



Brief Report

Isolation of *FLOWERING LOCUS C* and Preliminary Characterization in the Floral Transition of Xinjiang Precocious Walnut

Qiang Jin ^{1,2,3} , Rui Zhang ^{1,3}, Liping Chen ^{4,*} and Zhengrong Luo ^{2,*}

- ¹ College of Horticulture and Forestry, Tarim University, Alar 843300, China; qiangjin@taru.edu.cn (Q.J.); zhrqsh@163.com (R.Z.)
- ² National Key Laboratory for Germplasm Innovation & Utilization of Horticultural Crops, Huazhong Agricultural University, Wuhan 430070, China
- ³ Xinjiang Production & Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin, Alar 843300, China
- ⁴ Key Laboratory of Tarim Oasis Agriculture of Education Ministry of China, Information Engineering School of Tarim University, Alar 843300, China
- * Correspondence: mr.chenliping@gmail.com (L.C.); luozhr@mail.hzau.edu.cn (Z.L.)

Abstract: Walnut (*Juglans regia* L.) plants typically flower after eight to ten years of juvenile growth. Precocious germplasm, also known as early-flowering or early-mature genotypes, have shortened juvenile phases of one to two years and are therefore crucial for enhancing breeding efficiency. However, such precocious germplasms are very limited. Here, we isolated and characterized the key flowering-time gene *FLOWERING LOCUS C* (*FLC*) in the precocious walnuts of the Xinjiang Uygur Autonomous Region. Sequence alignment showed that *Juglans regia* *FLC* (*JrFLC*) contained a conserved MINICHROMOSOME MAINTENANCE 1 (MCM1), AGAMOUS (AG), DEFICIENS (DEF), and SERUM RESPONSE FACTOR (SRF) (MADS)-box domain. Analysis of an *FLC*–green fluorescent protein (GFP) fusion protein revealed that *JrFLC* was localized to the nucleus. Gene expression analysis showed that *JrFLC* was specifically expressed during the bud dormancy stage of precocious walnut, and that expression levels gradually decreased as the ambient temperature warmed. Exogenous *JrFLC* overexpression in *Arabidopsis thaliana* delayed flowering and increased the total leaf number, suggesting a similar function of *JrFLC* as a floral repressor in walnut and in other plants. Together, these results showed that *JrFLC* played an important role in regulating the floral transition of Xinjiang precocious walnut. Further studies, including a detailed characterization of *JrFLC*, are expected to validate *JrFLC* as a strong target for genetic improvement in flowering time in walnut.

Keywords: *FLC*; floral transition; flowering time; precocious germplasm; walnut



Citation: Jin, Q.; Zhang, R.; Chen, L.; Luo, Z. Isolation of *FLOWERING LOCUS C* and Preliminary Characterization in the Floral Transition of Xinjiang Precocious Walnut. *Horticulturae* **2023**, *9*, 582. <https://doi.org/10.3390/horticulturae9050582>

Academic Editor: Júlia Halász

Received: 28 April 2023

Revised: 12 May 2023

Accepted: 12 May 2023

Published: 14 May 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Walnut, also known as Persian walnut (*Juglans regia* L.; $2n = 2x = 32$), originate from Central Asia [1]. It is as a nut crop that is rich in a variety of microelements and polyphenols [2,3], but has additional value in medicine and other industrial fields [4–6]. Walnut trees have a relatively long juvenile stage (defined as the time from the vegetative to the reproductive phase), usually taking 8–10 years to undergo floral transition. In plants, the floral transition is regulated by several exogenous and endogenous stimuli to ensure the correct timing [7,8]. In the model plant *Arabidopsis thaliana*, flowering is controlled by various pathways including the photoperiod, vernalization, autonomous, and gibberellin pathways. The critical reproductive phase of flowering is regulated by a network of flowering genes. The most important and well-studied among them are *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T* (*FT*) [9,10]. Some walnut germplasms have the short-juvenile (precocious) trait, which is stably inherited and has been successfully applied

to shorten the breeding cycle in selecting new varieties. However, the floral transition is poorly understood in walnut due to a genetic bottleneck that has limited the number of precocious walnut germplasm.

The Xinjiang Uygur Autonomous Region of China, which is near the region in Central Asia in which walnut originated, has a large population of previously unutilized wild-type walnut resources. These wild germplasms are naturally preserved in situ in Gongliu County, at the northern portion of the Tianshan Mountains. Southern Xinjiang contains ancient walnut trees that have grown for hundreds to thousands of years. The main cultivated varieties are Xinjiang precocious thin-skinned varieties, which are abundant in the Xinjiang region [11–13]. A previous study from our lab revealed the genetic factors underlying floral transition in early-flowering walnut using RNA sequencing and microRNA sequencing [12]. In this brief report, we isolated the key floral transition gene *FLC* and measured its dynamic expression levels at multiple growth stages. Furthermore, we confirmed its molecular function via transgenic expression in wild type (Col-0) of *A. thaliana*. The discovery of *FLC*-driven precocious germplasm will allow us to answer critical questions about the floral transition and could be applied in future breeding efforts to shorten juvenility in Xinjiang walnuts.

2. Materials and Methods

2.1. Walnut Germplasm

The precocious walnut varieties 'Xinwen 179' and 'Xinwen 81' were planted in the field in the Xinjiang Uygur Autonomous Region, China (40°32'27.74239'' N, 81°18'3.40166'' E, altitude 957.37 m). Floral buds were collected from each variety during the growth stage in 2021 and 2022, with at least three biological replicates per genotype. Samples were flash-frozen in liquid nitrogen, then stored at -80°C prior to further analyses.

2.2. Walnut Morphological Analysis

Walnut buds were fixed in formaldehyde–alcohol–acetic acid (FAA), then dehydrated in an alcohol gradient. After dehydration, the samples were treated with a xylene gradient (2:1 alcohol:xylene for 20 min, 1:2 alcohol:xylene for 20 min, then 100% xylene for 10 min) to render the samples transparent. These samples were embedded in paraffin, sectioned with an RM2235 microtome (Leica, Wetzlar, Germany), and stained with fast green (1% *w/w*) and safranin (1% *w/w*). The samples were observed and photographed using an Olympus DP71 microscope (Olympus, Tokyo, Japan).

2.3. RNA Extraction

Total RNA was extracted from walnut buds with the RNA Easy Fast Plant Tissue Kit (TIANGEN, Beijing, China) as previously described [12]. RNA concentration and purity were detected with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis. Only high-integrity RNA was used in further experiments.

2.4. Identification of a Putative FLC Protein in Xinjiang Walnut

In plants, FLC belongs to the MINICHROMOSOME MAINTENANCE 1 (MCM1), AGAMOUS (AG), DEFICIENS (DEF), and SERUM RESPONSE FACTOR (SRF) (MADS)-box gene family. FLC-like proteins with a MADS domain were identified in the walnut genome [14] via BLAST. Other candidates were identified with the NCBI Conserved Domain Search database (NCBI CDD) (<https://www.ncbi.nlm.nih.gov/cdd/>, accessed on 2 November 2022) and the MultAlin software [15] (<http://multalin.toulouse.inra.fr/multalin/>, accessed on 25 November 2022) based on the presence of a MADS-box domain and AtFLC protein sequence.

2.5. Subcellular Localization

To construct an FLC–green fluorescent protein (GFP) fusion protein, the full-length coding sequence (CDS) of *FLC* was inserted between the 35S promoter and the sequence

encoding GFP in the pMDC85-GFP vector. The subcellular localization of FLC in *Arabidopsis* protoplasts was determined as previously described [16]. Briefly, protoplasts were isolated from 14-day-old *Arabidopsis* seedlings and the protoplasts were transformed with the JrFLC-GFP construct. GFP fluorescent signals were observed and images captured on an LSM 700 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

2.6. Quantitative Reverse Transcription (qRT)-PCR

qRT-PCR was performed with SYBR Green Master mix on a Light Cycle 96 Real-Time PCR system (Roche, Basel, Switzerland). Relative gene expression levels were calculated with the $2^{-\Delta\Delta C_t}$ method [17], using our previously published internal reference gene [12]. Primers of qRT-PCR are shown in Table S1.

2.7. Plasmid Construction and *Arabidopsis* Transformation

To generate the *35Spro:JrFLC* plasmid, the full-length *JrFLC* CDS without the stop codon was placed into the pK2GW7 vector using Gateway technology. The vector was inserted into *Agrobacterium tumefaciens* GV3101, and *Arabidopsis* plants were stably transformed with an *Agrobacterium*-mediated floral dip method [18]. Flowering time and total leaf number were measured in transgenic *35Spro:JrFLC* lines. Downstream target gene expression was determined with qRT-PCR using previously published primers for *Arabidopsis FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)*, and *LEAFY (LFY)* [19]. For measurement of flowering time and total leaf number, at least 10 *Arabidopsis* plants for each line were scored. Significant differences between the means of the indicated genotypes were calculated by a two-tailed Student's *t*-test (Excel 2019, accessed on 15 November 2022).

3. Results and Discussion

3.1. *JrFLC* Isolation and Expression in Precocious Walnut

In plants, the critical reproductive phase (flowering) is regulated by a network of flowering genes, including *FLC* [9,10]. The precocious walnut varieties found in Xinjiang have very short juvenility, typically blooming and bearing fruit within 3 years; some even flower within a single year [12]. Based on the known essential roles of *FLC* homologs in plants, we hypothesized that the key regulatory gene *FLC* may contribute to the short juvenility of Xinjiang precocious walnuts, and that this locus could be used to shorten breeding cycles. Thus, we isolated *FLC* and explored *FLC* expression patterns at several growth stages of precocious walnut.

We first cloned the full-length CDS of *FLC* from the precocious variety 'Xinwen 81' (Table S1). Sequence alignment showed that JrFLC had moderate similarity with AtFLC, and we identified a conserved N-terminal MADS-box domain in these two proteins (Figure 1A). Next, we conducted a subcellular localization assay. Protoplasts isolated from the leaves of 14-day-old *Arabidopsis* seedlings were transformed with the JrFLC-GFP construct. An examination with a confocal laser scanning microscope showed that the JrFLC-GFP fusion protein was localized to the nucleus (Figure 1B), comparable to *FLC* proteins in *Arabidopsis* [20] and other species. The conserved MADS-box domain and consistent subcellular localization indicated that JrFLC may have a similar molecular function as previously characterized plant *FLCs*.

To understand the dynamics of *JrFLC* expression throughout precocious walnut development, we collected samples at several growth stages. We examined overall bud morphology during its formation in precocious walnut (Figure 2A–D) and further assessed internal bud morphology at two key developmental stages, the dormant bud stage and the floral bud stage (Figure 2E,F). In apple, *FLC* is subject to dynamic regulation during the growth stage. There is some evidence that it is accumulated during the bud dormancy stage due to epigenetic modifications [21]. We hypothesized that *JrFLC* may have comparable expression patterns in precocious walnut. This hypothesis was tested by analyzing *JrFLC* expression at multiple stages via qRT-PCR. *JrFLC* was most highly expressed in the dormant buds, and gradually decreased when ambient temperatures

increased (Figure 2G and Figure S1). These results indicated that *JrFLC* expression was responsive to temperature signals, which may therefore have an impact on precocious walnut germplasm.

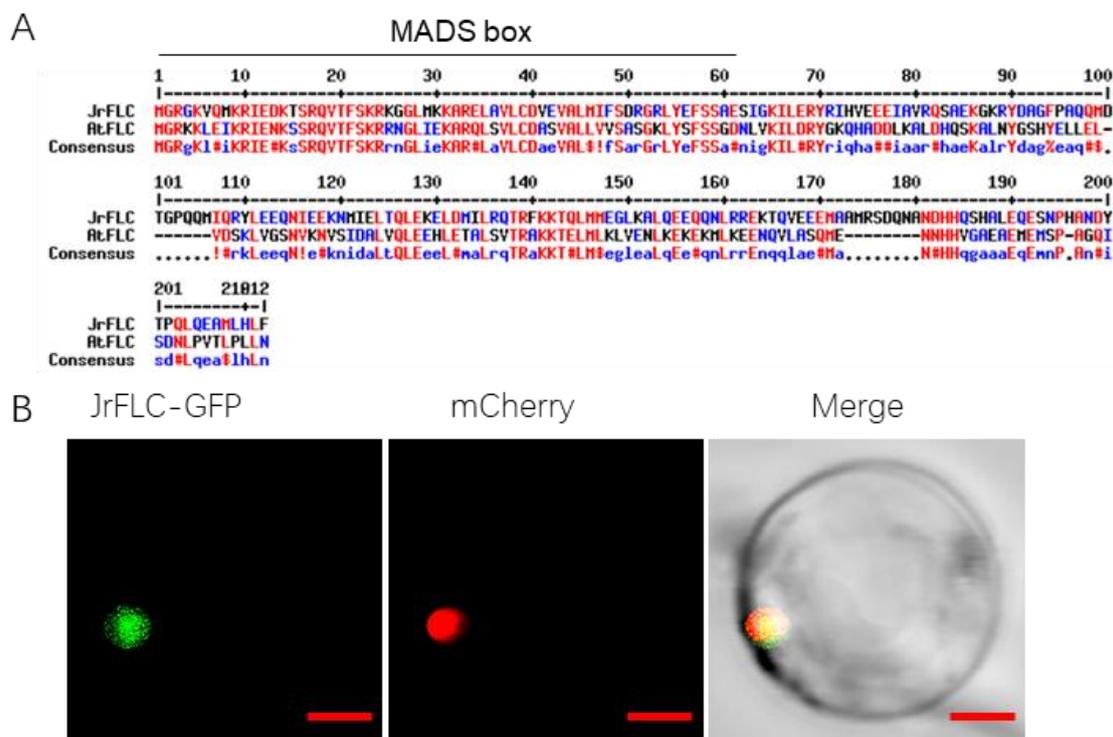


Figure 1. (A) Sequence alignment of *Juglans regia* and *Arabidopsis thaliana* FLC proteins. The N-terminal regions of JrFLC and AtFLC showed a conserved MINICHROMOSOME MAINTENANCE 1 (MCM1), AGAMOUS (AG), DEFICIENS (DEF), and SERUM RESPONSE FACTOR (SRF) (MADS)-box domain. Sequence identity between JrFLC and AtFLC was 45.5%. (B) Subcellular localization of JrFLC. JrFLC tagged with green fluorescent protein (GFP) co-localized with a nuclear marker. Bars in (B) were 50 μ m.

3.2. *JrFLC* Overexpression Delayed Flowering in *Arabidopsis*

To analyze the molecular function of JrFLC, we created transgenic *Arabidopsis* plants overexpressing *JrFLC* (*35Spro:JrFLC*) (Figure 3A). We first verified the successful construction of the overexpression lines by quantifying *FLC* expression patterns in transgenic plants (Figure 3B). We then analyzed the phenotypes of the validated *35Spro:JrFLC* plants. Compared to the wild-type Columbia-0 plants, *JrFLC* overexpressors showed delayed flowering and increased total leaf number (Figure 3C,D). Moreover, *JrFLC* overexpression reduced the expression levels of downstream floral transition genes, including *AtFT*, *AtLFY*, and *AtSOC1* (Figure 3E). Together, these results indicated that *JrFLC* and *AtFLC* had similar functions as floral repressors.

Walnut trees are known to have originated in Central Asia [1,22,23]. In this region, previous reports have revealed that some germplasm display an early-flowering phenotype characterized by short juvenility; such plants may produce flowers within three years (or even within the same year) of germination [24,25]. In these early-flowering germplasm, orthologs of the *Arabidopsis* MADS-box genes *AGAMOUS* and *APETALA 3* may be responsible for the early transition from the juvenile to the adult phase [24]. In walnut germplasm from the Xinjiang Uygur Autonomous Region, near Central Asia, we here revealed that another MADS-box gene, *FLC*, was involved in this transition. However, the mechanism by which MADS-box genes regulate the floral transition in early-flowering walnuts remains unclear.

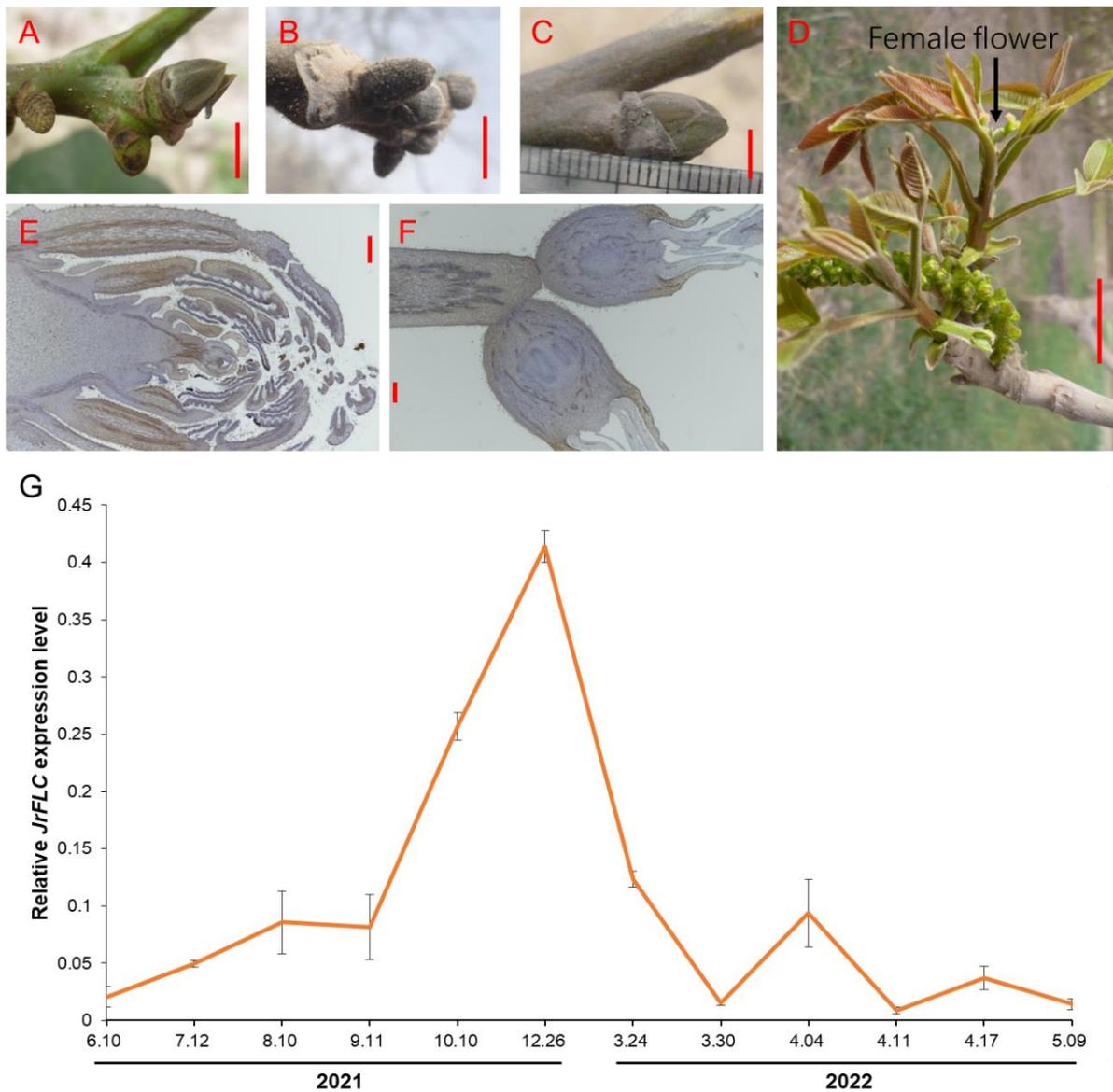


Figure 2. *FLC* accumulation in precocious walnut variety ‘Xinwen 81’ during bud dormancy. (A–D) Representative images of buds at several growth stages of precocious walnut: (A) bud differentiation, (B) bud dormancy, (C) bud sprout, and (D) floral formation and leaf expansion. (E–F) Internal morphological analysis of (E) dormant buds and (F) floral buds. (G) Dynamic expression levels of *JrFLC* in ‘Xinwen 81’. Bars in (A–C) were 0.5 cm; bar in (D) was 1 cm; bars in (E,F) were 50 nm. For qRT-PCR assays, bars in (G) indicated the standard deviations.

Notably, with the improvement in sequence technologies, we have previously performed RNA sequencing and microRNA sequencing for analyzing transcriptome-wide expression of genes and miRNAs in the hybrids between precocious walnut ‘Xinwen 81’ and a local later-flowering walnut germplasm [12]. In a previous study, we identified a large amount of differentially expressed genes (DEGs), including these MADS-box genes [12]. Guided by this work with a strategy for the molecular identification of a MADS-box gene *FLC*, our further study will isolate more MADS-box genes related to the regulation of floral transition in Xinjiang precocious walnuts.

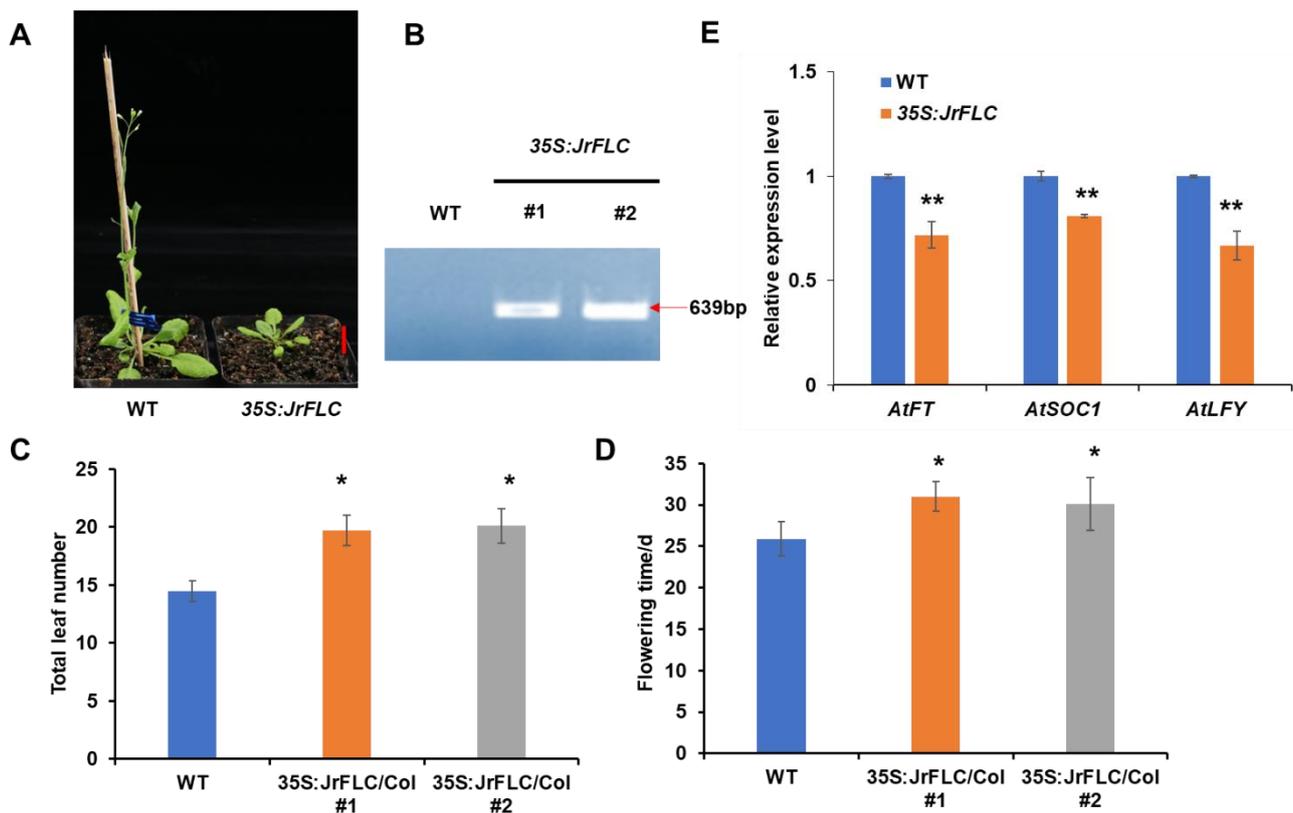


Figure 3. Functional validation of *Juglans regia* *FLC* via overexpression in *Arabidopsis*. (A) Representative image of wild-type (WT) and *JrFLC*-overexpression (*35Spro:JrFLC*) plants of the same growth stage. (B) Validation of *JrFLC* overexpression in transgenic plants via quantitative reverse transcription PCR. (C,D) Quantification of (C) total leaf number and (D) flowering time in WT and *35Spro:JrFLC Arabidopsis* plants. (E) Expression levels of flowering genes downstream of *FLC* (*FT*, *SOC1*, and *LFY*) in WT and *35Spro:JrFLC Arabidopsis* plants. Bar in (A) was 2 cm. At least 10 *Arabidopsis* plants for each line were scored with flowering time and total leaf number; significant differences between the means of the indicated genotypes were revealed by a two-tailed Student's *t*-test (* $p < 0.05$; ** $p < 0.01$). For qRT-PCR assays and measurement of flowering time, bars indicated the standard deviations.

In addition to MADS-box genes, flowering time in plants is also regulated by epigenetic mechanisms [26], including DNA methylation, non-coding RNAs, and histone modifications. These modifications control growth and development by regulating gene expression. In *Brassicaceae* species, the transcription factor *FLC* represses flowering until a plant is exposed to extended cold conditions (i.e., winter) in a process known as vernalization [10,27]. The prolonged chilling of winter leads to higher histone H3 lysine 27 trimethylation (H3K27me3) and lower histone H3 lysine 4 trimethylation (H3K4me3) levels at the *FLC* locus, stably repressing *FLC* transcription even after plants are returned to warmer temperatures [10,27]. It is therefore likely that temperature signals such as these contribute to the rapid floral transition among Xinjiang precocious walnuts under natural low-chilling, long-day conditions. Future studies should explore this dynamic epigenetic regulation of the *FLC* locus in walnut in depth.

4. Conclusions

This brief report summarizes our investigation into juvenility among two precocious varieties of Xinjiang walnut. We identified the key flowering-time gene *FLC* as a driver of precocity in these varieties. *JrFLC* had a high sequence identity with *AtFLC* and, like *AtFLC*, was shown to be nuclear-localized. In addition, *JrFLC* overexpression in *Arabidopsis*

delayed flowering time. Together, the results of this study indicate that *JrFLC* played an important role in regulating the floral transition among Xinjiang precocious walnuts. This gene is therefore a strong candidate for future genetic improvement in flowering time in walnut.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9050582/s1>, Table S1: Full-length FLC coding sequence and list of primers used in this study; Figure S1: Dynamic expression levels of *JrFLC* in ‘Xinwen 179’.

Author Contributions: Conceptualization, Q.J.; methodology, Q.J.; software, Q.J. and R.Z.; validation, Q.J. and L.C.; formal analysis, R.Z. and L.C.; investigation, Q.J.; resources, Q.J. and R.Z.; writing—original draft preparation, Q.J.; writing—review and editing, Z.L.; visualization, L.C.; supervision, L.C. and Z.L.; project administration, L.C. and Z.L.; funding acquisition, L.C. and Q.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Major Scientific and Technological Projects of XPCC (2017DB006), the National Natural Science Foundation of China (31260463, 32160698), and the Agricultural AI Application and Innovation Team of Tarim University (TDZKCX202308).

Data Availability Statement: Not applicable.

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

References

- Bernard, A.; Lheureux, F.; Dirlewanger, E. Walnut: Past and future of genetic improvement. *Tree Genet. Genomes* **2018**, *14*, 1–28. [[CrossRef](#)]
- Jahanban-Esfahlan, A.; Ostadrahimi, A.; Tabibiazar, M.; Amarowicz, R.; Tabibiazar, M.; Amarowicz, R. A comprehensive review on the chemical constituents and functional uses of walnut (*Juglans* spp.) husk. *Int. J. Mol. Sci.* **2019**, *20*, 3920. [[CrossRef](#)]
- Sheng, F.; Hu, B.; Jin, Q.; Wang, J.; Wu, C.; Luo, Z. The analysis of phenolic compounds in walnut husk and pellicle by UPLC-Q-Orbitrap HRMS and HPLC. *Molecules* **2021**, *26*, 3013. [[CrossRef](#)]
- Hardman, W.E. Walnuts have potential for cancer prevention and treatment in mice. *J. Nutr.* **2014**, *144*, 555S–560S. [[CrossRef](#)]
- Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [[CrossRef](#)]
- Trandafir, I.; Cosmulescu, S.; Nour, V. Phenolic profile and antioxidant capacity of walnut extract as influenced by the extraction method and solvent. *Int. J. Food Eng.* **2017**, *13*, 1–8. [[CrossRef](#)]
- Gu, X.; Wang, Y.; He, Y. Photoperiodic regulation of flowering time through periodic histone deacetylation of the florigen gene *FT*. *PLoS Biol.* **2013**, *11*, e1001649. [[CrossRef](#)] [[PubMed](#)]
- Gu, X.; Jiang, D.; Wang, Y.; Bachmair, A.; He, Y. Repression of the floral transition via histone H2B monoubiquitination. *Plant J.* **2009**, *57*, 522–533. [[CrossRef](#)]
- Jiang, D.; Gu, X.; He, Y. Establishment of the winter-annual growth habit via *FRIGIDA*-mediated histone methylation at *FLOWERING LOCUS C* in *Arabidopsis*. *Plant Cell* **2009**, *21*, 1733–1746. [[CrossRef](#)] [[PubMed](#)]
- He, Y.; Amasino, R.M. Role of chromatin modification in flowering-time control. *Trends Plant Sci.* **2005**, *10*, 30–35. [[CrossRef](#)] [[PubMed](#)]
- Wang, G.A.; Zhang, Q.; Huang, M.M.; Yakup, A. The breeding of six Xinjiang dwarf walnut cultivars. *Acta Hort.* **2014**, *1050*, 151–160. [[CrossRef](#)]
- Jin, Q.; Mo, R.; Chen, W.; Zhang, Q.; Sheng, F.; Wu, C.; Zhang, R.; Luo, Z. Identification and comparative analysis of genes and microRNAs involved in the floral transition of the Xinjiang early-flowering walnut (*Juglans regia* L.). *Horticulturae* **2022**, *8*, 136. [[CrossRef](#)]
- Yuan, X.; Niu, J.; Zhou, L.; Quan, S.; Ma, L.; Xu, H. Evolutionary analysis of *MIR156/157* family during floral induction in walnut (*Juglans regia* L.). *J. Hortic. Sci. Biotechnol.* **2022**, *97*, 697–707. [[CrossRef](#)]
- Zhu, T.; Wang, L.; You, F.M.; Rodriguez, J.C.; Deal, K.R.; Chen, L.; Li, J.; Chakraborty, S.; Balan, B.; Jiang, C.Z.; et al. Sequencing a *Juglans regia* × *J. microcarpa* hybrid yields high-quality genome assemblies of parental species. *Hortic. Res.* **2019**, *6*, 55. [[CrossRef](#)]
- Corpet, F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **1988**, *16*, 10881–10890. [[CrossRef](#)]
- Zhang, P.; Zhu, C.; Geng, Y.; Wang, Y.; Yang, Y.; Liu, Q.; Guo, W.; Chachar, S.; Riaz, A.; Yan, S.; et al. Rice and *Arabidopsis* homologs of yeast chromosome transmission fidelity protein 4 commonly interact with Polycomb complexes but exert divergent regulatory functions. *Plant Cell* **2021**, *33*, 1417–1429. [[CrossRef](#)] [[PubMed](#)]
- Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
- Clough, S.J.; Bent, A.F. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **1998**, *16*, 735–743. [[CrossRef](#)]

19. Zhang, P.; Li, X.; Wang, Y.; Guo, W.; Chachar, S.; Riaz, A.; Geng, Y.; Gu, X.; Yang, L. PRMT6 physically associates with nuclear factor Y to regulate photoperiodic flowering in *Arabidopsis*. *aBIOTECH* **2021**, *2*, 403–414. [[CrossRef](#)]
20. Jiang, D.; Yang, W.; He, Y.; Amasino, R.M. *Arabidopsis* relatives of the human lysine-specific demethylase1 repress the expression of *FWA* and *FLOWERING LOCUS C* and thus promote the floral transition. *Plant Cell* **2007**, *19*, 2975–2987. [[CrossRef](#)]
21. Chen, W.; Tamada, Y.; Yamane, H.; Matsushita, M.; Osako, Y.; Gao-takai, M. H3K4me3 plays a key role in establishing permissive chromatin states during bud dormancy and bud break in apple. *Plant J.* **2022**, *111*, 1015–1031. [[CrossRef](#)] [[PubMed](#)]
22. Hassankhah, A.; Rahemi, M.; Ramshini, H.; Sarikhani, S.; Vahdati, K. Flowering in Persian walnut: Patterns of gene expression during flower development. *BMC Plant Biol.* **2020**, *20*, 136. [[CrossRef](#)] [[PubMed](#)]
23. Vahdati, K.; Mohseniazar, M. Early bearing genotypes of walnut: A suitable material for breeding and high density orchards. *Acta Hort.* **2016**, *1139*, 101–105. [[CrossRef](#)]
24. Breton, C.; Cornu, D.; Chriqui, D.; Sauvanet, A.; Capelli, P.; Germain, E.; Jay-Allemand, C. Somatic embryogenesis, micropropagation and plant regeneration of “Early Mature” walnut trees (*Juglans regia*) that flower in vitro. *Tree Physiol.* **2004**, *24*, 425–435. [[CrossRef](#)] [[PubMed](#)]
25. Ebrahimi, A.; Zarei, A.; Zamani Fardadonbeh, M.; Lawson, S. Evaluation of genetic variability among “Early Mature” *Juglans regia* using microsatellite markers and morphological traits. *PeerJ* **2017**, *5*, e3834. [[CrossRef](#)]
26. He, Y. Chromatin regulation of flowering. *Trends Plant Sci.* **2012**, *17*, 556–562. [[CrossRef](#)]
27. He, Y.; Li, Z. Epigenetic environmental memories in plants: Establishment, maintenance, and reprogramming. *Trends Genet.* **2018**, *34*, 856–866. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.