisic acid (ABA), benzyladenine (BA), cytokinins, and gibberellic acid (GA), along with redox reactions, showed differential expression among different populations (Additional file 2). Among the hormone pathways, the ABA and GA pathways exhibited the highest abundance of DEGs, while the IAA pathway had the fewest DEGs. However, the MapMan analysis revealed a limited number of DEGs enriched in the transcription factor (TF) category, while no DEGs were identified in the protein modification and protein degradation categories.

To identify TFs involved in the regulation of flowering time in *A. artemisiifolia*, we used the iTAK software for analysis of the DEGs. Notably, a substantial proportion of these DEGs were found to be associated with TF families including WRKY, AP2/ERF, bZIP, C2H2, bHLH, NAC, and MYB (Additional file 3), thereby highlighting their potential influence on the regulation of flowering time.

Identification of candidate DEGs related to differentiation of flowering time

Based on the Flowering Interactive Database, which comprises genes associated with flowering time in *A. thaliana*, and a mapping file containing annotations of flowering-related genes in *A. artemisiifolia*, we have successfully identified potential candidate genes accountable for variation in flowering time among

populations in *A. artemisiifolia*. The results suggest that the genes including *CONSTANS* (*CO*), *LEAFY* (*LFY*), *FLOWERING LOCUS VE* (*FVE*), *FLOWERING LOCUS KH DOMAIN* (*FLK*), *SHORT VEGETATIVE PHASE* (*SVP*), *GA INSENSITIVE* (*GAI*), *FRIGIDA-ESSENTIAL 1* (*FES1*), *FLOWERING LOCUS* (*FD*) all may contribute to variation in flowering time across different populations and therefore involve pathways such as photoperiod, autonomous, temperature, gibberellin and vernalization (Fig. 5). Notably, a substantial proportion of the candidate genes were found to be associated with the autonomous pathway. For samples collected in July, both *SVP* and *FD* exhibited differential expression in the comparisons of DD vs. FK and DD vs. HG (Fig. 5A). *FES1* exhibited differential expression in the comparisons of DD vs. HG and HG vs. FK. In the samples collected in August, we identified four candidate genes related to flowering time (Fig. 5B). Among them, *SVP* and *FD* were differentially expressed in both comparisons of DD vs. FK and DD vs. HG. For samples collected in September, only *SVP* and *FD* were identified to exhibit differential expression in the comparison of DD vs. FK, due to the unavailability of HG sample during that period (Fig. 5C). It is noteworthy that certain candidate genes exhibited different functionalities compared to those observed in *A. thaliana*. For example, *FY*, *FVE* and *FLK*, belonging to the autonomous pathway, displayed elevated expression levels in the delayed flowering population FK. This implies that these genes exert a suppressive effect on the flowering process in *A. artemisiifolia*, whereas their homologous counterparts play a promotive role in regulating flowering in *A. thaliana*. The expression of *GAI* in the gibberellin pathway was found to be upregulated in the early flowering population DD, indicating that *GAI* plays a role as a promoter of flowering induction in *A. artemisiifolia*, which contrasts with its function in *A. thaliana*. Noticeable, some DEGs involved in flower development, as identified through GO annotation, lack homologous counterparts in *A. thaliana*. A total of 15 transcripts were identified and may represent novel genetic elements that contribute to differentiation of flowering time in *A. artemisiifolia*.

To validate the impact of these potential genes on flowering, the expression levels of all the samples were quantified using FPKM values and subsequently subjected to comparative analysis. The expression levels of numerous genes exhibited a substantial correlation with latitude (Fig. 6). In July, during the vegetative development stage of all samples, the expression level of *CO* exhibited a significantly positive correlation with the latitude of origin, whereas there was a notable negative correlation observed between the expression levels of *FY* and *FVE* and the latitude of origin (Fig. 6). The expression level of *SVP* showed a negative correlation with latitude of origin in September (Fig. 6).

qRT-PCR validation of expression of genes related to differentiation of flowering time

To validate the efficacy of RNA-seq in this experiment, qRT-PCR analysis was conducted on six genes, *SOC1*, *GI*, *FKF1*, *GASA5*, *GAI*, and *AP2*, selected from the DEGs associated with flowering induction. For the majority of genes, the expression patterns obtained from the qRT-PCR assays were found to be consistent with their observed trends in the RNA-seq analysis (Fig. 7), thereby providing support for the reliability and accuracy of the RNA-seq data in reflecting gene expression.

Discussion

The differentiation of flowering time in Chinese invasive populations of *A. artemisiifolia*

Multiple studies have provided evidence that *A. artemisiifolia* displays a remarkable capacity for local adaptation, enabling it to thrive and reproduce effectively in novel environments [4, 5, 27, 38]. Flowering time, being a pivotal trait, has been observed to be negatively correlated with the latitude of origin in *A. artemisiifolia* [4, 7], and our findings from investigating flowering time across seven populations in China substantiate this perspective. The MDJ and DD populations, which originate from the northernmost regions, also exhibited the earliest onset of flowering, whereas the FK population, from the southernmost site, displayed the latest flowering time, approximately sixty days later than that observed in the northernmost populations (Figs. 1 and 2). This observed variation in flowering time is likely to be associated with adaptation to local environments. Notably, despite the abundance of studies examining flowering time variation among accessions, only a limited number of investigations have successfully revealed the genetic mechanisms underlying such variation, particularly in non-model plants [27]. One reason is the high cost associated with traditional approaches to ascertain gene function. Another reason is the prevalent tendency to assume conservation of genetic functions in model plants and extrapolate their molecular mechanisms to non-model plants, despite a considerable proportion of genes exhibiting distinct functionalities compared to those observed in model plants. Fortunately, transcriptome sequencing technology has emerged as a widely used method for investigating gene expression, as well as screening and predicting candidate genes, even in the absence of a reference genome [28, 29, 39]. Additionally, invasive plants offer optimal materials for studying evolution and adaptation. Elucidating the molecular mechanism underlying flowering time adaptation in invasive plants holds significant value as it aids in comprehending the factors contributing to their global distribution and successful invasions at the molecular level.

Differential expression of genes among the populations with different flowering time

Limited knowledge exists regarding the transcriptional and gene expression mechanisms governing the regulation of flowering time variation among populations in *A. artemisiifolia* due to a dearth of comprehensive genomic data. Several studies have demonstrated genetic differentiation in diverse life history traits among different populations of *A. artemisiifolia* [38, 40]. In this study, we employed transcriptome data from three populations with distinct flowering times to investigate the molecular mechanisms underlying the differentiation of flowering time in *A. artemisiifolia*. The DD, HG and FK populations, representing the early-flowering, intermediate-flowering and late-flowering groups respectively, encompass a broad range of latitudes across China. These populations may provide valuable insights into the overall characteristics of Chinese populations in terms of flowering time variation. We hypothesized that environmental adaptation plays a critical role in flowering time variation. The comparison of DD vs. FK populations displayed the greatest number of DEGs, irrespective of the sample time, followed by the comparison of DD vs. HG populations and ultimately the comparison of HG

vs. FK. The DD and FK populations originate from the northern and southern regions of China, respectively, thereby experiencing significantly divergent climatic conditions in their indigenous habitats. Plants situated in the northern regions of China typically encounter lower temperatures and earlier frost events compared to their counterparts in southern regions. Given the sensitivity of fruit development in *A. artemisiifolia* to frost, only early-flowering plants have the opportunity to produce viable seeds [41]. A study conducting reciprocal transplant experiment on *A. artemisiifolia* revealed that the northern population exhibited earlier flowering and greater production of female flowers and seeds compared to their southern counterparts, suggesting local adaptation in the former through enhanced female fitness [7].

Differential expressions in genes with various biological functions, regulation and metabolism-related functions among populations with different flowering time

A GO annotation of DEGs provided significant insights into the biological functions associated with variation in flowering among populations. During the vegetative stage of all samples in July, we observed a higher enrichment of upregulated DEGs related to DNA replication, methylation, and cell activity in the FK population compared to both the DD and HG populations. Conversely, the DD and HG populations exhibited a greater enrichment of upregulated DEGs associated with photosynthesis and light systems when compared to the FK population. These findings suggest that populations with earlier flowering exhibit elevated expression of genes associated with growth and development. Notably, the DEGs pertaining to DNA methylation were discerned in the comparisons between the DD and FK populations during July, primarily linked with the vernalization and autonomous pathways within the flowering induction network. During August, when the DD and HG populations had already flowered while the FK population remained in a vegetative stage, a higher abundance of upregulated DEGs related to biosynthesis and metabolism was observed in the FK population. This finding reflects the ongoing active growth in the FK population. During September, when all populations had undergone flowering with the FK population exhibiting the latest flowering time, the DEGs identified in the comparison between DD and FK were found to be significantly enriched in light system, photosynthesis, and sugar transport. These findings suggest that the regulation of floral transition in the FK population is intricately linked to crucial roles played by sugar metabolism pathways. In conclusion, our findings suggest that the light, sugar, vernalization, and the autonomous pathways may collectively influence flowering time variation among populations.

Furthermore, the biological regulation and metabolism-related functions of DEGs were examined using Mercator4 protein categories. Overall, it has been observed that during the sample time of July and August, a significant downregulation of genes associated with hormone regulation and redox regulation was evident in populations exhibiting earlier flowering compared to those displaying later flowering phenotypes. In contrast, a greater number of downregulated genes in the FK populations compared to DD were related to abscisic acid (ABA) regulation in September. The flowering of the FK population prior to the collection of the September samples implies a potential inhibitory role of ABA in triggering the floral transition in *A. artemisiifolia*. This inference is consistent with findings in other species such as

roses [42] and *Mikania micrantha* [39]. In September, a higher number of upregulated genes, associated with gibberellin (GA) regulation, were observed in the late flowering FK population compared to earlier time points. This finding implies that GAs exerts a beneficial impact on the induction of floral development in *A. artemisiifolia*. GAs have been reported to promote the initiation of flowering in various plant species, such as chrysanthemum [43] and peony [44], while inhibiting flower induction in certain fruit trees like apple [45] and loquat [46]. Furthermore, more downregulated genes in the comparison of DD vs. FK were observed to be related to cytokinin in July compared to both August and September, indicating that the cytokinin pathway exerts a negative regulatory effect on flower induction in *A. artemisiifolia*. This finding is consistent with previous studies that have shown the antagonistic effects of GAs and cytokinin on leaf formation and meristem maintenance [47]. Additionally, no discernible trends were observed in the levels of other hormones across different populations, indicating that their impact on the regulation of flowering in *A. artemisiifolia* may be negligible.

Differential expressions in transcription factors among populations with different flowering time

Given the potential significance of transcription factors (TFs) in regulating flower differentiation [48, 49], it was imperative to identify TFs related to flowering time differentiation in *A. artemisiifolia*. Although only a restricted subset of TFs was identified using the Mapman analysis (Fig. 5), further investigation using the iTAK software revealed the presence of several TFs associated with the differentiation of flowering time in *A. artemisiifolia*, including WRKY, AP2/ERF, bZIP, C2H2, bHLH, NAC, and MYB-related TFs. Some of these TFs have previously been confirmed to impact the timing of flowering. For instance, the WRKY family of TFs is well-established for its regulatory role in plant development and flowering [50, 51]. In *Arabidopsis*, under short daylength conditions, *WRKY12* and *WRKY13* exhibit divergent effects on flowering, with *WRKY12* promoting flowering while *WRKY13* inhibiting it [50]. These TFs govern the regulation of flowering through the gibberellin and age pathways [50, 51]. The bZIP TFs contribute to the process of floral induction by participating in a sophisticated network of transcriptional interactions. The *FD* bZIP TF, expressed in the stem apical meristem, forms a transient complex with *FT/TSF* to promote floral induction [52]. The *AP2* TFs, on the other hand, exert a negative regulatory effect on flowering through their interaction with other TFs within the intricate network of gene expression. For example, *AP2* and *SMZ* directly bind to *SOC1* and *FT* promoters, thereby inhibiting their expression [53, 54]. *C2H2* TFs influence flowering induction through participating in histone modification at *FLC* sites and regulating *FT* expression via the photoperiodic regulatory pathway [55]. The bHLH TFs are involved in GA biosynthesis and action during the flowering transition [56]. Under long-day conditions, GAs inhibit the activity of the *MYC3* bHLH TF, which is stabilized by DELLAs [57]. *MYC3* acts as a direct inhibitor of *FT* and antagonizes *CO* in the regulation of flowering [57]. These important TFs are likely to play a pivotal role in the differentiation of flowering time also in *A. artemisiifolia* through various pathways, including the photoperiod, autonomous, vernalization, and GA pathways. The identification of these TFs provides valuable insights into the regulatory mechanisms underlying the variation in flowering time within this species.

Differential expressions in flowering-related genes among populations with different flowering time

Based on the gene networks involved in controlling flowering time in *A. thaliana* and the mapping file of genes related to flowering in *A. artemisiifolia*, we have identified eight potential candidate genes that contribute to differentiation of flowering time in *A. artemisiifolia*. The expression levels of *CO*, *GAI*, *FD*, and *FES1* genes were found to be significantly upregulated in early-flowering populations (Fig. 5), thereby serving as promoters of flowering in *A. artemisiifolia*. Conversely, *FY*, *FVE*, *FLK*, and *SVP* genes exhibited elevated expression levels in populations with delayed flowering (Fig. 5), exerting inhibitory effects on the floral transition process. *CO* plays a vital role in the photoperiod pathway as a transcription factor that facilitates flowering by inducing the expression of key downstream genes, namely *FT* [58] and *SOC1* [59]. Acting as integrators, *FT* and *SOC1* receive upstream signals and promote flowering by activating downstream genes involved in the initiation of flowering [60]. In our study, our results suggest that *CO* functions as a positive regulator of flowering induction in *A. artemisiifolia*, akin to its role in *A. thaliana*. In the autonomous pathway of *A. thaliana*, seven regulators (*FCA*, *FY*, *FPA*, *FVE*, *FLD*, *FLK*, and *LD*) act in parallel to suppress the expression of *FLC* and facilitate flowering [61]. In our study, we found that the *FY*, *FVE* and *FLK* genes exerted inhibitory effects on flowering in *A. artemisiifolia*. *GAI*, a DELLA protein known for its inhibitory role in flowering through the gibberellin pathway in *A. thaliana* [62], exhibited a promotive function on flowering in *A. artemisiifolia*. *SVP* delays flowering by suppressing the transcription of *FT* and *SOC1* in the temperature pathway in *A. thaliana* [63]. The suppressive effect of *SVP* on flowering was also observed in *A. artemisiifolia*, as evidenced by the higher expression observed in the population with delayed flowering (Fig. 5). *FES1*, involved in the *FRI*-mediated vernalization pathway, plays a crucial role in the upregulation of *FLC* expression, and its inactivation leads to the complete suppression of *FRI*-mediated delayed flowering in *A. thaliana* [64, 65]. The results in this study suggest that *FES1* acted as a positive factor in flowering induction in *A. artemisiifolia*. The *FD* protein collaborates with *FT* in the meristem to facilitate the activation of *SOC1* and the floral meristem identity genes, thereby triggering flowering in *A. thaliana* [66]. The promotive effect of *FD* on flowering was also evident in *A. artemisiifolia* in this study. In conclusion, the genes involved in the photoperiodic, vernalization, gibberellin, temperature, and autonomous pathways collectively contribute to the differentiation of flowering time in *A. artemisiifolia*. Our findings are thus consistent with a previous study which suggested that genetic variation within the autonomous, temperature, and photoperiodic pathways enable *A. artemisiifolia* to synchronize flowering with favorable climatic conditions [67].

The genetic mechanisms involved in the regulation of flowering in non-model species have been predominantly investigated by referencing the model plant *A. thaliana*. The regulatory network governing flowering time is typically conserved across plant species, yet variations in regulation also exist among different taxa. For example, a study conducted on strawberries showed that *GAIP* exhibited a promotive effect on flower induction in strawberry plants, while it functioned as an inhibitor agent in *A. thaliana* [13]. Similarly, our study revealed that the roles of *FY*, *FVE*, *FLK*, and *GAI* in flowering regulation exhibited inconsistencies between *A. artemisiifolia* and *A. thaliana*, highlighting the nuanced complexities

underlying this process. In addition, the identification of novel genes further highlighted variation in the regulation of flowering across different taxa. Some species have identified novel genes that lack homologous counterparts *A. thaliana*. In our study, we employed GO annotation and successfully identified 13 novel transcripts (TRINITY_DN50167_c0_g2_i1, TRINITY_DN51257_c1_g3_i1, etc.) associated with flower development in *A. artemisiifolia* that lack homologs in *A. thaliana*. Therefore, we speculated that these 13 newly discovered transcripts may possess distinct advantageous roles in the specific regulation of flowering in *A. artemisiifolia*.

Conclusions

In this research, we conducted a common garden experiment and performed transcriptome analysis to investigate the variation in flowering time among populations of *A. artemisiifolia* from different latitudes in China and elucidate the genetic mechanisms underlying such differentiation. We observed a substantial number of differentially expressed genes (DEGs) between populations with differential flowering times. The analysis of GO annotation and bioregulation annotation on DEGs unveiled the involvement of light-related pathways, the autonomous pathway, sugar metabolism, vernalization, and hormone signaling in modulating the differentiation of flowering time in *A. artemisiifolia*. Additionally, we have identified several families of transcription factors, including WRKY, AP2/ERF, bZIP, C2H2, bHLH, NAC, and MYB-related TFs that are likely to contribute to the observed variation in flowering time in *A. artemisiifolia*. Importantly, we have identified eight candidate flowering-related genes that may exert substantial influence on the differentiation of flowering time among different populations of *A. artemisiifolia*. Specifically, it was observed that *CO*, *FES1*, *FD* and *GAI* functioned as positive regulators while *FY*, *FVE*, *FLK*, and *SVP* acted as negative regulators in the context of flowering regulation in *A. artemisiifolia*. Additionally, we have discovered 13 previously uncharacterized transcripts that appear to be involved in the regulation of flowering in *A. artemisiifolia*. These findings provide valuable insights into the molecular mechanisms that underlie the differentiation of flowering time in *A. artemisiifolia*, thereby enhancing our comprehension of the mechanisms driving this successful invasion.

Abbreviations

TFs

Transcription factors

LFY

LEAFY

FT

FLOWERING LOCUS T

SOC1

SUPPRESSOR OF OVEREXPRESSION OF CO 1

PHYs

PHYTOCHROME s

GI

GIGANTEA
BTC1
BOLTING TIME CONTROL 1
FLC
FLOWERING LOCUS C
PRR37
Pseudo-Response Regulator 37
FRL3a
FRI-LIKE3a
GI1
GIGANTEA 1
CO
CONSTANS
LFY
LEAFY
FVE
FLOWERING LOCUS VE
FLK
FLOWERING LOCUS KH DOMAIN
SVP
SHORT VEGETATIVE PHASE
GAI
GA INSENSITIVE
FES1
FRIGIDA-ESSENTIAL 1
FD
FLOWERING LOCUS
RNA-Seq
RNA sequencing
DEGs
Differentially expressed genes
FPKM
Fragments Per Kilobase Million
ORFs
Open reading frames
BUSCO
Benchmarking Universal Single-Copy Orthologs
CDS
Coding domain sequences
FDR

False discovery rate
GO
Gene Ontology
FLOR-ID
Flowering Interactive Database
RT-qPCR
Real-time quantitative PCR
ANOVA
Analysis of variance
BP
Biological process
CC
Cellular component
MF
Molecular function
ABA
Abscisic acid
GAs
Gibberellins.

Declarations

Ethics approval and consent to participate

NO specific permission was needed to collect *Ambrosia artemisiifolia* materials for research.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. 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 SAMN40142471 (<http://www.ncbi.nlm.nih.gov/bioproject/1080606>).

Competing interests

The authors declare that they have no competing interests.

Funding

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

Authors' contributions

YXF, LXM and LWJ designed the research. LXM, XC and LWJ performed common garden experiment, and YXF performed the quantitative real-time experiments, 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.

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 seed collections of *A. artemisiifolia*, and we extend our appreciation to Quan-Bao Zhang for his assistance in the data analysis for transcriptome.

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Figures

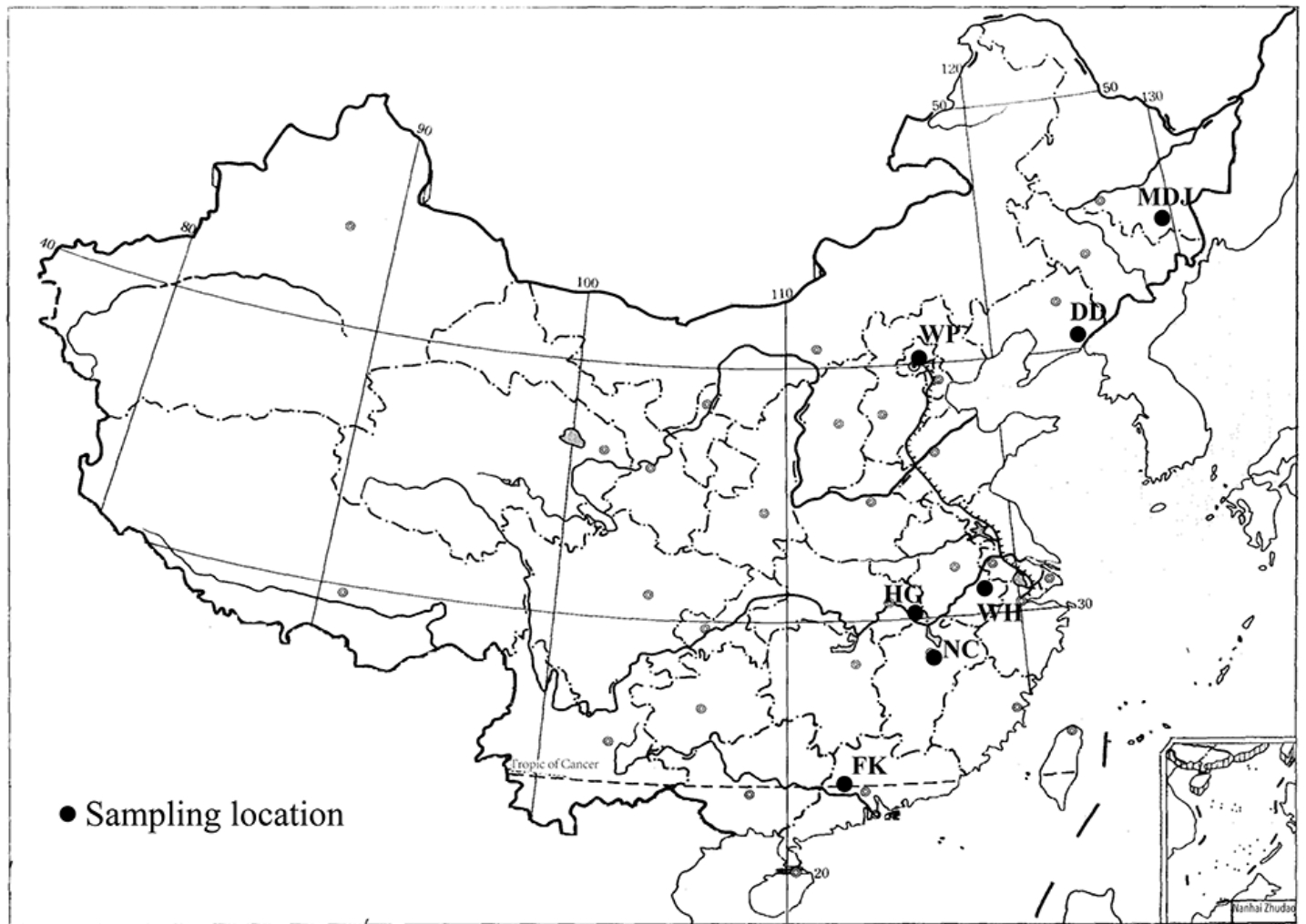


Figure 1

The locations of *Ambrosia artemisiifolia* populations included in this study. MDJ, DD, WP, WH, HG, NC and FK represent populations from Mudanjiang, Dandong, Wangping, Wuhu, Huanggang, Nanchang and Fengkai, respectively.

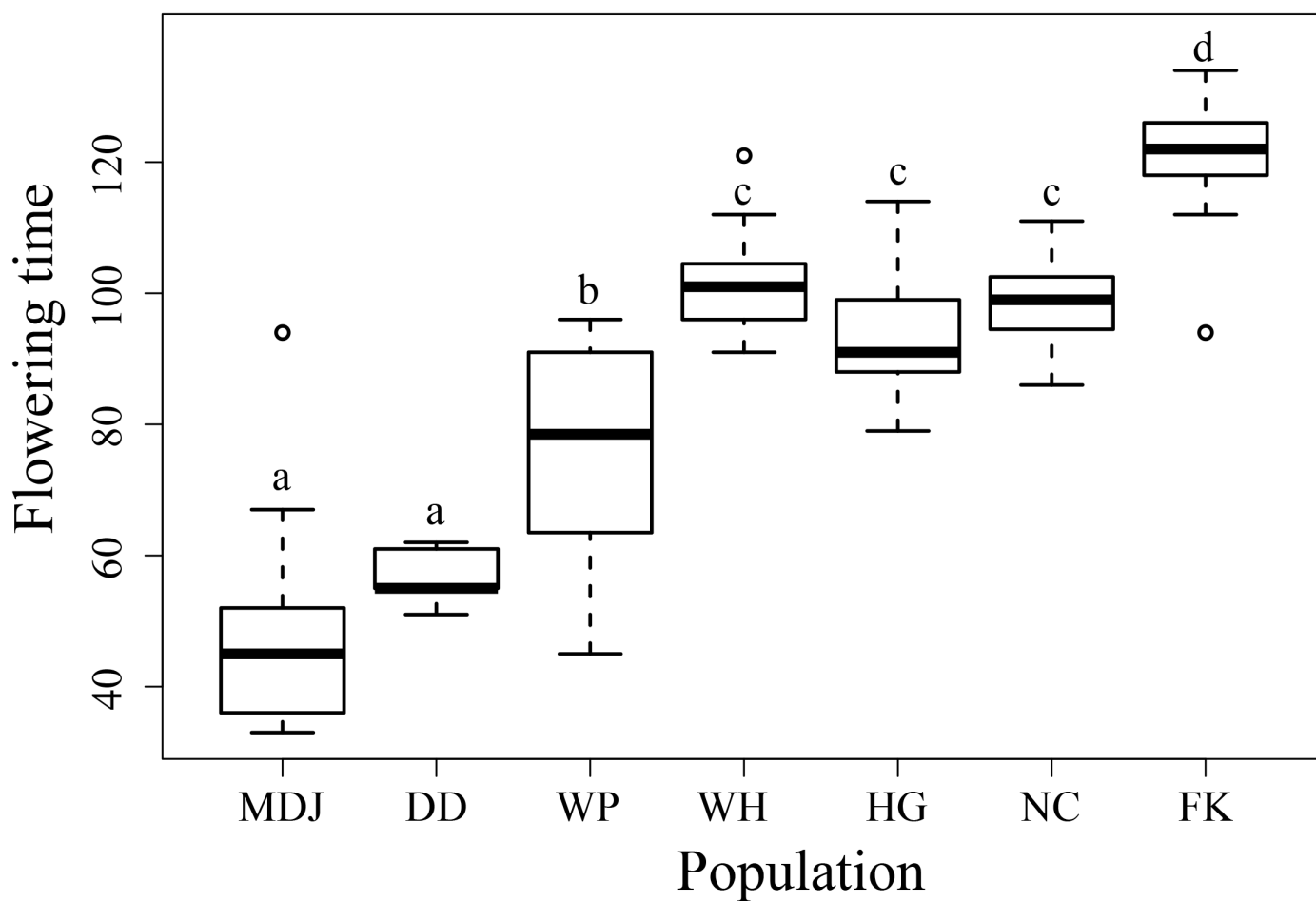


Figure 2

The box plot showing the flowering time of different populations of *Ambrosia artemisiifolia* in China. The central line in the box marks the median value. The bars represent the ranges of data. The distinct characters displayed above the box indicate a statistically significant distinction.

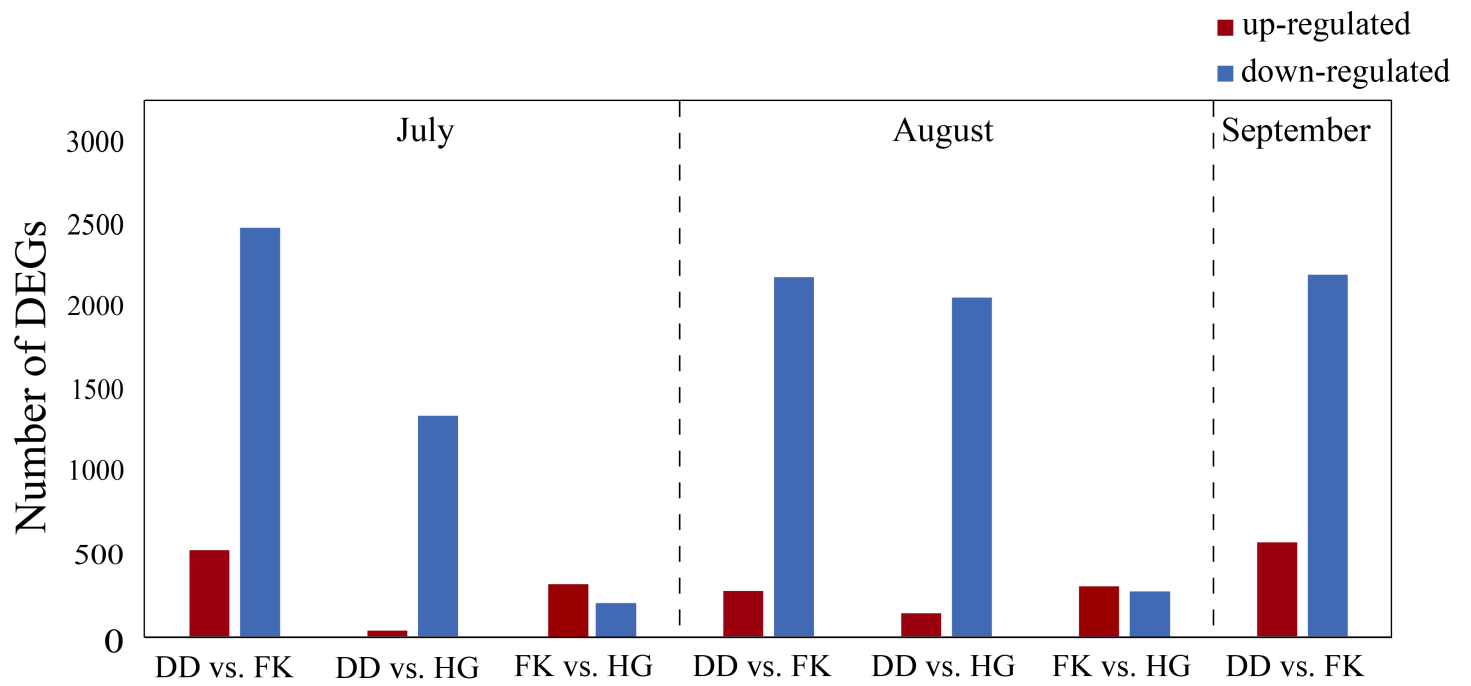


Figure 3

Number of up-regulated and down-regulated DEGs between different populations of *Ambrosia artemisiifolia* in China.

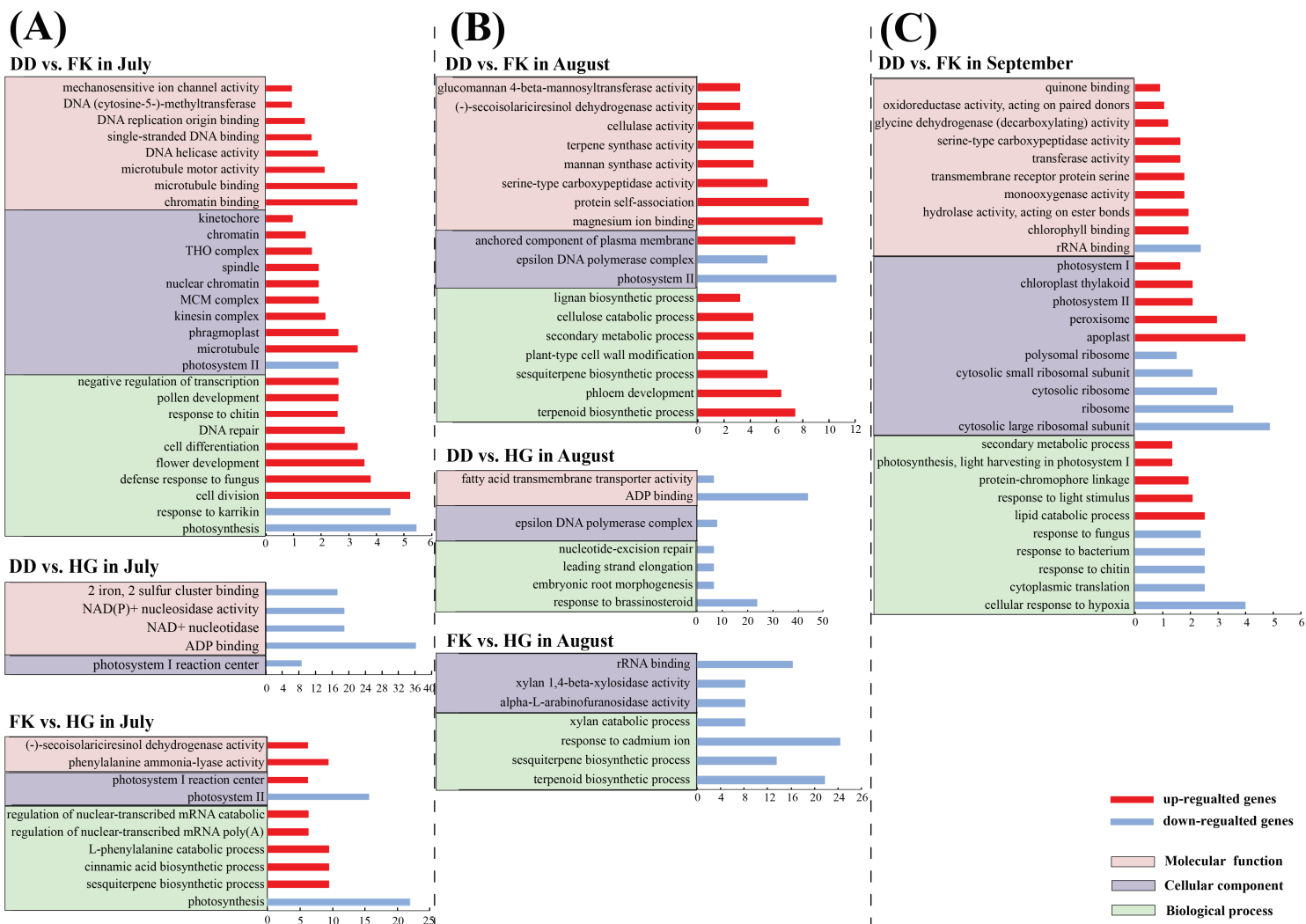


Figure 4

GO functional classification of differential expressive genes among the samples with different flowering time. The horizontal axis represents the percentage of genes, and the vertical axis represents the GO terms. The GO terms were sorted based on corrected P -values. (A) Significantly enriched GO terms for samples of July. (B) Significantly enriched GO terms for samples of August. (C) Significantly enriched GO terms for samples of September.

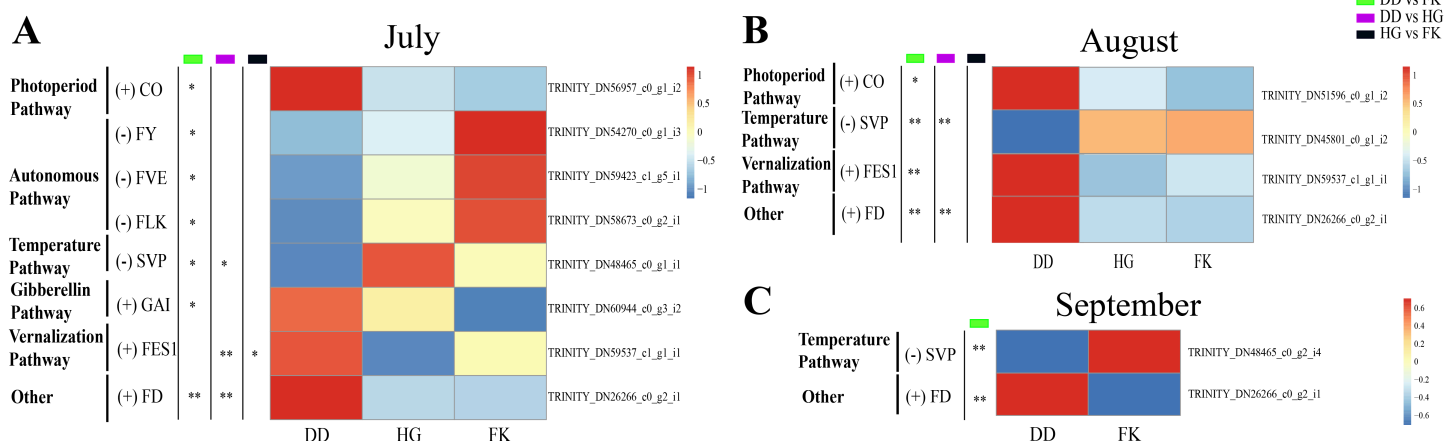


Figure 5

Heatmaps illustrating the FPKM values of flowering related DEGs across populations with different flowering time. The bar represents the range of expression levels for each gene in every population, with blue rectangles indicating low expression and red rectangles denoting high expression. The symbols (+) and (-) are employed to denote the positive and negative regulators, respectively, of flower initiation in *A. artemisiifolia*. The asterisk (*) indicates a statistically significant difference in the expression levels observed for each comparison, as determined by the student's t-test analysis (* $p < 0.05$, ** $p < 0.01$). (A-C) show the heatmaps for samples of July, August, September, respectively.

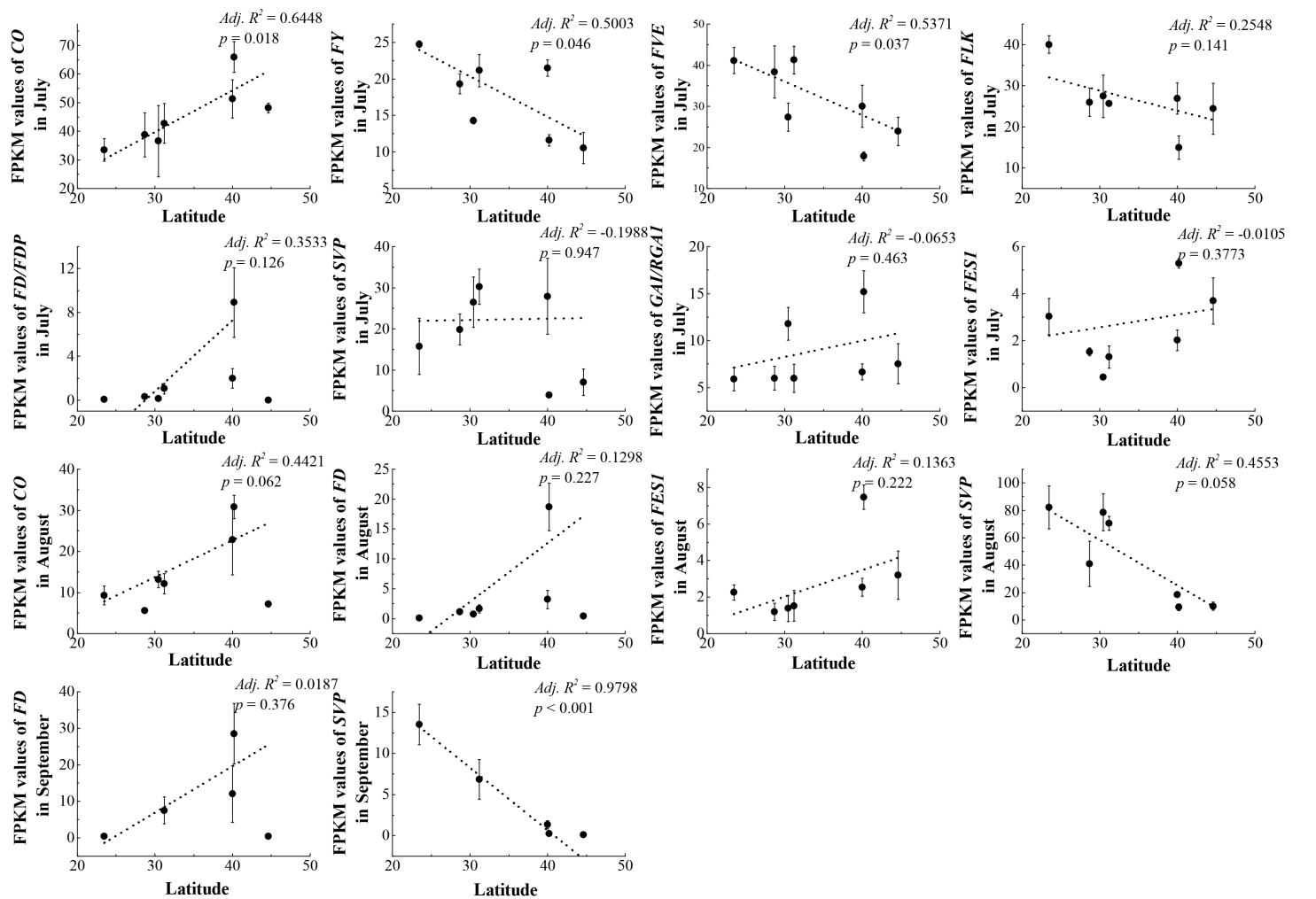


Figure 6

The correlations between the FPKM values and latitudes of original populations for several flowering-related genes expressed in July, August and September, respectively.

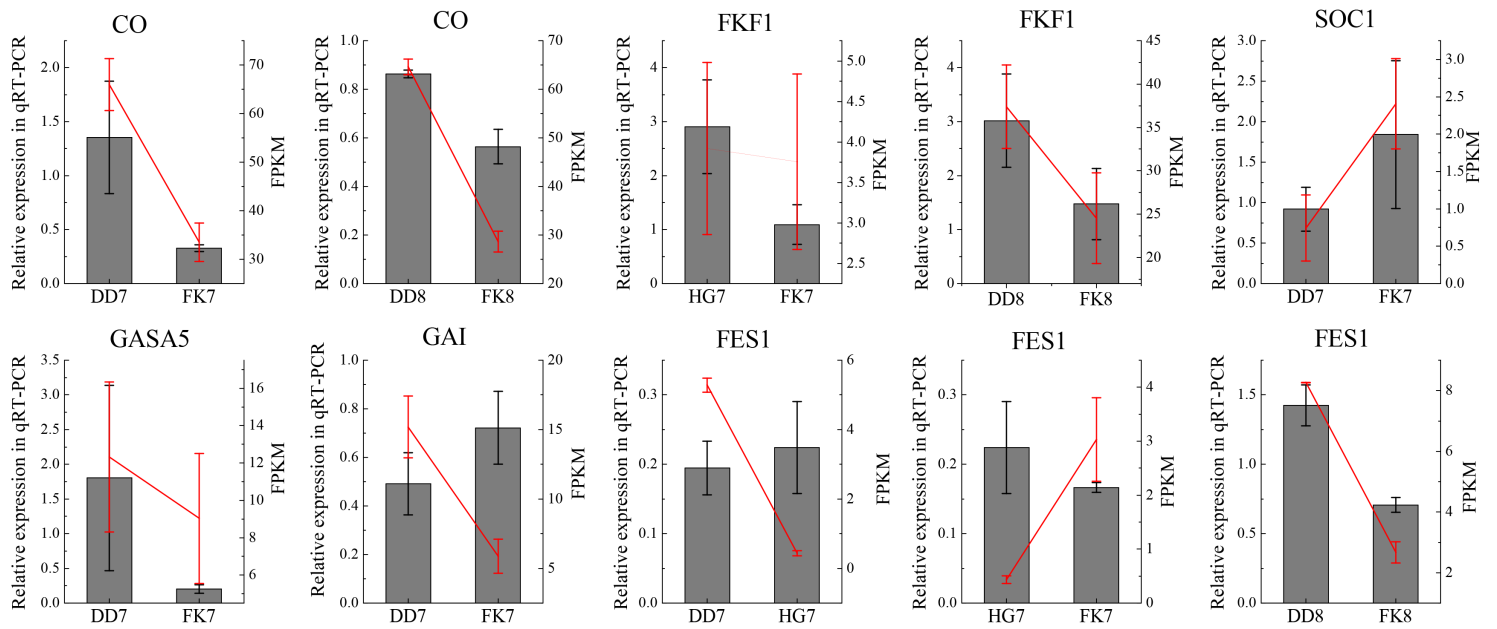


Figure 7

Expression levels of candidate genes related to flowering time differentiation measured using qRT-PCR. DD7, DD8, HG7, FK7, and FK8 represent the samples of Dandong in July, Dandong in August, Huanggang in July, Fengkai in July, and Fengkai in August, respectively.

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