Integrated Transcriptome and Proteome Analysis Provides insights into CpFPA1 for Floral Induction in Chimonanthus praecox (Magnoliidae) without FLC in genome

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Research Article

Keywords: transcriptome and proteome analysis, FPA, Chimonanthus praecox, RNA binding protein, autonomous pathway, flower bud differentiation

Posted Date: October 4th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3381230/v1

Version of Record: A version of this preprint was published at Plant Cell Reports on February 10th, 2024. See the published version at https://doi.org/10.1007/s00299-024-03145-7.

Abstract

Wintersweet (*Chimonanthus praecox*), a rare winter-flowering woody plant, is well known for its unique blooming time, fragrance and long flowering period. However, the molecular mechanism of flowering in *C. praecox* remains poorly unclear. In this study, we used transcriptomic and proteomic association analysis to reveal the critical genes/proteins at three key flower bud (FB) differentiation stages (FB.Apr, FB.May and FB.Nov) in *C. praecox*. The results showed that a total of 952 DEGs and 40 DEPs were identified. Gene ontology (GO) enrichment revealed that DEGs in FB.Apr/FB.May comparison group were mainly involved in metabolic of biological process, cell and cell part of cellular component and catalytic activity of molecular function. In the KOG functional classification, DEPs were predicted mainly in the function of general function prediction only (KOG0118), post-translational modification, protein turnover and chaperones. The autonomous pathway genes play an essential role in the floral induction. Based on transcriptome and proteome correlation analysis, six candidate genes associated with the autonomous pathway were identified, including *FPA1*, *FPA2a*, *FPA2b*, *FCA*, *FLK*, *FY.* The fold change of unigene0031805 FPA1 in mRNA and protein level reached over 5 and 1.5 in FB.Nov/FB.Apr and FB.Nov/FB.May; and that of which reached over 2.5 and 1.2 in FB.May/FB.Apr, respectively. Furthermore, *CpFPA1* was isolated and functionally characterized, and ectopic expression of *CpFPA1* in *Arabidopsis* Columbia (Col-0) resulted in earlier flowering. These data could contribute to understand the function of *CpFPA1* for floral induction and provide information for further research on the molecular mechanisms of flowering in wintersweet.

Key Message

We used transcriptomic and proteomic association analysis to reveal the critical genes/proteins at three key flower bud differentiation stages and overexpression of *CpFPA1* in *Arabidopsis* resulted in earlier flowering.

1. Introduction

The flowering process generally consists of three stages: floral induction, floral evocation and floral development. Floral induction is a particularly critical part of life cycle for plants, also a complex process controlled by external environment and endogenous factors, which refers to the transition from vegetative to the reproductive phase (Kim et al. 2013). This process leads to the transition of the shoot apical meristem (SAM) into the floral meristem (FM) (Latif Ahmad et al. 2021; Bernier and Perilleux 2005). Previous studies have shown six major pathways that are involved in flowering, including photoperiod, vernalization, ambient temperature, aging, gibberellin (GA), and autonomous pathways (Wellmer and Riechmann 2010). Finally, these pathways form a regulatory network that comes together and interacts, integrating the flowering pathways mainly through flowering integrator genes, such as *FLOWERING LOCUS T* (*FT*), *SUPPERSSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *APETALA1* (*AP1*) and *LEAFY* (*LFY*) (Castro Marin et al. 2011; Zhang et al. 2011).

A massive number of photoperiod and vernalization pathways have been reported in plants. However, the autonomous pathway has not been intensively studied. It was found that floral transition could be induced by the autonomous pathway paralleled to photoperiod and vernalization pathways upstream of *FLOWER LOCUS C* (*FLC*) (Abou-Elwafa et al. 2011; Srikanth and Schmid 2011). The autonomous pathway is not affected by external environmental factors and regulates flowering by responding to internal factors (Mouhu et al. 2009; Wellmer and Riechmann 2010). The autonomous pathway promotes flowering by inhibiting the expression level of the *FLC* gene by the RNA-based controlled mechanism and/or chromatin modification (Winichayakul et al. 2005; Abou-Elwafa et al. 2011). *FLC*, a member of MADS-box gene family, acted as a repressor of flowering (Rouse et al. 2002). In *Arabidopsis*, there are several autonomous pathway genes: *FLOWERING TIME CONTRO*L *PROTEIN FPA* (*FPA*), *FLOWERING LOCUS D* (*FLD*), *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS K* (*FLK*), *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS VE* (*FVE*), *FLOWERING TIME CONTROL PROTEIN FY* (*FY*), *REF6*, etc. (Khan et al. 2014; Lu et al. 2011). Three RNA-binding proteins (RBPs), *FPA*, *FCA* and *FLK* and one 3'-end RNA-processing factor, *FY*, were involved in RNA processing (Schomburg et al. 2001; Sonmez et al. 2011; Simpson et al. 2004; Quesada et al. 2005). *FLD*, *FVE* and *LD* could regulate chromatin modification state (Hu et al. 2014). *FPA*, belonged to the split ends (SPEN) family, which contained three RRM (RNA recognition motif) motifs and a SPOC (spen paralog and ortholog C-terminal) domain at the C terminus (Solis-Guzman et al. 2017; Candela et al. 2016). The *fpa* mutant exhibited a late-flowering phenotype (Schomburg et al. 2001). *FPA* played a functionally redundant role with *FLD*, *FVE*, *LD* and *FY* in the process of growth and development (Veley and Michaels 2008; Wu et al. 2020). *FPA* and *FCA* functioned redundantly through the accumulation of *FLC* mRNA, but the activation of *FPA* and *FCA* might require *FLD* (Hornyik et al. 2010; Steffen et al. 2019). Other autonomous pathway genes have been discovered in recent years, including *AGL28*, *CK2*, *DBP1*, *ATPRP39-1*, *KHZ1*, *KHZ2* and others. (Cheng et al. 2017; Wang et al. 2007; Yan et al. 2020). Furthermore, the autonomous pathway could also be involved in regulating the seed germination (Auge et al. 2018).

Chimonanthus praecox (wintersweet), a traditional flower in China, is popular for its flowering time, color and fragrance with high ornamental and economic value (Sui et al. 2015; Kamran et al. 2020). It is also used as a traditional Chinese medicinal plant to treat rheumatic arthritis, measles and vomit (Xiang et al. 2010). In wintersweet, previous studies have found that FM was generally formed from March to early April, and perianth segments including calyxes and tepals primordium formed in early May, stamen primordium formed in mid-May, and pistil primordium formed in late May (Jiang et al. 2016). June to September was considered as the dormancy period, followed by recovery and ovary development in October, followed by experiencing cold induction in late November, and finally bloomed in late December to early January in Chongqing City, China (Li et al. 2020; Jiang et al. 2016). The floral organ (perianth segment, stamen and pistil) primordium differentiation from early April to May is the pivotal period of flower bud (FB) differentiation, which is the transition from vegetative growth into reproductive growth and affect the quantity and quality of flowering. Therefore, it is a need to conduct several research during this period to understand the mechanism involved in the floral induction.

Recently, proteome and transcriptome analysis have become a novel mean to analysis the growth and development process of plants. So far, wintersweet transcriptomic and proteomic analysis has mainly focused on the fragrance genes (Tian et al. 2018), break dormancy (Li et al. 2020), floral development, etc. (Liu et al. 2014). However, little is known about the complete mechanism of floral induction in *C. praecox*. In this study, we utilized a comparative proteome and transcriptome analysis by RNA-Seq, TMT, bioinformatics and molecular biology methods to obtained the number, type and predicted function of differentially expressed genes (DEGs) and proteins (DEPs) in response to floral induction at three critical FB differentiation stages of *C. praecox*. In addition, several genes related to the autonomous pathway were screened, and ectopic of *CpFPA1* in *Arabidopsis* had been carried out to explore the function. These data could provide a basis for selecting candidate genes in the process of floral induction and lay a foundation for to reveal the molecular mechanisms of flowering in *C. praecox*.

2. Materials and Methods

2.1 Plant materials

In this study, *C. praecox* was grown on the campus of Southwest University in Chongqing City, China (106°43E, 29°83N). As described in previous study (Li et al. 2020), FBs samples in April, May and November (FB.Apr, FB.May and FB.Nov) in 2019, and FBs exposed to different chill units (CU), such as FB150, FB300, FB450, IB570, and nF16 for RNA-sequencing; FB.Apr, FB.May, FB.Nov, IB570, and nF16 for TMT-sequencing were collected, respectively. In which, FB.Nov, nF16, FB150, FB300, FB450, and IB570 RNA- and/or TMT-sequencing data have been used for revealing the chilling-induced dormancy breaking in deep Winter (Li et al. 2020); here, FB.Apr, FB.May and FB.Nov RNA- and TMT-sequencing data would be used for FB initiation in early Spring in *C. praecox*. The FBs morphology in April, May and November was shown in Supplementary Fig.S1. FBs and flowers samples from 8. March 2018 to 7. January 2019 were harvested for quantitative real-time PCR (qRT-PCR) analysis for the expression patterns analysis of *CpFPA1*. All the samples were immediately frozen using liquid nitrogen and stored at -80°C for subsequent experiments.

The seeds of *Arabidopsis* Cloumbia-0 (Col-0) were obtained from our laboratory. The seeds were sowed on solid Murashige and Skoog (MS) medium and held at 4°C for 3 d, followed by grown in a controlled greenhouse for an 8/16 of dark/light period at 22 ± 1°C for 12 d, and then transplanted into the mixture (vermicculite: soil = 1: 1) and grown under the same condition.

2.2 RNA Extraction, purification, cDNA library preparation and sequencing

Total RNA was extracted from FB.Apr, FB.May and FB.Nov using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The eukaryotic and prokaryotic mRNA were enriched by Oligo(dT) beads and Ribo-ZeroTM Magnetic Kit (Epicentre), respectively, and fragmented into short length. The first-strand cDNA synthesis using random hexamers, followed by the second-strand cDNA

synthesis. Then the cDNA fragments were purified with QiaQuick PCR extraction kit, end repaired, adding poly (A), and ligated to Illumina sequencing adapters. Next the size of ligated product was selected by agarose gel electrophoresis, and PCR amplification was applied. The sequencing was performed using Illumina HiSeqTM 4000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

2.3 Functional annotation, differential expression analysis

Reads contained adapters, more than 10% of unknown nucleotides (N) and more than 40% of low quality (Q-value ≤ 20) bases were removed before analysis. Basic annotation of the unigenes were performed based on BLASTx program (http://www.ncbi.nlm.nih.gov/BLAST/) with an e-value threshold of 1e − 5 to NCBI non-redundant protein (Nr) database (http://www.ncbi.nlm.nih.gov), the Swiss-Prot protein database (http://www.expasy.ch/sprot), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg), and the COG/KOG database (http://www.ncbi.nlm.nih.gov/COG). The expression level of unigenes was calculated at Reads Per kb per Million reads (RPKM) method (Mortazavi et al. 2008). In order to identify DEGs across sample, the edgeR package (http://www.r-project.org/) was used. We identified genes with a fold change (FC) ≥ 2 and a false discovery rate (FDR) < 0.05 in a comparison as significant DEGs. Subsequently, DEGs were analyzed for the enrichment analysis of GO functions and KEGG pathways. Principal component analysis (PCA) was used with R package software (http://www.r-project.org/) in this experience.

2.4 Protein extraction, digestion and TMT labeling

Total protein was extracted from FB.Apr, FB.May and FB.Nov samples by cold acetone method as following described: briefly, the samples were ground to power in liquid nitrogen and added to the ice-cold acetone at -20 ℃ overnight to precipitate proteins. Then the proteins mixed with 2 mL lysis buffer (8M urea, 2% SDS, 1x Protease Inhibitor Cocktail (Roche Ltd. Basel, Switzerland)), followed with ultrasonic on ice for 30 min and centrifugation at 13 000 rpm for 30 min at 4 ℃. The protein quality and concentration of samples was determined by SDS-PAGE and The BCA protein assay kit, respectively. Proteins were then tryptic digested with sequence-grade modified trypsin (Promega, Madison, WI) at 37° C for overnight. Finally, the resulting peptide mixture was labeled with various TMT 10-plex isobaric labeling tags (ThermoFisher Scientific, MA, USA) at room temperature for 2 hours.

2.5 LC-MS/MS, protein identification, quantification, functional annotation and enrichment analysis

LC-MS/MS analysis was performed on Easy-nLC 1000 system (Thermo Fisher Scientific, MA, USA) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) equipped with an online nano-electrospray ion source. Protein identification was performed using Mascot search engine (Matrix Science, London, UK; version 2.3.2). In this study, mascot database was set up based on *C. praecox* reference transcriptome. The protein is accepted for the identification with an FDR less than 1.0% by the Scaffold Local FDR algorithm. We identified proteins with FC > 1.2 or < 0.83 and unadjusted significance level p < 0.05 as being considered DEPs. Proteins were annotated against GO, KEGG and COG/KOG database to obtain their predicted functions with p value ≤ 0.05.

2.6 Integrated transcriptome and proteome analysis

Association analysis of transcriptome and proteome data was carried out to determine the correlation between protein and mRNA by calculating the Pearson correlation coefficient. In order to identify DEGs and DEPs between samples, the edgeR software package (http://www.r-project.org/) was used. Venn diagrams were used to calculate the numbers of DEGs and DEPs in various comparisons. For those interested DEGs and DEPs, we used hierarchically clustered heatmaps to investigate the relative expression levels of them in different samples.

2.7 Cloning, phylogenetic, expression pattern and functional analysis of CpFPA1

The CDS, protein and gene sequences of *CpFPA1* are list Supplementary Table S1. GSDS2.0 software was used for visualization of gene structure of *CpFPA1*. The conserved domain of CpFPA1was searched using CD-Search. The wintersweet genome information was provided by Nanjing Agricultural University and Huazhong Agricultural University. The primers used for cloning and expression analysis were designed for Primer 5.0 software and listed in Supplementary Table S2. The total RNA was extracted from different stages (FB and flowering) following the previously described. The PCR program was carried out with a 94°C at 5 mins, followed by 28 cycles of 94°C at 30 s, 59°C at 30 s and 72°C at 3 mins 15 s, 72°C at 10 mins. Multiple protein sequence alignment of the FPA1-like proteins was performed with the BioEdit software. The phylogenetic tree of CpFPA1 and other plants FPA-like proteins was constructed using MEGA 6.0 software with neighbor-joining (NJ) with 1000 replications. The proteins sequences of other species are listed in Supplementary Table S3. The vector construct of *CpFPA1* was digested with KpnI and XbaI restriction enzymes and connected with pCAMBIA1300 containing the CaMV 35S promoter. The vector *35S::CpFPA1* was transformed into wild-type (WT) *Arabidopsis* (Col-0) by the floral-dip method. The *35S::FPA1* transgenic lines were screened by HygromycinB (Hyg). qRT-PCR was performed to analysis the expression level of *CpFPA1*, and *CpActin* and *Cptublin* gene were used as the internal reference gene to standard expression levels. To further investigate the expression level of endogenous gene (*AtAP1* and *AtFLC*) in 14-day-old transgenic plants, and the *AtActin* gene was used as an internal reference gene (Li et al. 2020). qRT-PCR conditions were implemented in 94°C for 30 s, 40 cycles of 95°C at 5 s, 60°C at 5 s and 65–95°C for melting curve analysis for each 0.5°C using Bio-Rad Manager TM Software (Version 1.1).

2.8 Statistical analysis

The qRT-PCR results and phenotype analysis of *35S::CpFPA1* transgenic plants and wild type (WT) of *Arabidopsis* were analyzed by Excel (2010) and SPSS 20.0 software based on One-way Analysis of Variance (ANOVA) with LSD (L) and Duncan (D) with $p < 0.05$. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative changes in the gene expression levels. All experiments were conducted with three replicates for each sample.

3. Results

3.1 Transcriptomic analysis

Nine cDNA libraries were constructed, and RNA-seq was executed using the Illumina Hiseq 4000 platform. A total of 3173 (1471 upregulated and 1702 downregulated), 41891 (25444 upregulated and 16447 downregulated) and 39109 (23974 upregulated and 15135 downregulated) in FB.May/FB.Apr, FB.Nov/FB.Apr and FB.Nov/FB.May comparison groups were identified as DEGs, respectively (Fig. 1a). The number of upregulated DEGs in FB.Nov/FB.May and FB.Nov/FB.May were higher than those of downregulated DEGs. The correlation coefficient were 0.34–0.35 among FB.Apr, FB.May and FB.Nov and there is a strong correlation higher than 0.99 in three biological repetition (Fig. 1b). Sample clustering analysis indicated that samples of every period come together (Fig. 1c). The above information ensured the credibility of this study. Principal component analysis (PCA) showed that PC1 98.8% and PC2 1.2% (Fig. 1d). All the biological replicates set three times.

3.2 Functional classification of identified DEGs

GO and KEGG enrichment analysis were used to classify the functions of the per unigenes. The results indicated that a total of 805 sequences were successfully distributed to one or more GO annotation in FB.Apr/FB.May group. They were involved in the biological process (404, 50.2%), cellular component (189, 23.5%) and molecular function (202, 25.1%), including 32 functional groups (Fig. 1e). For biological process category, 105 and 98 DEGs were mainly participated in metabolic process and cellular process. There were 51 DEGs involved in cell and cell part, respectively, and were most functional in cellular component. In the molecular function category, 105 and 67 DEGs were mainly involved in catalytic activity and binding, followed by transporter activity (11, 1.3%).

3.3 Proteome analysis

To supplement the transcriptome study, proteome analysis based on TMT technology was carried out to confirm the differentially expressed proteins (DEPs) between any two stages. A total of 403 (166 upregulated and 237 downregulated), 626 (273 upregulated and 353 downregulated) and 343 (127 upregulated and 216 downregulated) DEPs in FB.May/FB.Apr, FB.Nov/FB.Apr and FB.Nov/FB.May, respectively (Fig. 2a). The results indicated that most DEGs were observed in FB.Nov/FB.Apr. Through KOG database for function class prediction, the results showed that a total of 7344 proteins were classified into 25 KOG categories (Fig. 2b). General function prediction only (KOG0118) (1079, 14.7%) is the function with the most quantity prediction, followed by posttranslational modification, protein turnover and chaperones (796, 10.8%), translation ribosomal structure and biogenesis (591, 8.0%), signal translation mechanisms (536, 7.3%), RNA processing and modification (6.4%). Cell motility (8, 0.1%) and extracellular structures (10, 0.1%) were the smallest groups.

3.4 Association analysis of proteomics and transcriptomics

In order to further screen out the key genes, we performed an association analysis of proteomics and transcriptomics. According to the results, profiles 4, 3, 7, 2, 0, 6, 5, 1 from the trend analysis of FB.Apr, FB.May and FB.Nov were significantly enriched (Fig. 3a). The overall trend of profiles 4 and 7 were upregulated with 17462 and 5589 DEGs, respectively. The overall trend of profiles 3, 0 and 1 were downregulated with 15705, 2141 and 892 DEGs. To further know about the association between the DEGs and DEPs, using the proteome and transcriptome correlation analysis to determine the key genes. Venn diagrams were shown as Fig. 3b, the results showed that a total of the common 40 DEPs and 952 DEGs were observed during three stages (Fig. 3b). Unigene0031805 FPA1 protein expression of FB.Apr was significantly higher than FB.May and FB.Nov. At the mRNA level, Unigene0031805 *FPA1* expression in FB.Apr was significantly higher than FB.May and FB.Nov (Fig. 3c). In order to explore the overall correlation between DEGs and DEPs, all identified mRNA were matched with DEPs from three FBs development stages, and then the volume ratios of DEPs and these transcript ratios between any two stages were converted into $\log^2$. There is a negative correlation. The Pearson correlation were 0.0303, 2e-04 and − 0.0282 of proteome and transcriptome analysis in FB.May/FB.Apr, FB.Nov/FB.Apr and FB.Nov/FB.May, respectively. It suggested that most DEGs and DEPs have almost little corresponding correlation. To identify the autonomous pathway genes, we constructed a scatter plot of the nine-quadrant association analysis diagram base on log2 for proteins. The results showed that only unigene0031805 *FPA1* in quadrant 7 showed difference in FB.May/FB.Apr, unigene0009412 FCA in quadrant 2 in FB.Nov/FB.Apr and unigene 009412 *FCA* in quadrant 2, unigene0031805 *FPA1* in quadrant 7, unigene0016660 *FLK*, unigene0068303 *FPA2a*, unigene 0068304 *FPA2b* in quadrant 5, unigene0056002 *FY* in quadrant 4 in FB.Nov/FB.May (Fig. 3d-f, Supplementary Table S4). *FPA1*, *FPA2a*, *FPA2b*, *FCA*, *FLK* and *FY*, were screened base on the transcriptome and proteome association analysis.

Only Unigene0031805 *FPA1* displayed a significant difference in any two stages comparison. In the mRNA level, Log_2 FC in FB.Nov/FB.Apr (-3.710) and FB.Nov/FB.May (-2.339); and Log_2FC in FB.Nov/FB.Apr (-0.953) and FB.Nov/FB.May (-0.614) in the protein level showed differences, respectively (Supplementary Table S4). Therefore, we selected *CpFPA1* gene for subsequent functional analysis to explore its role in flowering.

3.5 Identification, phylogenetic and expression analysis of *CpFPA1*

An *FPA*-like sequence from *C. praecox* was successfully isolated and named *CpFPA1* gene. *CpFPA1* was located on Chromosome 04 of *C. praecox* and contained seven exons (Fig. 4a-b). We found that *CpFPA1* had an open reading frame (ORF) 3099 bp and encoded 1032 amino acid residues. The result of multiple sequence alignment showed that the protein domain of CpFPA1 was conservative, containing three RRM motifs and a SPOC domain, suggesting that it belonged to the SPEN family (Fig. 4c, e). To know about the evolutionary relationship between CpFPA1 and other plants, the phylogenetic tree was constructed based on protein sequences, the result showed that CpFPA1 clustered close to CmFPA in *C. micranthum* (Fig. 4d).

To assess the expression level of the *CpFPA1* gene in different tissues and stages (FBs and flowering), qRT-PCR method was applied. The results revealed that the highest expression level of *CpFPA1* in vegetative organs was found in the leaves. *CpFPA1* was expressed strongly in the stamens and was detected with low level in stems and fruits (Fig. 4f). These stages were divided into five group based on the previous study: floral organ primordium differentiation, summer dormancy, ovary development, chilling accumulation, and flowering stage (Li et al. 2020). As shown in Fig. 4g, *CpFPA1* was expressed in all the tested period and indicated a variety of expression levels. In sum, the overall trend of *CpFPA1* expression was first increased and then induced and then increased. In the floral organ primordium formation stage, the expression level of *CpFPA1* was first induced and increased and then induced. The expression level of *CpFPA1* was peaked on 16-Jul (dormancy period). In the dormancy stage and the ovary development stage, the expression of *CpFPA1* showed a relatively low level than other stages. After entering the flowering stage, the expression level of *CpFPA1* was gradually increased.

3.6 Overexpression of *CpFPA1* in *Arabidopsis*

A total of eight independent lines were obtained after Hyg-resistance selection and PCR identification. High (OE 21 − 4), moderate (OE 5 − 3) and low (OE 13 − 5) expression level were chosen for endogenous gene expression analysis and phenotypic observations under long day (LD) conditions. The high-expression OE 21 − 4 showed a faster growth state from the phenotypic observation (Fig. 5a-c). The rosette leaf numbers of *35S::CpFPA1* transgenic lines were reduced than that of WT (12.13) (Fig. 5d). The flowering time and the bolting time of 35S::CpFPA1 transgenic lines were prior to that of WT (Fig. 5d). Whereas, the cauline leaf numbers of transgenic plants were increased (Fig. 5d). The time of inflorescence formation and flowering of *35S::CpFPA1* were earlier than those in the WT, respectively (Fig. 5d). In addition, qRT-PCR showed that ectopic of *35S::CpFPA1* in *Arabidopsis* upregulated the expression level of *AtAP1* and downregulated the expression level of *AtFLC* under LD conditions (Fig. 5c). These data implied that ectopic expression of *CpFPA1* in *Arabidopsis* accelerated flowering.

4. Discussion

Floral induction is a critical process of plant development, involving multiple pathways that are both independent and interactional. Usually, we know about the mechanism of flowering time regulation mainly through the model plants such as *Arabidopsis*. Most flowering studies have focused on photoperiod pathway, however, there have a few studies on autonomous genes in *C. praecox*. Consequently, the use of emerging technological tools to know about the floral induction mechanism of wintersweet is of great significance for artificial regulation of flowering time, which might be improve ornamental and economic value of *C. praecox*.

4.1 Transcriptome and proteome association analysis

In this present study, we investigated DEGs and DEPs at FB.Apr, FB.May and FB.Nov base on an integrated proteomic and transcriptomic analysis. The RNA-Seq analysis showed that a total of 3173

DEGs (1471 upregulated and 1702 downregulated) were identified. GO analysis showed that main enriched function of these DEGs at FB.May/FB.Apr were cellular process, metabolic process, single-organism process, cell, cell part, binding and catalytic activity. It suggested that flowering is related to cell and metabolic process, which is closely associated with the growth and development of plants. The proteome analysis stated that a total of 403 DEPs were identified in FB.May/Apr comparison group. Transcriptome and proteome association analysis was carried out on the flower buds among FB.May/FB.Apr, FB.Nov/FB.Apr and FB.Nov/FB.May groups. A total of the common 40 DEPs and 952 DEGs were identified, and KOG revealed that these proteins were mainly involved in function prediction only (KOG0118), posttranslational modification, protein turnover and chaperones. The correlation analysis of transcriptome and proteome association analysis quantitative proteins and genes showed a poor correlation, which may be due to the inconsistent expression levels of mRNA and corresponding proteins.

Previous studies indicated that an integrating genome-wide and transcriptome related to blooming time in *P. mume* were screened out 191 gene candidates across cultivars (Zhang et al. 2021). Comparative transcriptome analysis of floral development in loquat showed that DEGs were mainly enriched in metabolic pathways of hormone signal transduction, starch and sucrose metabolism (Jing et al. 2020). A comprehensive transcriptomic and proteomic analyses were performed in *Agapanthus* at three different time points including the vegetative, induced, and reproductive period, a total of 374 DEGs and 72 DEPs were identified about the molecular mechanisms of floral initiation (Zhang et al. 2013).

4.2 The function of CpFPA1 gene

In this study, six candidate genes (*FPA1*, *FPA2a*, *FPA2b*, *FLK*, *FY* and *FCA*) in the flower bud differentiation process of were screened by the integrated transcriptome and proteomic analysis. Finally, *CpFPA1* exhibited difference in FB.Mar FB.Apr, and FB.Nov in mRNA level and in FB.Apr in protein. In order to verify the role, a candidate gene *CpFPA1* was successfully isolated from wintersweet. CpFPA1, belonged to the SPEN family gene, a RBP protein contained three RRM domains and a SPOC domain. RBPs protein play an important role in flowering time, floral development, ABA signal, stress response, chromatin modification (Ambrosone et al. 2012; Lorkovic 2009). *CpFPA1* was found to be involved in all the tested period (8-March to 7-Jan of the following year). In this present study, *35S::CpFPA1* transgenic plants appeared early flowering phenotypic. Overexpression of *CpFPA1* downregulated the floral inhibition *AtFLC* expression and upregulated the *AtAP1* gene expression. These finding suggested that *CpFPA1* promoted flowering. *FLC* could inhibit the expression of *AP1*. *MtFPA* in *M. truncatula* accelerated flowering time under LD conditions. *35S::MtFPA* transgenic lines regulate flowering by down-regulating the expression level of *FLC* (Hwang et al. 2018), which was consisted with the present study. In *Arabidopsis*, *FPA* showed an early flowering (Schomburg et al. 2001). *FPA* controlled flowering time by regulating the alternative 3'-end processing of *FLC* antisense RNA (Candela et al. 2016). *FPA* affects many aspects of development, not just flowering (Veley and Michaels 2008). *FPA* promote proximal polyadenylation of a large number of Arabidopsis transcripts genome wide (Duc et al. 2013). It was found that *FPA* mediated extensive

premature cleavage and polyadenylation of *NLR* transcripts, controlling their functional expression and influencing immunity (Parker et al. 2021). In summary, the function of *CpFPA1* need to further study.

4.3 Autonomous pathway and without *FLC* gene in *C. praecox* genome

The autonomous pathway is mainly independent of environmental factors to promote flowering. A vast number of studies have shown that autonomous pathway genes are mainly regulated flowering time by inhibiting *FLC* expression. *GmFLD*, a soybean orthologous of *FLD* gene, accelerated flowering by upregulating the expression level of *FT* and *SOC1* and recused the late flowering phenotype of *fld* mutant in *Arabidopsis* (Hu et al. 2014). Overexpression of *DhFVE* gene from *Doritaenopsis* in *Arabidopsis* delayed flowering 2–3 days, and *DhPRP39* delayed flowering 6–7 days, which indicated they played a role in the flowering regulation (Sun et al. 2012b, a). The previous study revealed that the floral induction in apple was regulated by endogenous developmental cues (age, hormones, and sugars) through an autonomous pathway (Mimida et al. 2013). In monocots, transcriptome profiles of some candidate flowering time genes of maize revealed that photoperiod and autonomous pathways act independently (Minow et al. 2018). It was found that autonomous genes regulated flowering by affecting the circadian clock genes (Huang and Nusinow 2016).

FLC, as a flowering repressor integrating autonomous and vernalization pathways, play an essential role in plants flowering process (Zhang et al. 2011). The FLC subfamily was lost in the *C. praecox* genome (Hou et al. 2023; Shang et al. 2020). Previous studies have shown that wintersweet genome undergone two whole-genome duplication (WSD) events (Shen et al. 2021). In addition to that, there were several plants that were absent of *FLC* gene. In monocots, *FLC-like* gene in rice was absent, but the ectopic of *AtFLC* from *Arabidopsis* in rice could delay flowering (Tadege et al. 2003). *BdODDSOC2*, a *FLC* ortholog in *Brachypodium*, was found to be as a vernalization-regulated flowering repressor (Sharma et al. 2017a). Similarly, *TaAGL33* and *TaAGL22* in wheat as *FLC* orthologs exhibited an expression pattern similar to *FLC* in response to vernalization (Kennedy and Geuten 2020; Sharma et al. 2017b). Previous studies had shown that *FLC* genes appear to have been lost in several lineages of flowering plants (Ruelens et al. 2013). In strawberry, it was not able to found *FLC* and *FLC*-like sequence (Mouhu et al. 2009). In the *Rosaceae*, it was proposed that *TFL1* function as a major flowering repressor, similar to *FLC* in *Arabidopsis*. In the absence of poplar *FLC*, the function of *FLC* is replaced mainly by the response of *FT1* to reproductive growth in winter and *FT2* to vegetative growth and the inhibition of bud set (Hsu et al. 2011). *FLC* is lost in apple (Guitton et al. 2012), but two *FLC*-like genes, *MdMADS135* and *MdMADS136* in apple, played a role as repressor regulator of flowering (Kumar et al. 2016). Sugar beet *BvFL1* (a *FLC*-like gene) also repressed flowering (Abou-Elwafa et al. 2011). *FLC* was not found in *M. sinostellata*, but *FY*, *FPA* and *FRI* with the high expression level (Fan et al. 2018).

The previous studies showed that the temperature was independent for floral bud formation and flowering of *C. praecox* in Yunnan Province, China (Li et al. 2022). Moreover, the lack of *FLC* in the wintersweet genome, it is speculated that pathways other than these two pathways contribute to

involving FB differentiation, also suggested that upstream unknown genes of the vernalization pathway. The autonomous pathways, vernalization pathways and CpFUL/SEP/AGL6 genes in *C. praecox* were shown in Fig. 6a. The previous study was found that *SEP*-, *AGL6*-, *SQUA*- and *FLC*-like genes lineages were clustered into a strongly supported superfamily, SEP3-FLC and SEP1-SQUA tandems in core eudicots had also occurred. (Ruelens et al. 2013). According to our previous studies, *CpFUL/SEP/AGL6* play a role in vegetative meristem to floral meristem, and overexpression of *CpFUL* in *Arabidopsis* resulted in earlier flowering (Hou et al. 2023). For further, we proposed a model for the mechanism of how autonomous pathway genes regulate flowering in the absence of *FLC* gene in *C. praecox* genome (Fig. 6b). These findings implicated that there is a uniquely *FLC*-independent flowering regulation pathway in *C. praecox*. Therefore, further researches are needed to be investigated exactly how *CpFPA1* regulates flowering in the absence of *FLC* and which genes replaces the role of *FLC*.

5. Conclusion

In this study, an integrated analysis based on proteomic and transcriptomic were performed for three critical FB differentiation stages to screen DEGs and DEPs. A total of 40 DEPs and 952 DEGs were observed during three stages. DEGs in FB.Apr/FB.May were mainly involved in metabolic of biological process, cell and cell part of cellular component and catalytic activity of molecular function. DEPs were mainly engaged in general function prediction only (KOG0118), posttranslational modification, protein turnover and chaperones, translation ribosomal structure and biogenesis, signal translation mechanisms, RNA processing and modification. Here, *CpFPA1* was isolated and identified functional. Overexpression of *CpFPA1* in *Arabidopsis* could accelerate flowering through upregulating the expression level of *AtAP1* and downregulating the expression level of *AtFLC*. These data are significant for further revealing the molecular mechanism of floral induction and enriching the theory of flowering about autonomous pathway genes in *C. praecox*.

Declarations

Funding

The work was supported by grants from the Natural Science Foundation of Chongqing (CSTB2023NSCQ-MSX0236, cstc2020jcyj-msxmX1014); Fundamental Research Funds for the Central Universities (XDJK2020B059).

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Authors' Contributions Statement

Z.L. designed the research. C.W., N.L. Y.J and H.H. performed the experiments. Z.L. and C.W. performed graphic drawing. H.H. and Z.L. wrote the article. C.W, Z.L., J.H. and S.S. analyzed the data. All authors

read and approved the manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

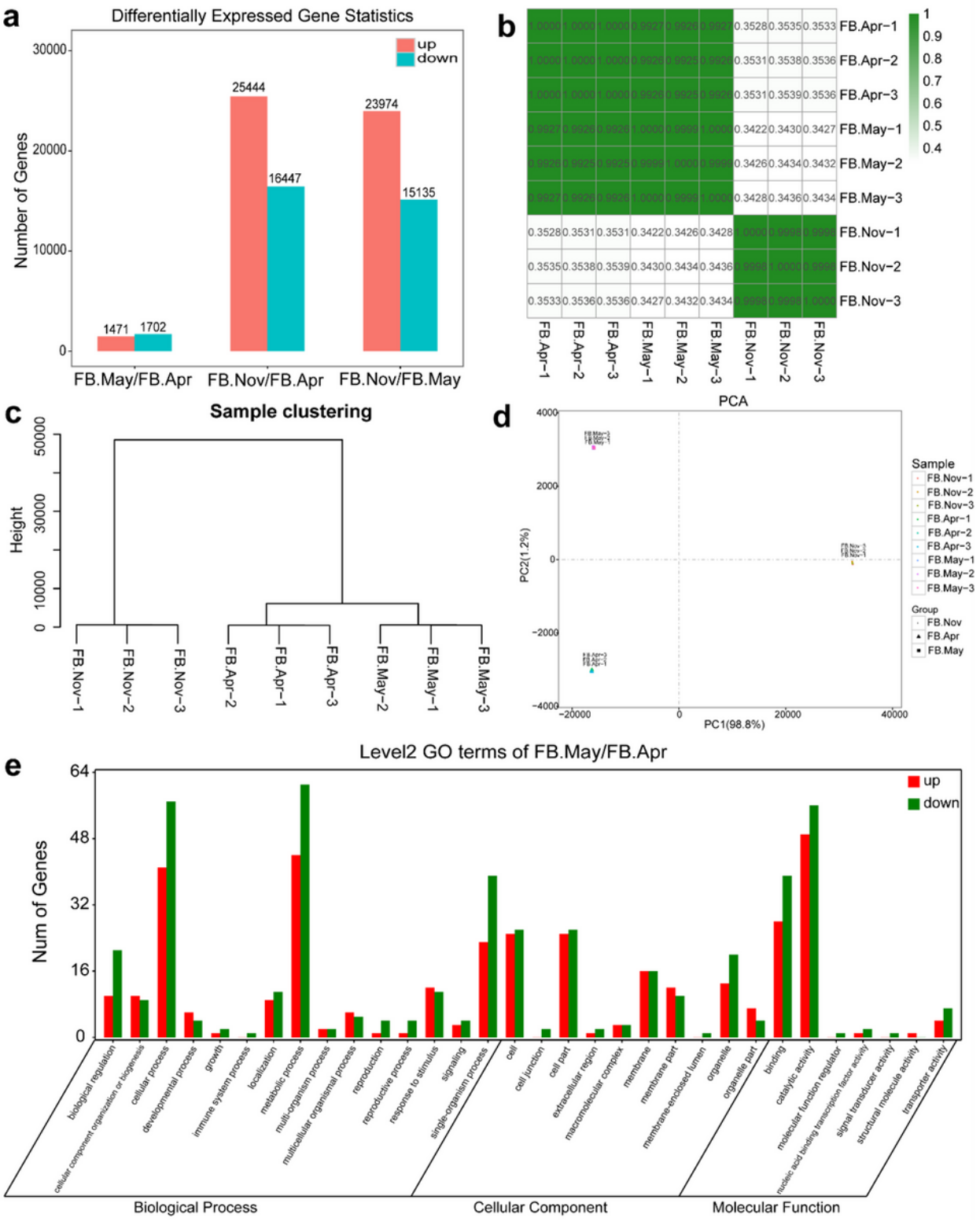

Figure 1

DEGs, Pearson correlation, hierarchical cluster, PCA and GO analysis of 9 RNA-seq samples a Differentially expressed genes (DEGs). **b** Pearson correlation among all the samples. **c** Hierarchical cluster tree. **d** PCA analysis plot of the 9 RNA-seq libraries. **e** GO assignments for the 9 samples up-/down-regulated DEGs in FB.May/FB.Apr.

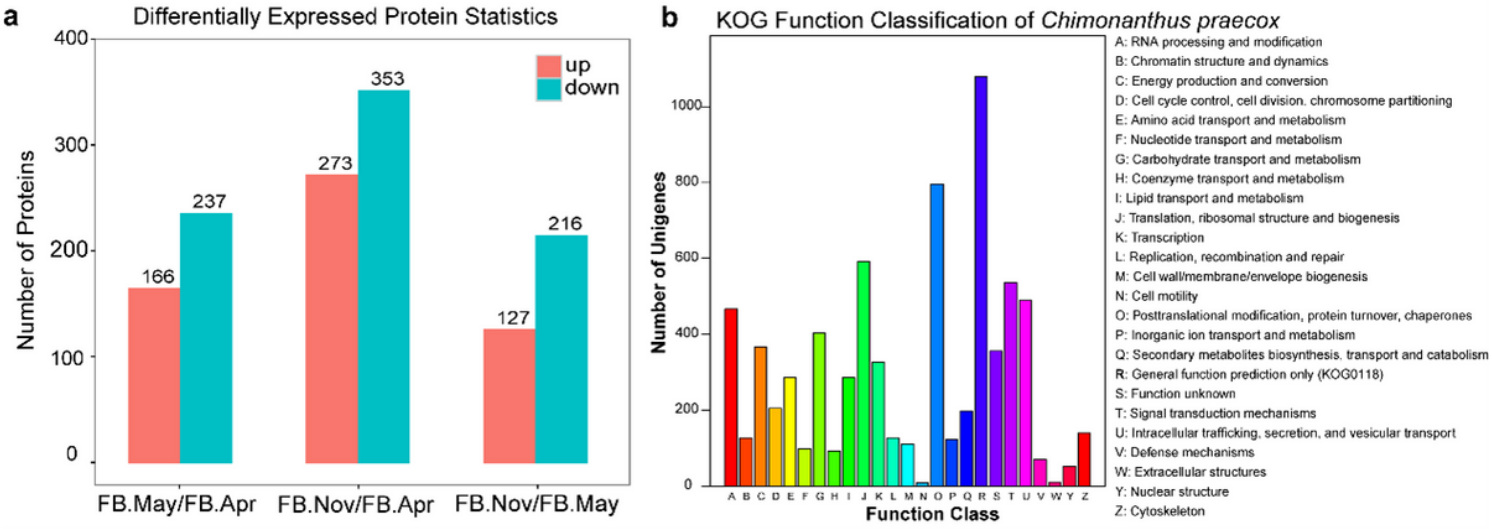

Figure 2

The number of DEPs in any two stages and KOG function classification of proteins a The numbers of DEPs statistics. **b** KOG function classification. The capital letters indicated KOG function prediction in detail.

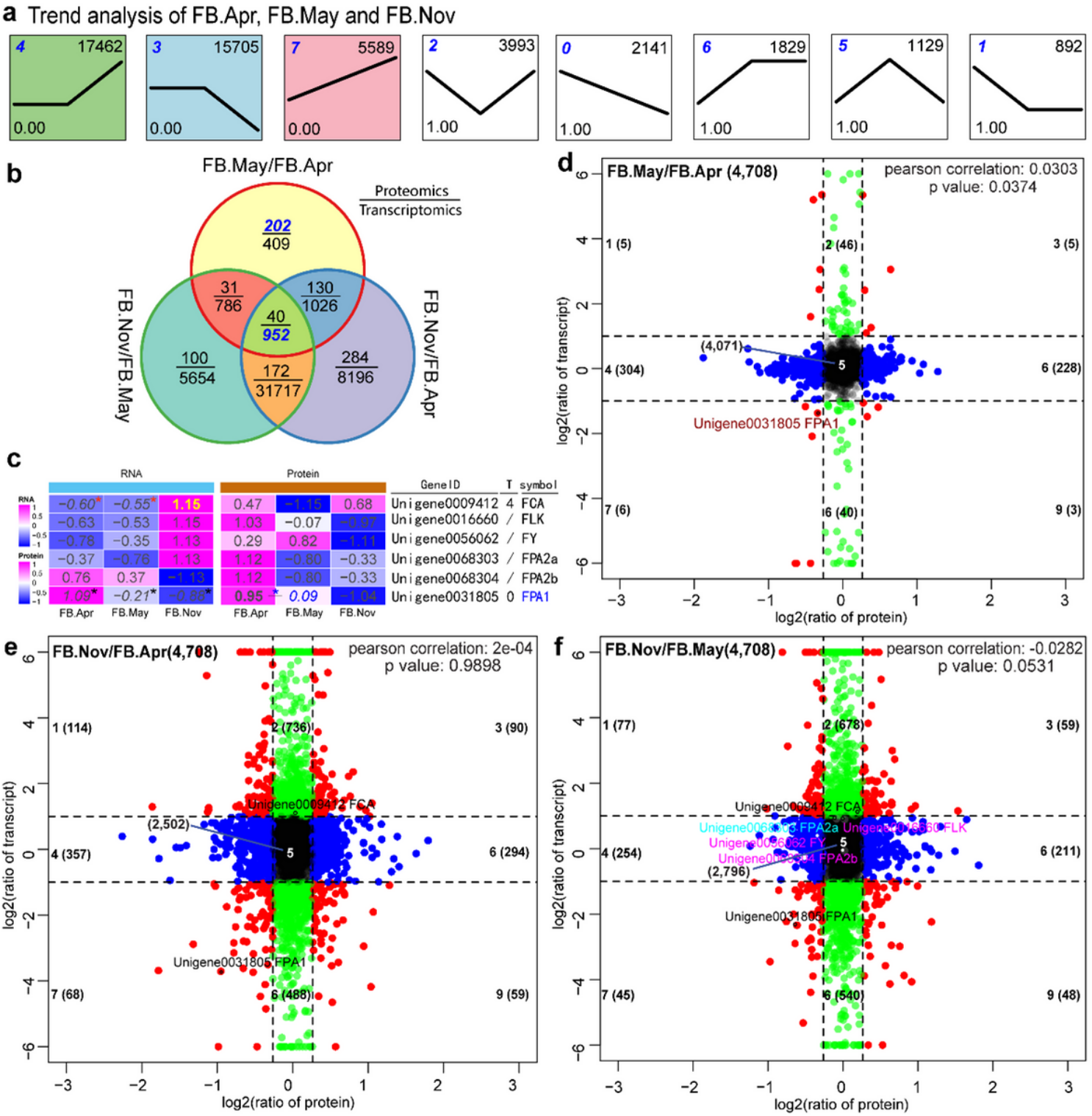

Figure 3

Trend, venn, heat map and nine quadrant associate analysis a Trend analysis of FB.Apr, FB.May and FB.Nov. **b** Venn diagram of DEGs and DEPs from FB.May/FB.Apr, FB.Nov/FB.May and FB.Nov/FB.Apr. **c** Heat map analysis of normalized RPKM scaled by Z score method from 6 differentially expressed proteins, including FCA, FLK, FY and FPA. **d-f** Scatter plot of 9-quadrant associate analysis of DEGs and DEPs from log_2FC during FB.May/FB.Apr **(d)**, FB.Nov/FB.Apr **(e)** and FB.Nov/FB.May **(f)**. Number 1-9,

quadrant NO., respectively. The number of points in each quadrant is shown in parentheses. Unigene ID, highlighted protein in **(c)**. Red dots represented significant up-regulated, green dots represented down-regulated and blue dots represented non-significant proteins.

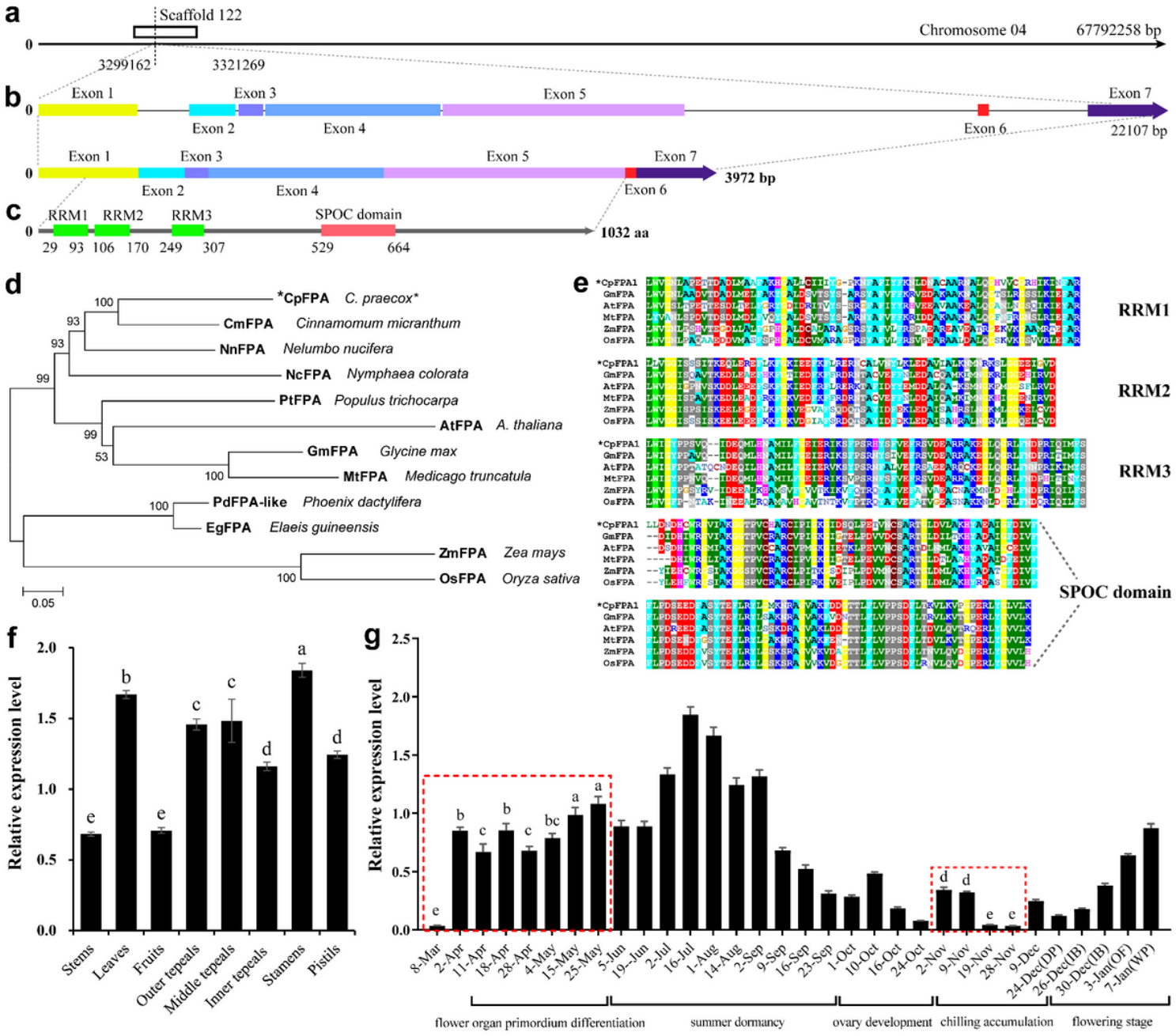

Figure 4

Phylogenetic analysis and expression analysis of CpFPA1 a The chromosomal localization of CpFPA1. **b** The exon-intron of CpFPA1. **c** The conserved domain of CpFPA1. **d**Phylogenetic analysis of CpFPA1. CpFPA1 was represented by the asterisk. Bootstrap values were indicated. **e** Multiple sequences alignment of GmFPA (*G. max*), AtFPA (*A. thaliana*), MtFPA (*M. truncatula*), ZmFPA (*Z. mays*), OsFPA (*O. sative*) and CpFPA1. Three RRM sequence motifs and one SPOC domain were shown. CpFPA1 was marked with the asterisk. **f**Expression analysis of *CpFPA1* in different tissues. Lowercase letters

represented significant differences (p<0.05). **g** Relative expression analysis of *CpFPA1* in FBs and flowering from 8-March 2018 to 7-Jan 2019. The samples from 8-Mar 2018 to 25-May 2018 were performed for difference analysis, and lowercase letters represented significant differences (p<0.05).

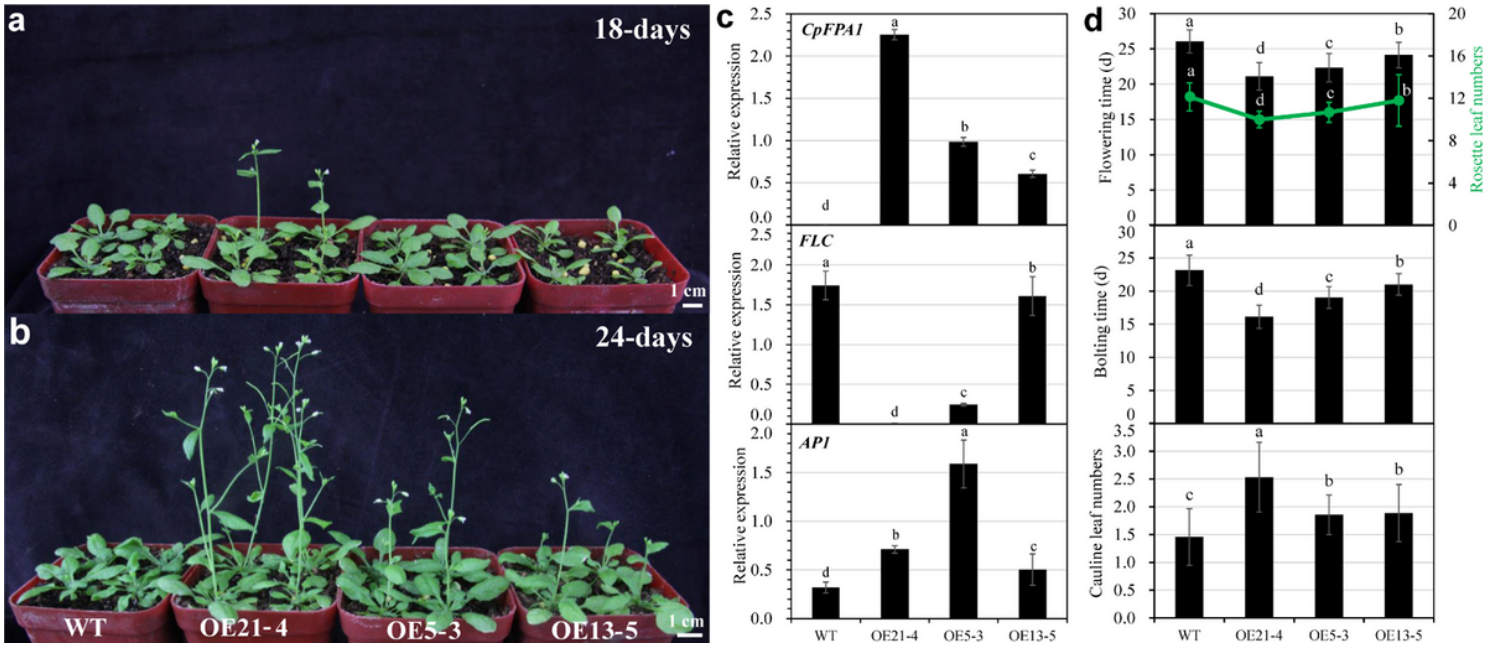

Figure 5

Ectopic expression analysis of *CpFPA1* in *Arabidopsis*. a-b Phenotypic observations of *35::CpFPA1/*Col-0 T2 plants and WT for 18- (**a**) and 24- day (**b**). **c** Relative expression analysis of *CpFPA1*, *AtFLC* and *AtAP1* in 35S::*CpFPA1* transgenic lines and WT. **d** Phenotype analysis in T_2 generation of *35S::CpFPA1*/Col-0 and WT. a, b, c and d mean the difference was significant (p<0.05).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- SupplementarymaterialCpFPA1ProRNA.docx