Characterization of the MADS-Box Gene CmFL3 in Chrysanthemum

Kunkun Zhao, Song Li, Diwen Jia, Xiaojuan Xing, Haibin Wang, Aiping Song, Jiafu Jiang, Sumei Chen, Fadi Chen and Lian Ding *

State Key Laboratory of Crop Genetics and Germplasm Enhancement, Key Laboratory of Landscape Architecture, Ministry of Agriculture and Rural Affairs; Key Laboratory of Biology of Ornamental Plants in East China, National Forestry and Grassland Administration, College of Horticulture, Nanjing Agricultural University, Weigang No. 1, Nanjing 210095, China; 2016204028@njau.edu.cn (K.Z.); 2020104090@stu.njau.edu.cn (D.J.); xingxiaojuan@njau.edu.cn (X.X.); hb@njau.edu.cn (H.W.); aiping_song@njau.edu.cn (A.S.); jiangjiafu@njau.edu.cn (J.J.); chensm@njau.edu.cn (S.C.); chenfd@njau.edu.cn (F.C.)

* Correspondence: dinglian@njau.edu.cn; Tel.: +86-25-84395291; Fax: +86-25-84432420

Abstract: *Chrysanthemum morifolium* is one of the four major cut flowers in the world, with high ornamental and economic value. Flowering time is an important ornamental characteristic of chrysanthemum that affects its value in the market. In Arabidopsis, the *FRUITFULL* (*FUL*) gene plays a key role in inducing flowering. Here, we isolated an *FUL* clade MADS-box gene, *CmFL3*, from chrysanthemum inflorescence buds. *CmFL3* localized in the cellular membrane and nucleus, and showed no transcriptional activity in yeast. The qRT-PCR assay showed that *CmFL3* was strongly expressed in the leaves, receptacles, and disc floret petals. Furthermore, *CmFL3* was mainly detected in the inflorescence meristem and bract primordia using in situ hybridization. Similar to Arabidopsis, overexpression of *CmFL3* in chrysanthemum induced early flowering. Particularly, the expression level of *CmAFT* was downregulated, whereas that of *CmFTL3* was upregulated in the leaves of transgenic chrysanthemum lines. Meanwhile, the overexpression of *CmFL3* in Arabidopsis also led to earlier flowering. Furthermore, the expression of *AtIFT*, *AtAPI1*, *AtLFY*, and *AtFUL* was significantly increased in *CmFL3* transgenic Arabidopsis. The present study verified the function of *CmFL3* in regulating flowering time and further revealed that it could affect the expression of other flowering-related genes—*CmAFT* and *CmFTL3*. Therefore, the *CmFL3* gene may be an important candidate for genetic breeding aimed at regulating flowering.

Keywords: *Chrysanthemum morifolium*; MADS box; AP1/FUL-like genes; Flowering time; *CmFTL3*; *CmAFT*

1. Introduction

MADS-box genes are involved in many developmental processes in plants [1–7]. *APETALA1/FRUITFULL* (*AP1/FUL*)-like MADS-box genes play a conserved role in the specification of floral meristem (FM) identity [8–13] and are involved in the induction of flowering in many species [14–18]. Their expression is induced by *FLOWERING LOCUS T* (*FT*) upon the induction of flowering [19–22]. *AP1/FUL*-like genes are divided into three distinct clades: euAPETALA1 (euAP1), euFRUITFULL (euFUL), and euFUL-like, which are believed to have evolved from a common eudicot through several duplication events [13,23]. These genes have been shown to possess novel functions. For example, *AP1* and *FUL* function redundantly in specifying floral meristem identity, but function independently in specifying sepal and petal identities (*AP1*) [10,24–26] and proper fruit development and determinacy (*FUL*) [27,28]. In addition, *AP1* and *FUL* clade genes encode conserved MADS-, I- (intervening), and K-domains (keratin-like), but differ with respect
to the C-motifs [13,16,29]. The C-terminal domains of the AP1 lineage proteins include a farnesylation domain [30], whereas those of the FUL group proteins contain six hydrophobic amino acids [13,31,32].

The euFUL group of genes plays multiple roles in leaf development, branching, flowering, inflorescence, and carpel development [33–35]. FUL has been reported to regulate flowering time and affect the determinacy of meristems [35]. FUL, together with SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), LEAFY (LFY), and FLOWERING LOCUS D (FD), acts downstream of FT in meristems, whereas SOC1 can be expressed in leaves independently of FT during the vegetative stage [35,36].

Several AP1/FUL-like genes have been functionally characterized in the Asteraceae [14,16,20,37,38]. For example, AP1-like genes (HAM75 and HAM92) in Helianthus annuus have been identified [39], and their independent ectopic expression in Chrysanthemum morifolium results in early flowering [37]. Overexpression of Chrysanthemum Denranaanthema grandiflorum MADS (CDM) 111 (API-like) in C. morifolium leads to early flowering, whereas its overexpression in Arabidopsis partially rescues the phenotype of ap1-1 mutants [32,37]. In Gerbera, overexpression of GSQUA2 (homologous gene of FUL) caused dwarfism, abnormal vegetative growth, early flowering, and fewer florets [14]. In C. seticuspe, marked changes were detected in the expression of an FUL clade gene, CsAFL1 [20], and two other members [40] under short-day conditions. CsAFL1 was predicted to induce the expression of CSFIL3 via positive feedback regulation, and overexpression of CsFDL1 increased the expression of CsAFL1 [20]. However, in chrysanthemum, the function of the CsAFL1 homolog, CmAFT3, remains unknown.

Chrysanthemum morifolium has high ornamental value and cultural connotations. Under short-day conditions, flowering can be induced in most chrysanthemum cultivars [41,42]. Here, we identified and characterized the FUL gene CmFL3. Transgenic C. morifolium ‘Jinba’ plants overexpressing CmFL3 showed early flowering. Furthermore, we found that the expression of CmFTL3 was upregulated and that of CmAFT was downregulated in the leaves of 35S::CmFL3 transgenic lines. The overexpression of CmFL3 in Arabidopsis also led to early flowering. Moreover, the flowering-related genes, AtFT, AtLFY, AtAP1, and AtFUL, were also significantly upregulated in transgenic Arabidopsis plants.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Chrysanthemum morifolium ‘Jinba’ was obtained from the Chrysanthemum Germplasm Resource Preservation Center (Nanjing Agricultural University, Nanjing, China). Wild-type (WT) plants and transgenic lines were cultivated in a multispan greenhouse under optimum relative humidity and temperature conditions.

Arabidopsis thaliana (Col-0) seeds were plated on solidified Murashige and Skoog (MS) [43] medium and stored at 4°C for 3 d, and then transferred to a growth chamber (23°C, 16 h photoperiod, 80–100 μmol/m²/s). After 10 d, the seedlings were transplanted into a vermiculite/peat moss (1:2) mixture and cultured under long-day conditions.

2.2. Gene Sequence Analysis

RNA was extracted from young inflorescence buds using an RNAiso reagent (TaKaRa Bio Inc., Tokyo, Japan) as previously described [16]. The coding sequence (CDS) of the target gene was amplified from the cDNA template using primers designed according to the transcriptome sequence (CL9895.Contig2_All-nr) [44]. AP1/FUL-like protein sequences were downloaded from GenBank (www.ncbi.nlm.nih.gov, accessed on 1 October 2021) and subjected to phylogenetic analysis using MEGA v5.0 [45]. Sequence alignment was conducted using DNAMAN 5.2.2 software. The primers used are listed in Table S1.
2.3. qRT-PCR and RNA in Situ Hybridization

To prepare single-stranded cDNA as a template for qRT-PCR, 1 μg of RNA was reverse-transcribed using PrimeScript 	extsuperscript{®} Reverse Transcriptase (TaKaRa Bio Inc, Tokyo, Japan). Primers were designed according to specific regions using Primer 5.0 software (www.bbioso.com/Soft/2005/114.htm). Each qRT-PCR analysis included three biological and three technical replicates. Relative expression levels were determined using the 2\(^{-ΔΔCt}\) method [46]. Chrysanthemum EF1a (GenBank accession number: AB548817) was selected as the reference gene [47]. Primer sequences are listed in Table S1.

Inflorescence buds (diameter < 2 mm) were fixed in a 3.7% formalin-acetic acid-alcohol (FAA) solution, dehydrated by passing through an ethanol series, and embedded in paraffin. Gene-specific probes were amplified using specific primers, including the SP6 and T7 RNA polymerase binding sites, and labeled using a DIG RNA Labeling Kit (SP6/T7) (Roche, Switzerland). In situ hybridization was performed as previously described [48]. Primer sequences used for probe synthesis are listed in Table S1.

2.4. Subcellular Localization of CmFL3

The LR reaction was used to recombine the pENTR 1A-CmFL3 plasmid with the binary vector pMDC43 (p35S::GFP) to generate a fusion plasmid (pMDC43-CmFL3) harboring the p35S::GFP-CmFL3 construct. Fusion and control (p35S::GFP) plasmids were introduced into onion epidermal cells using a helium-driven PDS-1000 particle accelerator (Bio-Rad, Hercules, CA, USA). The bombarded tissues were cultured in the dark for 16 h at 22°C, and GFP activity was determined using confocal microscopy (Carl Zeiss AG, Oberkochen, Germany).

2.5. Transactivation Assay

The Clontech yeast assay system [49] was used to determine the transactivation activity of CmFL3. CmFL3 CDS was introduced into the pGBK7 vector using SalI and NotI cloning sites. The resulting pGBK7-CmFL3 construct, along with pGBK7 (negative control) and pCL1 (positive control), was inserted independently into the yeast strain Y2H. Transformants were cultured on SD/-His-Ade medium containing either 20 or 0 mg mL\(^{-1}\) x-α-gal and evaluated after 3 d of growth at 28°C.

For the luminescence assay, CmFL3 CDS was introduced into 35S::GAL4DB using the LR reaction (Invitrogen). The transient expression assay was conducted by independently transfecting Arabidopsis mesophyll protoplasts with 10 μg of 35S::GAL4DB-AtARF5, 35S::GAL4DB, or 35S::GAL4DB-CmFL3 constructs as previously described [16]. The protoplasts were cultured in 1.5-mL tubes for 18 h in light at 23°C. LUC images were obtained using a low-light cooled CCD imaging apparatus (DU934P Andor, UK) in a 96-well plate. Luciferase activity was detected with a 10 s integration period (Promega, Madison, WI, USA) as previously described [50].

2.6. CmFL3 Genetic Transformation

The pMDC43-CmFL3 vector was introduced into the chrysanthemum ‘Jinba’ using Agrobacterium-mediated genetic transformation as previously described [48]. The pMDC43-F (forward primer) for the vector and CmFL3-RT-R (reverse primer) for the open reading frame (ORF) of the CmFL3 gene were used to identify the transgenic lines using RT-PCR. The expression levels of the transgenic plants were detected using qRT-PCR (primers are listed in Table S1).

In addition, the pMDC43-CmFL3 vector was introduced into the Agrobacterium tumefaciens strain EHA105 and from thence into Arabidopsis [51]. Transformed progeny were screened by culturing on a solid medium containing 1/2 MS salts and 20 μg mL\(^{-1}\) hygromycin. Thereafter, the progeny was self-pollinated to obtain the T1 generation. The PCR assay was used to identify transgenic Arabidopsis plants. The primer sequences (pMDC43-F/CmFL3-RT-R) are listed in Table S1.
3. Results

3.1. Sequence Analysis of CmFL3

The euFUL clade gene CmFL3 was isolated from the inflorescence buds of ‘Jinba’ chrysanthemum using RT-PCR based on the available transcriptome sequence (CL9895.Contig2_All-nr) [44]. The CmFL3 gene encodes a 237-amino-acid polypeptide that is 53.52% and 57.85% identical to AtAPI and AtFUL, respectively. The predicted polypeptides included the MADS-, I-, and K-box domains and the C-terminal domain containing an MPPWMV motif (Figure 1A). Subsequently, an ML-based phylogenetic tree was constructed, and API/FUL-like subfamily proteins in angiosperms were observed to be clustered into three clades: API, FUL, and FUL-like (Figure 1B). CmFL3 belonged to the eudicot FUL cluster, wherein it shared the closest relationship with CDM8, followed by CsAFL1 and GSQUA2 (Figure 1B).

![Figure 1. Sequence analysis of CmFL3. (A) Sequence alignment of CmFL3 and homologous proteins. The black color indicates 100% identity; red, 80% identity. Gaps are shown by dots; FUL motif is shown by the black box, and other key motifs are underlined. (B) Phylogenetic tree of API/FUL-like subfamily proteins and CmFL3 (black box). Detailed information is shown in the Supplementary Materials. The GenBank accession number of CmFL3 is ON959211.](image-url)
3.2. Expression Patterns of CmFL3

qRT-PCR was used to examine the expression patterns of CmFL3 in various organs and tissues of ‘Jinba’. At the vegetative stage, CmFL3 was strongly transcribed in leaves (Figure 2A). At the reproductive stage, CmFL3 transcripts mainly accumulated in the receptacle, followed by the leaves, bracts, and disc floret petals (Figure 2B).

In situ hybridization was used to detect the spatial distribution of CmFL3 expression in young inflorescence buds (diameter < 2 mm). The CmFL3 signal was detected in the inflorescence meristem (IM), flower meristem (FM), receptacle, and bracts (Figure 2C).

Figure 2. Transcriptional profiling of CmFL3. The relative expression level of CmFL3 in (A) vegetative stage and (B) reproductive stage under short-day conditions quantified using qRT-PCR. In situ localization of CmFL3 in (C) inflorescence bud (diameter < 2 mm). Rt, root; Ste, stem; Le, leaf; Sa, shoot apex; Rpe, ray floret petal; Rpi, ray floret pistil; Dpe, disc floret petal; Dpi, disc floret pistil; Dst, disc floret stamen; IM, inflorescence meristem; FM, flower meristem; Br, bract; Re, receptacle. The IM, FM, and Br (C) are indicated by red arrows. Values (A,B) represent mean ± SE (n = 3). Significant differences were analyzed using Duncan’s multiple range test. Different letters above the bars represent significant differences (p < 0.05). Bar = 200 μm.

3.3. Subcellular Localization and Transactivation Activity of CmFL3

GFP activity in onion epidermal cells that were transformed with the control pMDC-43 plasmid was detected throughout the cells. However, GFP fluorescence was restricted to the cellular membrane and nucleus after the introduction of p35S::GFP-CmFL3 plasmid (Figure 3), implying that CmFL3 localizes in the cellular membrane and nucleus.
Figure 3. Subcellular localization of CmFL3. GFP, green fluorescence protein; DIC, differential interference contrast; Merged, overlay plots. Bars = 50 μm.

To further examine whether CmFL3 functions as a transcriptional activator, a transactivation assay was conducted in yeast. pCL1 and pGBKT7 were used as the positive and negative controls, respectively. Yeast cells harboring the pCL1 vector grew freely on the SD/-His-Ade medium independent of x-α-gal. In contrast, cells that harbored an empty pGBKT7 vector or either of the pGBKT7-CmFL3 plasmids did not grow on either medium (Figure 4A). Therefore, these experiments indicate that CmFL3 shows no transactivation activity in yeast.

Figure 4. Transcriptional activity of CmFL3. (A) Transcriptional activity of CmFL3 in yeast. pCL1 and pGBKT7 are the positive and negative controls, respectively. -AH: SD/-His-Ade lacking x-α-gal; -AH+x-α-gal: SD/-His-Ade containing x-α-gal. (B) Arabidopsis mesophyll protoplasts captured after luciferin addition using a charge-coupled device camera. (C) Luciferase activity measured after the introduction of 35S::GAL4DBCmFL3 into Arabidopsis mesophyll protoplasts. Values are represented as mean ± SE (n = 3). Significant differences were analyzed using Duncan’s multiple range test, and bars with different letters are significantly different from each other (p < 0.05).

A series of equivalent experiments were further performed in Arabidopsis protoplasts; 35S::GAL4DB-AtARF5 and 35S::GAL4DB were used as the positive and negative controls, respectively. 35S::GAL4DB-CmFL3 (5×GAL4-LUC, luciferase reporter) was
transfected into Arabidopsis protoplasts. The results showed that the LUC activity of CmFL3 was lower than that of 35S::GAL4DB (Figure 4B,C). These results imply that CmFL3 may function as a transcriptional repressor in Arabidopsis protoplasts.

3.4. Overexpression of the CmFL3 Gene Accelerated Flowering in Chrysanthemum

To characterize the biological function of CmFL3 in chrysanthemum, the construct carrying the CaMV 35S promoter followed by the CDS of CmFL3 was transformed into ‘Jinba’ [52]. Three transgenic lines (Ox-CmFL3) were selected, which were confirmed using the forward primer from the pMDC-43 vector and reverse primer from the CDS. Their relative expression levels were determined through qRT-PCR using CmFL3 specific primers (Figure 5A,B), and the phenotype was observed at different stages (Figure 5C,D).

In the present study, the flowering time of the transgenic lines differed significantly from that of the WT plants (Figure 5C,D). The Ox-CmFL3 lines 2#, 3#, and 5# reached the FM stage alongside the formation of bract primordia at 78, 78, and 80 d, respectively, after transplantation, whereas in WT plants, these events occurred at 89 d under the same conditions (Figure 5D). At 127 d after transplantation, inflorescences of the three transgenic lines had reached the early opening (EO) stage; however, the WT plants did not reach the EO stage until 139 d after transplantation (Figure 5D). Additionally, the Ox-CmFL3 transgenic plants were shorter than the WT plants.

Figure 5. Phenotypes of CmFL3 transgenic ‘Jinba’ plants. (A) RT-PCR analysis of CmFL3 in WT plants and Ox lines at the cDNA level; the forward primer was from pMDC-43 vector and reverse primer from the CDS. (B) The relative expression levels of CmFL3 in the WT and Ox transgenic
plants were determined using qRT-PCR. CmEF1a was the control gene. The values represent means ± SE (n = 3). Significant differences were determined using Student’s t-test (** p < 0.01). Each sample included three biological and three technical replicates. (C) Flowering phenotype of WT and Ox transgenic lines. The photographs were captured at 116 d after transplantation. (D) Development of inflorescence in WT ‘Jinba’ and Ox plants. FM stage, flower meristem stage; FBD stage, flower bud development stage; VC stage, visible color stage; EO stage, early opening stage; OF stage, opened flower stage; FO, fully opened flower stage; SF stage, senescing flower stage. Bars = 3.0 cm in the upper panel, whereas bars = 13.5 cm in the lower panel. Bar = 3 cm in (D) except for the FM stage, where bar = 500 μm.

In chrysanthemum, many genes, such as CmAFT and CmFTL3, are involved in flowering [21,53]. We further examined the expression levels of these genes in the Ox-CmFL3 transgenic lines (2#, 3#, and 5#) and WT plants using qRT-PCR. The results showed that the flowering promoter gene CmFTL3 was upregulated, whereas the flowering repressor factor CmAFT was downregulated in the leaves of the Ox lines (Figure 6A,B). Thus, the data showed that CmFL3 inducers flowering by upregulating the expression of CmFTL3 and downregulating the expression of CmAFT in chrysanthemum leaves.

3.5. Ectopic Expression of CmFL3 Induced Early Flowering in Arabidopsis

In the present study, 16 independent T3 Arabidopsis lines constitutively expressing CmFL3 were produced, and three transgene homozygotes (2#, 6#, and 20#) were selected to observe the phenotypic effect of CmFL3 expression in Arabidopsis (Figure 7A). The transgenic Arabidopsis lines showed early flowering (Figure 7B) and generated fewer rosette leaves (Figure 7C) than the WT (Col-0) plants. The transgenic plants flowered approximately 14 d earlier than the WT plants (Figure 7B). During bolting in WT plants, the number of rosette leaves formed was approximately 14, whereas it was approximately 7 in transgenic lines (Figure 7C). This shows that CmFL3 is involved in controlling flowering in Arabidopsis (Figure 7D). To further explore how CmFL3 controls flowering, the transcription of four key flowering-related genes—AtFUL, AtAPI1, AtFT, and AtLFY—was detected in 14-day-old Arabidopsis seedlings. The qRT-PCR data showed that these four genes were induced in Ox transgenic Arabidopsis plants (Figure S1).
4. Discussion

In Arabidopsis, loss of FUL function causes a slight delay in flowering time, whereas overexpression of FUL leads to an early flowering phenotype [54]. Genes of the FUL group have also been shown to regulate flowering in other species, such as VRN1, FUL2, and FUL3 in wheat [12]; BdVRN1, BdFUL2, and BdFUL3 in Brachypodium distachyon [55]; and OsMADS15 in rice [56]. In Asteraceae, the function of FUL has not been completely explored, although overexpression of FUL homologous genes has been reported to induce flowering in C. lavandulifolium [29], C. morifolium [16], and Gerbera [14]. Our previous study showed that several FUL genes were identified in chrysanthemum ‘Jinba’ transcriptome [44], but their functions need to be explored.

4.1. CmFL3 Is One of the FUL Group MADS-Box Genes

In model plants and crops, such as snapdragon, Arabidopsis, petunia, wheat, and rice, expression patterns and functions of AP1/FUL-like MADS-box genes have been well explored. However, information on AP1/FUL-like genes in ornamental plants, including those belonging to Asteraceae, is limited. In the present study, an euFUL clade MADS-box gene, CmFL3, was cloned from the chrysanthemum ‘Jinba’. Expression pattern analysis showed that CmFL3 had high expression levels in leaves at both vegetative and reproductive stages. In young inflorescence buds, the CmFL3 signal was also strongly detected in IM, similar to the GSQUA2 expression pattern [14].

Phylogenetic analysis revealed that CmFL3 merged with CDM8 first with 100% bootstrap support, and then with FUL and GOSQUA2 in the euFUL group. Moreover, CmAPl1L, CAL, and AP1 were grouped into the euAP1 clade and divided PapsFLL1 and Aquilegia coerulea AqFLL1A into the FUL-like group (Figure 1B). These results are consistent with those reported in previous studies [16]. The C-terminal domain of CmFL3 contained a well-conserved MPPWMV motif (Figure 1A). In addition, CmFL3 localizes in the nucleus and cellular membrane (Figure 4), which is similar to the localization of MADS-domain transcription factors [16,29,31,57]. However, these results need to be further verified in live plant tissues of Chrysanthemum morifolium, which will reveal more information on protein localization patterns [57]. The results indicate that CmFL3 may promote flowering in Arabidopsis. However, in chrysanthemum, the functions of euFUL clade genes, such as CmFL3, CDM8, and CDM41, remain unknown.
4.2. The Function of CmFL3 Is Conserved in the Regulation of Flowering, but Not in Flower Development

The function of the FUL gene ClM8 from *C. lavandulifolium* was explored in Arabidopsis, and the transgenic lines showed early flowering, lateral flowers, and composite terminal flowers [29]. In addition, ClM8 transgenic Arabidopsis plants were dwarfish and produce small rosette leaves. Moreover, the silique also showed some abnormalities as the style of the siliques was longer and thinner. However, ectopic overexpression of CmAFL3 in Arabidopsis led to early flowering (Figure 7). Furthermore, qRT-PCR data showed that the expression levels of *AtFT*, *AtAP1*, *AtLFY*, and *AtFUL* were significantly increased in 35S::ClM8 transgenic Arabidopsis [29]. These four genes were also upregulated in the Ox-CmFL3 transgenic Arabidopsis plants (Figure S1). Therefore, CmFL3 and ClM8 may play the same role in promoting flowering in Arabidopsis, except for in floral development.

In general, *API/FUL*-like subfamily genes are involved in regulating flowering [18,21,58], which function downstream of the flowering pathway [18,59–62]. *API* functions as the downstream target of the FT-FD complex in the shoot apical meristem of Arabidopsis [63,64]. In *Triticum aestivum*, the TaFT-TaFDL2 complex induces the expression of VRN1 (*API*-like gene) in leaves and shoot tips [65]. In chrysanthemum, euFUL group genes, such as GSQUA2, ClM8, and CmAFL1, are considered to control flowering time [20,29,66]. Notably, in 35S::CsFDL1 and 35S::CsFTL3 transgenic plants, the expression level of CsAFL1 increased in the leaves. CsAFL1 has also been predicted to regulate the expression of CsFTL3 via a positive feedback mechanism [20]. Indeed, it was confirmed in this study that the expression of CmFTL3 was upregulated in the leaves of 35S::CmFL3 transgenic chrysanthemum plants (Figure 6). Furthermore, the relative expression of CmAFT (a homologous gene of CsAFT) was downregulated in the leaves of Ox-CmFL3 transgenic plants (Figure 6).

In *Gerbera*, floret numbers of GSQUA2-overexpressing plants were lower than those of WT ‘Terra Regina’ plants [14]. However, the floret numbers showed no significant difference in CmFL3 transgenic lines (30.2 ± 4.2) and WT plants (295.1 ± 2.2). Therefore, during the evolution of plant species, the functions of euFUL clade members may have retained ancestral roles, swapped roles, or acquired novel roles [13,30,67].

5. Conclusions

Our findings revealed that the overexpression of CmFL3 induced early flowering, promoted the expression of CmFTL3, and inhibited the expression of CmAFT. Overall, our study demonstrates the function of CmFL3 in regulating flowering time, thus providing a framework for the development of molecular breeding programs in the future that are aimed at the flowering time of chrysanthemum. In the future, gene editing and other technologies will be used to genetically manipulate CmFTL3 to obtain early flowering chrysanthemums.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/article/10.3390/agronomy20171716/s1, Table S1. Primer names and sequences used in this study. Figure S1 The relative expression of flowering-related genes AtAPI, AtFUL, AtFT and AtLFY in WT (Col-0) and transgenic Arabidopsis seedlings. Supplement data: The information of *API/FUL*-like proteins.

**Author Contributions:** Conceptualization, F.C. and L.D.; methodology, X.X.; software, D.J.; validation, S.L., D.J. and X.X.; formal analysis, A.S.; investigation, X.X.; resources, J.J.; data curation, K.Z.; writing—original draft preparation, K.Z.; writing—review and editing, L.D.; visualization, H.W.; supervision, F.C.; project administration, S.C.; funding acquisition, X.X., L.D. and F.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was financially supported by the National Natural Science Foundation of China (32171855, 32002075), China Agriculture Research System (CARS-23-A18), Seed industry project of Jiangsu Province (JBCS [2021]020), the National Key Research and Development Program of China (2020YFD1000400) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

**Institutional Review Board Statement:** Not applicable.
Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References


