Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.022225

ARTICLE

# Overexpression of Wheat *TaELF3-1BL* Delays Flowering in Arabidopsis

**Junsheng Sun[#], Haozhen Zhang[#], Mingxia Zhang, Sishen Li and Yanrong An[*]**

State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, 271018, China

[*]Corresponding Author: Yanrong An. Email: 15053819853@163.com

[#]These authors contributed equally to this paper

Received: 28 February 2022 Accepted: 21 April 2022

## ABSTRACT

*EARLY FLOWERING 3* (*ELF3*), a light zeitnehmer (time-taker) gene, regulates circadian rhythm and photoperiodic flowering in Arabidopsis, rice, and barley. The three orthologs of *ELF3* (*TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*) have been identified in wheat too, and one gene, *TaELF3-1DL*, has been associated with heading date. However, the basic characteristics of these three genes and the roles of the other two genes, *TaELF3-1BL* and, *TaELF3-1AL*, remain unknown. Therefore, the present study obtained the coding sequences of the three orthologs (*TaELF3-1AL, TaELF3-1BL*, and *TaELF3-1DL*) of *ELF3* from bread wheat and characterized them and investigated the role of *TaELF3-1BL* in Arabidopsis. Protein sequence comparison revealed similarities among the three *TaELF3* genes of wheat; however, they were different from the Arabidopsis *ELF3*. Real-time quantitative PCR revealed *TaELF3* expression in all wheat tissues tested, with the highest expression in young spikes; the three genes showed rhythmic expression patterns also. Furthermore, the overexpression of the *TaELF3-1BL* gene in Arabidopsis delayed flowering, indicating their importance in flowering. Subsequent overexpression of *TaELF3-1BL* in the Arabidopsis *ELF3* nonfunctional mutant (*elf3* mutant) eliminated its early flowering phenotype, and slightly delayed flowering. The wild-type Arabidopsis overexpressing *TaELF3-1BL* demonstrated reduced expression levels of flowering-related genes, such as *CONSTANS* (*AtCO*), *FLOWERING LOCUS T* (*AtFT*), and *GIGANTEA* (*AtGI*). Thus, the study characterized the three *TaELF3* genes and associated *TaELF3-1BL* with flowering in Arabidopsis, suggesting a role in regulating flowering in wheat too. These findings provide a basis for further research on *TaELF3* functions in wheat.

## KEYWORDS

*TaELF3*; photoperiod; flowering time; Arabidopsis; *Triticum aestivum* L

## 1 Introduction

Bread wheat (*Triticum aestivum* L.) is an important food crop species. It is grown on more than 220 million hectares worldwide with an annual global production of 750 million tons. Nearly 50 billion dollars (US) worth of wheat are traded worldwide every year. It is the staple food for about 40% of the world population and is widely cultivated for other needs, including animal feed [1]. The timing of flowering is an important agronomic trait of wheat influencing its adaptation to specific cropping environments. A better understanding of the genes controlling flowering time will help breeders develop new cultivars with adaptive traits [2,3].

In wheat, flowering is determined by vernalization (*VRN*), photoperiod (*PPD*), and earliness per se (*EPS*) genes. Numerous quantitative trait loci (QTL) for flowering have been identified, and few of their target genes have been cloned in wheat. Characterization of four *VRN* genes (*VRN1*, *VRN2*, *VRN3*, and *VRN4*) controlling vernalization-mediated flowering has advanced our understanding of vernalization regulatory pathways [4–9]. Researchers have identified *PPD* genes, including *Ppd-A1*, *Ppd-B1*, and *Ppd-D1*, in wheat that contribute to photoperiod sensitivity and strongly influence flowering time [10–14]. Meanwhile, *EPS* genes influence flowering time, independent of vernalization and photoperiod responses [15,16]. The QTLs controlling *EPS* were detected on most chromosomes in wheat, but only a few genes were cloned [17–23]. Moreover, the complex regulatory mechanisms of flowering in wheat remain largely unknown.

Wheat is a long-day plant, similar to the model plant *Arabidopsis thaliana*. Several genes related to flowering time in wheat have been identified and characterized via cloning based on Arabidopsis homologs [24–27]. This strategy has accelerated studies on the molecular mechanisms of flowering in wheat. Among the various genes, *EARLY FLOWERING 3* (*ELF3*) is a zeitnehmer (time-taker) that regulates circadian clock and photoperiod flowering in Arabidopsis [28–35]. *OsELF3-1* and *OsELF3-2*, the rice homologs of *ELF3*, are essential for circadian rhythm regulation and photoperiodic flowering [36]. In barley, too, *HvELF3*, a homolog of *ELF3*, is responsible for early flowering [37,38]. Meanwhile, Wang et al. [39] identified three homologs of *ELF3* (*TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*) in wheat and associated *TaELF3-1DL* with heading date based on a QTL found segregating with this locus. However, no study has characterized the three genes and investigated the roles of *TaELF3-1BL* and *TaELF3-1AL*.Therefore, the present study aimed to analyze the characteristics of three *TaELF3* genes and the role of the *TaELF3-1BL* gene. We obtained the *ELF3* ortholog sequences from bread wheat, characterized them, and analyzed their expression patterns across tissues and under various day and night cycles. Furthermore, a transgenic approach was used to explore the functions of *TaELF3-1BL* in WT (wild-type) and *elf3* mutant of Arabidopsis. Collectively, the study will enrich our understanding of *TaELF3* genes and the role of *TaELF3-1BL* in photoperiod flowering, providing a basis for further research on *TaELF3* functions in wheat.

## 2 Materials and Methods

### *2.1 Experimental Materials and Growth Conditions*

The winter wheat cultivar Yannong15 was used in the present study. The coding sequences of *TaELF3* genes were obtained, and the tissue expression patterns were analyzed by growing Yannong15 in a field at the experimental station of Shandong Agricultural University (Taian, Shandong, China, 36.17°N, 117.17°E). For rhythmic expression analysis, the winter wheat cultivar Yannong15 was grown in chambers at 60% humidity under LD (long day; 16 h light at 23°C and 8 h darkness at 16°C), or SD (short day; 10 h light at 23°C and 14 h darkness at 16°C), or SL (standard light; 12 h light at 23°C and 12 h darkness at 16°C). The samples collected from these plants were used to analyze the rhythmic expression of the genes. The study used the WT Arabidopsis (Columbia ecotype) and the Arabidopsis *elf3* mutant for genetic transformation. The seeds of the WT and mutant plants were surface-sterilized in 70% ethanol for 1 min and kept at 4°C in the dark for 3 d and under LD conditions at 23°C for one week to germinate. The seedlings coming from seed germination were transplanted to vermiculite and grown under LD conditions. The seeds of the Arabidopsis *elf3* mutant were purchased from the Arabidopsis Biological Resource Center (ABRC).

### *2.2 TaELF3 Gene Amplification and Vector Construction*

The Arabidopsis *ELF3* protein sequence (AT2G25930) was used to query BLAST against the wheat genome database (http://plants.ensembl.org/index.html), and the three wheat sequences homologous (highest similarity) to Arabidopsis *ELF3* were extracted. Based on sequence alignment, three gene-

specific primers pairs (TaELF3-1A, TaELF3-1B, and TaELF3-1D) were designed by Primer Premier Version 5.0 and adjusted manually (Table 1). The amplicons were sequenced, and the predicted amino acid sequences were aligned and compared using DNAMAN software (version 7.212, Lynnon Corp., Quebec, Canada).

**Table 1:** Primers used in this study

| Primer | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| TaELF3-1A | TAGCTGGCCGCGATGAGGAG | CATACTCCCAAGCGCTAATTGGC |
| TaELF3-1B | CATGATGGCGGCCTAGCTGGCTG | GCATGCGGTCGCTCGGTCACG |
| TaELF3-1D | GCATGATGGCGTCCTAGCTAGCTG | GGAATGCTATTTGCTTGCTGCCTG |
| TaELF3-1A-1 | GCCAACGCCGCCAACACC | GTTGTTGTTGCTGTTATTGTCTCTACG |
| TaELF3-1B-1 | GAACAAGGAATGAGAATGC | CTCCGACAGATTAGAAGC |
| TaELF3-1D-1 | GAGACAATAACAGCAACAAC | CCACTCTTATACGGACTTG |
| AtCO | CCATCAGCGAGTTCCAATTCTAC | TCCTTATCACCTTCTTCACCTTCC |
| AtFT | CGCCAGAACTTCAACACTCG | CTTCCTCCGCAGCCACTC |
| AtGI | ATCTATTGAAGTGTCGTCTACCAG | GGCGGCGTTGAAGAATCG |
| TaACT | TATGCCAGCGGTCGAACAAC | GGAACAGCACCTCAGGGCAC |

Total RNA was extracted from the young wheat leaves using Trizol reagent (Invitrogen), and 2 μg of the RNA were used to synthesize the first-strand cDNA with the Superscript II First-Strand Synthesis Kit (Cat. No. 11904-018; Invitrogen). The cDNA was used to amplify *TaELF3* genes by PCR (20 μL; 80 ng of template DNA, 2 pmol of each primer, and 10 μl of 2 × Taq PCR Mix; HT-biotech, http://www.ht-biotech.net) performed at 94°C for 5 min, followed by 35 cycles of amplification at 94°C for 30 s, 60°C–66°C (depending on the primers) for 30 s, 72°C for 2 min 30 s, and a final extension at 72°C for 10 min. The PCR product was separated on 1% agarose gels, purified, cloned into the pEASY-T1 vector (TransGen Biotech Co., Ltd., Beijing; http://www.transgen.com.cn), sequenced at Sangon Biotech Co., Ltd. (Shanghai, China; http://www.sangon.com), and ligated into the expression vector pBI121 to generate the 35S::TaELF3-1BL expression construct.

### *2.3 Tissue and Rhythmic Expression Patterns of TaELF3 Genes*

Total RNA was extracted from the young root (Rt), stem/shoot (St), flag leaf (Fl), leaf sheath (Ls), internode (In), and young spikelet (Sl) of field-grown wheat plants at the booting stage using the Trizol reagent. Approximately 2 μg of the total RNA were used to synthesize the first-strand cDNA, and the expression level of the three *TaELF3* genes was analyzed by quantitative real-time PCR (qRT-PCR) using gene-specific primers (TaELF3-1A-1, TaELF3-1B-1, and TaELF3-1D-1, Table 1). The relative expression levels of the genes were quantified following the ΔΔCt method [40], using Actin (*TaACT*) as the reference gene and three biological replicates per sample.

For the rhythmic expression analysis, the leaves of 14-day-old wheat seedlings grown under various light-dark conditions (LD, 16 h light/8 h dark; SD, 10 h light/14 h dark; SL, 12 h light/12 h dark) were used. Leaves of the seedlings grown under LD or SD conditions were collected at zeitgeber time (time giver or time cue; environmental factor that acts as a circadian cue) 0 (ZT0, light on 6:00 AM) and every 4 h over a 24 h period. Meanwhile, seedlings grown under standard light (SL, 12 h light/12 h dark) for 14 d were divided into two groups and exposed to continuous light or continuous darkness in the morning (ZT0). Leaves were collected starting from the 15$^{th}$ day at 6:00 AM (ZT24) and every 4 h over a 24 h period, immediately frozen in liquid nitrogen and stored at −80°C until further analysis. Finally,

the expression levels of *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL* genes in these samples were analyzed by quantitative real-time PCR (qRT-PCR) following the described method.

### *2.4 Heterologous Transformation of TaELF3-1BL Gene in Arabidopsis*

The 35S::TaELF3-1BL expression construct was introduced into *Agrobacterium tumefaciens* (GV3101 strain) and transformed into *Arabidopsis thaliana* by the floc staining method. The transformants selected on an agarose plate containing Kanamycin (50 mg $L^{-1}$) were planted in vermiculite and watered regularly with nutrient solution. DNA was extracted from the leaves of these seedlings following the CTAB method [41], and the transgene integration was confirmed by PCR.

### *2.5 Analysis of the Phenotype and Flowering-Related Gene Expression in Transgenic Arabidopsis Plants*

Thirty transgenic plants expressing 35S::TaELF3-1BL and 30 WT plants were selected as the population for phenotypic and gene expression analyses. The flowering time and the number of rosette leaves at flowering were measured after growth under LD conditions.

Total RNA was extracted from the Arabidopsis leaves at bolting and flowering using Trizol reagent and reverse transcribed to obtain the first-strand cDNA. Then, qRT-PCR was carried out using this cDNA to analyze the expression of flowering-related genes, including *CONSTANS* (*AtCO*), *FLOWERING LOCUS T* (*AtFT*), and *GIGANTEA* (*AtGI*), under LD conditions. The primers used are shown in Table 1.

## 3 Results

### *3.1 Analysis of the Three Wheat TaELF3 Genes*

BLAST analysis against the Chinese genome v1.1 database using Arabidopsis *ELF3* protein sequence on the *EnsemblPlants* website (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast) identified three annotated genes, *TraesCS1A02G443200*, *TraesCS1B02G477400*, and *TraesCS1D02G451200*, with the highest homology, on chromosomes 1AL, 1BL, and 1DL, respectively. Wang et al. [39] had previously cloned these three genes and named them *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*. The present study initially obtained the coding sequences (CDS) of these three genes. Sequence analysis revealed that *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL* had an intact open reading frame (ORF) of 2304, 2304, and 2295 bp, encoding polypeptides with 768, 768, and 765 amino acid residues, respectively. These three *TaELF3* genes had similar amino acid sequences; however, the sequences were highly different from the Arabidopsis *ELF3* (Fig. 1).

The deduced amino acid sequences of wheat *ELF3* (*TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*) were aligned with Arabidopsis *ELF3* using the DNAMAN software. Pink boxes indicate conserved regions in the amino acid sequences of the three wheat genes, and black regions indicate conserved regions in amino acid sequences of the four genes.

### *3.2 Tissue Expression Pattern of TaELF3 Genes*

We analyzed the expression patterns of the three *TaELF3* genes in various wheat tissues at the booting stage by qRT-PCR. The analysis revealed expression of these three genes in all tissues, including young root (Rt), stem/shoot (St), flag leaf (Fl), leaf sheath (Ls), internodes (In), and spikelet (Sl). In each tissue, the expression patterns of these three genes were similar; however, the expression levels of each gene were different across the tissues (Fig. 2). The highest expression of *TaELF3* genes was detected in the young spikes.


**Figure 1:** Amino acid sequence alignment of *ELF3* from wheat and *Arabidopsis thaliana*

### *3.3 Rhythmic Expression Analysis of TaELF3 Genes*

Furthermore, the transcript levels of *TaELF3* genes in the leaves of wheat seedlings grown under four conditions, including LD, SD, continuous light (LL), and continuous darkness (DD), were examined every 4 h for 24 h to explore the role of the circadian clock in regulating the expression of the genes under all four conditions. The analysis revealed similar rhythmic expression patterns for *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL* genes. Under LD conditions, the expression levels of the three *TaELF3* genes were low at the beginning and gradually progressed as a wave at ZT8 and peaked at ZT20 (Fig. 3a). Meanwhile, under SD

conditions, the expression levels of the three *TaELF3* genes peaked after the light exposure at ZT4 and after darkness exposure at ZT20 (Fig. 3b). However, the rhythmic expression of the three *TaELF3* genes was disrupted under LL (continuous light) and DD (continuous darkness) conditions (Figs. 3c and 3d).

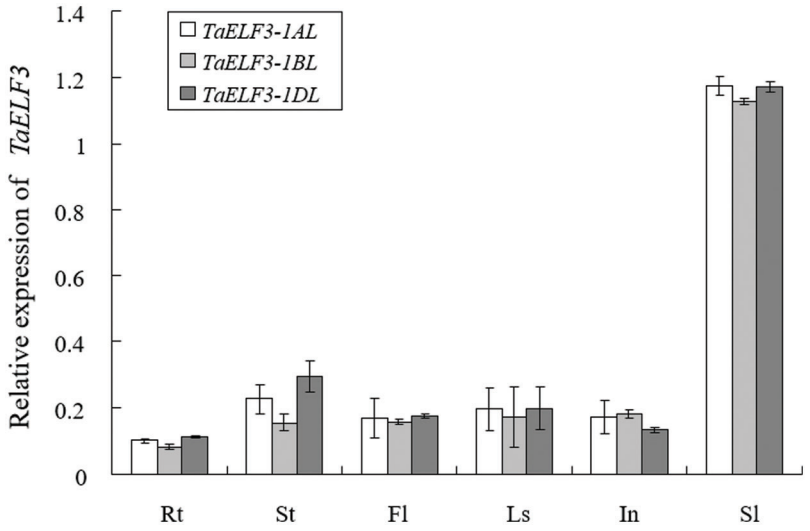


**Figure 2:** Expression pattern of the three *TaELF3* genes in various wheat tissues. The expression patterns of *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL* in the young root (Rt), stem/shoot (St), flag leaf (Fl), leaf sheath (Ls), internode (In), and young spikelet (Sl) of wheat plants at the booting stage were determined by qRT-PCR. Data represented are mean ± standard deviation (SD; n = 3)

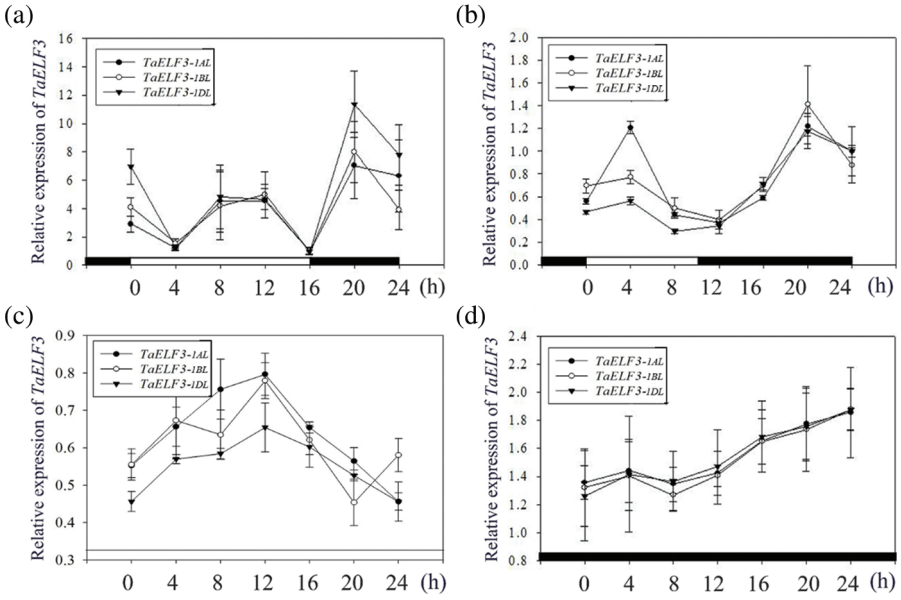


**Figure 3:** Rhythmic expression analysis of the three *TaELF3* genes in wheat under various light conditions. The expression patterns of *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL* genes in wheat seedlings grown under (a) long-day conditions; (b) short-day conditions; (c) continuous light; and (d) continuous darkness were analyzed by qRT-PCR. Data represented are the mean ± standard deviation (SD; n = 3)

### *3.4 Overexpression of Wheat TaELF3-1BL Delays Flowering in Arabidopsis*

To further investigate the role of *TaELF3* genes, one of the three genes, *TaELF3-1BL*, was overexpressed in Arabidopsis. PCR analysis of the transgenic lines confirmed the integration of the transgene *TaELF3-1BL* into the Arabidopsis genome (Fig. 4b). The overexpression of *TaELF3-1BL* in Arabidopsis delayed the flowering time under LD conditions (Fig. 4a). The WT plants flowered at 27 days after transplanting, while the transgenic plants flowered at about 32 days after transplanting (Fig. 4c). The transgenic Arabidopsis lines had an average of five rosette leaves more than the WT plants (Fig. 4d), indicating delayed flowering with *TaELF3-1BL* overexpression.

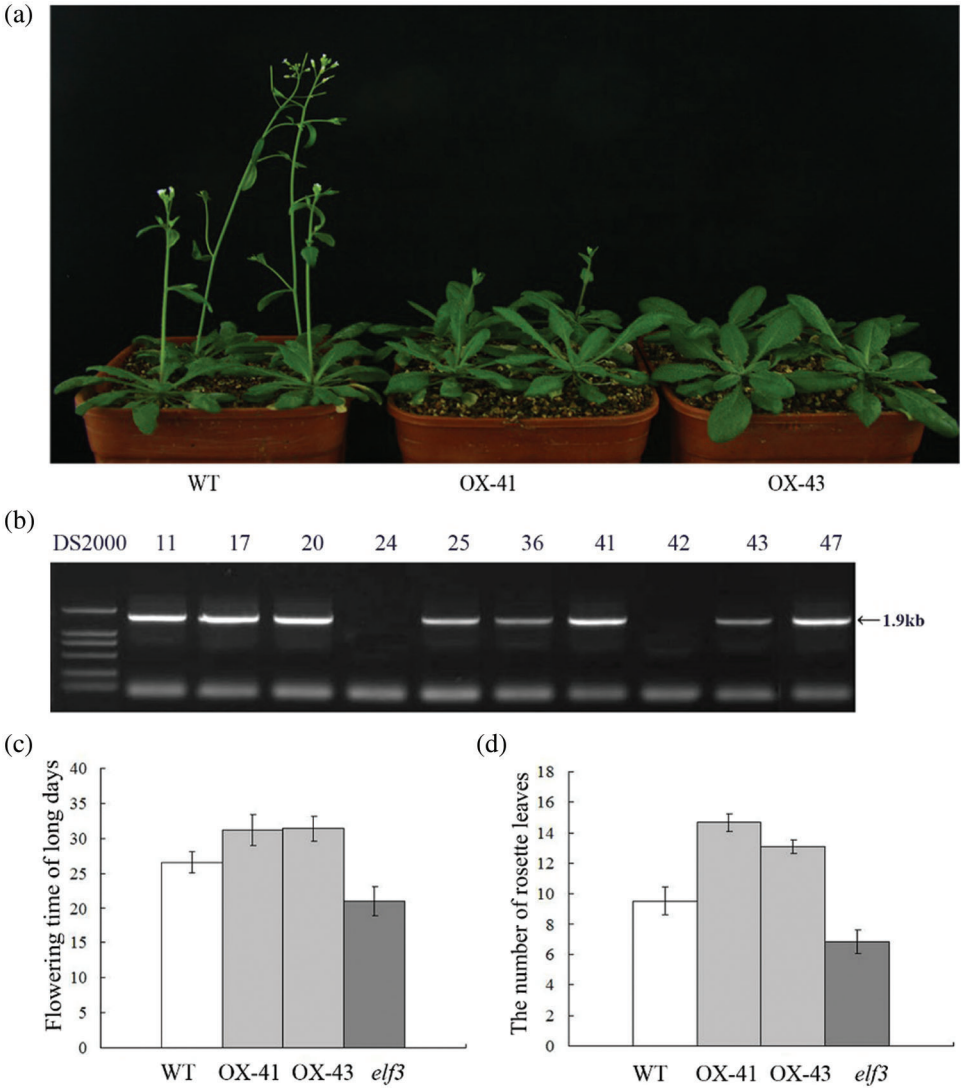


**Figure 4:** *TaELF3-1BL* overexpression delays flowering in *Arabidopsis thaliana*. (a) Flowering in wild-type (WT) and *TaELF3-1BL* overexpressing Arabidopsis plants; OX-41 and OX-43 are the two transgenic lines; (b) PCR confirmation of transgenic plants (11, 17, 20, 24, 25, 36, 41, 42, 43, and 47 represent the putative transgenic plants analyzed, and 11, 17, 20, 25, 36, 41, 43, and 47 represent the positive plants). The expected amplicon is 1.9 kb long; DS2000 indicates the marker. (c–d) Flowering time (number of days from transplanting to flowering) (c) and the number of rosette leaves (d) in the transgenic plants and the wild-type plants. Data represented in (c) and (d) are mean ± standard deviation (SD; n = 30)

To further confirm the function of *TaELF3-1BL*, the 35S::TaELF3-1BL construct was expressed in the Arabidopsis *elf3* mutant. The overexpression of *TaELF3-1BL* in the Arabidopsis *elf3* mutant slightly delayed flowering (Fig. 5a). The WT took 26 days to flower, while the *elf3* mutant took 22 days. Overexpression of *TaELF3-1BL* in the mutant, delayed the flowering to about 26 days. (Fig. 5b). Meanwhile, both the transgenic lines and the WT had more rosette leaves than the *elf3* mutant. The number of rosette leaves of the overexpressing plants was slightly more than on the WT, indicating retention of the WT flowering phenotype (Fig. 5c). These observations indicate that the overexpression of *TaELF3-1BL* in the Arabidopsis *elf3* mutant slightly delayed flowering compared with the WT, it is obtained from the number of rosette leaves.

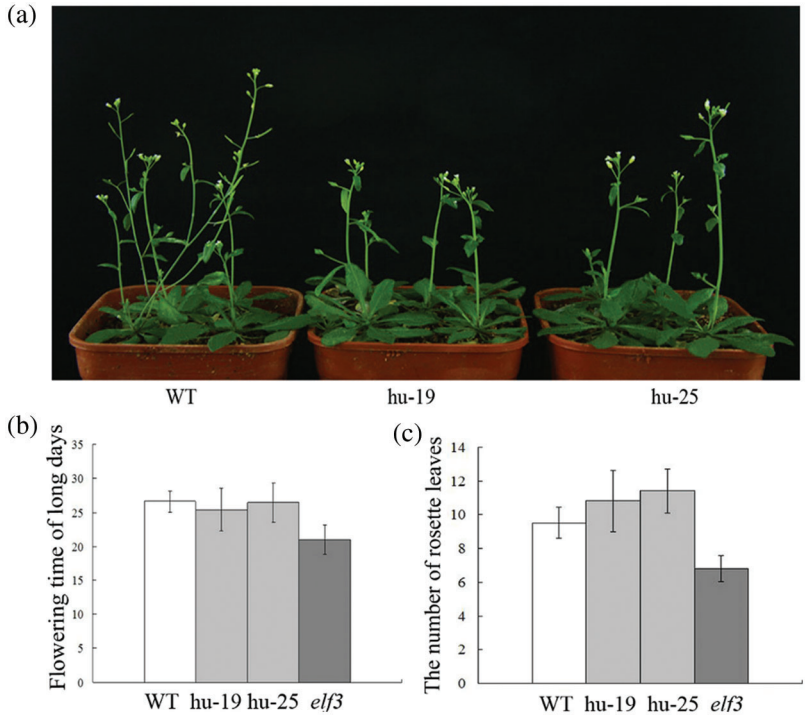


**Figure 5:** Overexpressing *TaELF3-1BL* delays flowering slightly in the Arabidopsis *elf3* mutant plants. (a) Phenotype; (b) flowering time; and (c) rosette leaf number of wild-type (WT), *elf3* mutant overexpressing *TaELF3-1BL* (hu-19 and hu-25), and *elf3* mutant plants. Data represented in (b) and (c) are mean ± standard deviation (SD; n = 30)

### *3.5 Expression Pattern of Flowering-related Genes in* TaELF3-1BL *Overexpressing Transgenic Arabidopsis Plants*

Further, the effects of *TaELF3-1BL* overexpression on the flowering-related genes were analyzed via qRT-PCR using RNA isolated from the WT and 35S::TaELF3-1BL overexpressing Arabidopsis plants. The results indicated that *AtCO*, *AtFT*, and *AtGI* were repressed in the transgenic plant (Fig. 6a). These observations suggest that the overexpression of *TaELF3-1BL* altered the expression of flowering-related genes, leading to delayed flowering in Arabidopsis.

## 4 Discussion

The *ELF3* gene shows a circadian rhythmic expression pattern and regulates photoperiodic flowering in Arabidopsis. Studies in bread wheat have identified three *TaELF3* genes and associated the *TaELF3-1DL* gene with heading date [39]. However, the characteristics of the three *TaELF3* genes and the roles of

*TaELF3-1AL* and *TaELF3-1BL* have not been fully explored. Therefore, the present study pulled out the three *TaELF3* genes (*TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*) from the winter wheat cultivar Yannong 15, analyzed their amino acid sequences, and found similarities among the three genes; however, these amino acid sequences were different from the Arabidopsis *ELF3*. Further analysis revealed that three genes were expressed in all wheat tissues tested, with the highest expression in young spikes, suggesting a significant role in developing young panicles and a constitutive expression of the three *TaELF3* genes. In addition, the three *TaELF3* genes showed similar rhythmic expression patterns under LD and SD conditions, which were disrupted under continuous light and continuous darkness conditions. Earlier, Hicks et al. demonstrated a rhythmic expression for *ELF3* in Arabidopsis after exposure to LD conditions, with a low expression at dawn and a peak expression at dusk [42]. Similarly, our experiments showed the rhythmic expression of these three *TaELF3* genes, similar to Arabidopsis *ELF3* under LD conditions, which suggests a probable rhythmic function of *TaELF3*.

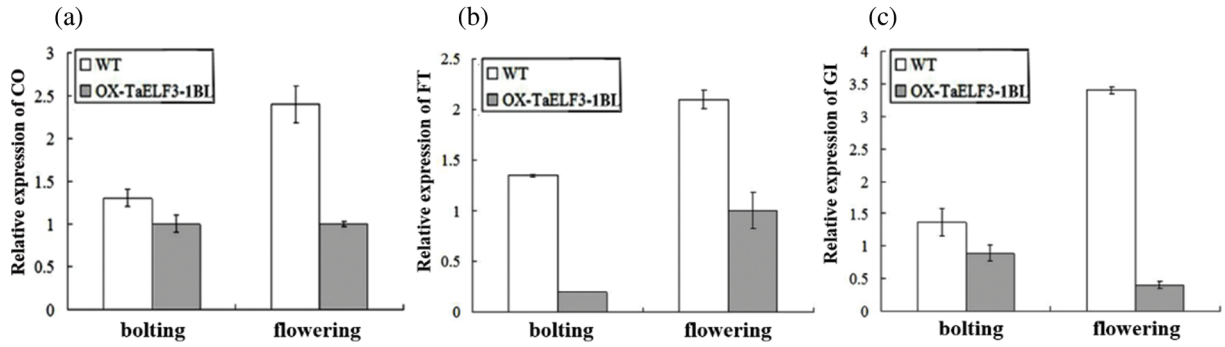


**Figure 6:** Overexpression of *TaELF3-1BL* influences the expression of flowering-related genes at bolting and flowering in Arabidopsis. The expression levels of *AtCO*, *AtFT*, and *AtGI* genes in wild-type (WT) and *TaELF3-1BL* overexpressing plants were determined by qRT-PCR. Data represented in (a), (b), and (c) are mean ± standard deviation (SD; n = 3)

Under LD conditions, loss-of-function mutations in the Arabidopsis (*elf3* mutant) gene caused early flowering, while overexpression of *ELF3* caused late flowering, with reduced expression of the flowering-related genes *AtCO*, *AtFT*, and *AtGI* [31,37,38,43–45]. Similarly, the present study found that the overexpression of *TaELF3-1BL* in Arabidopsis delayed flowering. Overexpression of the *TaELF3-1BL* gene in the Arabidopsis *elf3* mutant also slightly delayed flowering, confirming the role of the *TaELF3-1BL* gene in Arabidopsis flowering. These observations suggest that the *TaELF3-1BL* gene may also regulate wheat flowering, which needs to be further investigated. Further analysis of the expression of flowering-related genes at bolting and flowering in the WT Arabidopsis overexpressing the *TaELF3-1BL* gene revealed reduced expression of *AtCO*, *AtFT*, and *AtGI* genes. Thus, our study suggests that the overexpression of *TaELF3-1BL* delayed flowering in Arabidopsis by reducing the expression of the flowering-related genes. The findings on the characteristics of the three genes and the effect of *TaELF3-1BL* in Arabidopsis flowering provide a basis for further research on the three *TaELF3* genes.

**Authors' Contributions:** YA and SL designed the experiments. JS, HZ and MZ performed the experiments. JS and HZ analyzed the data. JS and YA wrote the manuscript.

**Ethics Approval and Consent to Participate:** Not applicable.

**Consent for Publication:** Not applicable.

**Acknowledgment:** Not applicable.

**Availability of Data and Materials:** All data generated or analyzed during this study are included in this published article.

**Funding Statement:** The authors received no specific funding for this study.

**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## References

1. Zhao, G. C., Chang, X. H., Wang, D. M., Tao, Z. Q., Wang, Y. J. et al. (2018). General situation and development of wheat production. *Crops, 34(4),* 1–7. DOI 10.16035/j.issn.10017283.2018.04.001.
2. Beniston, M., Stephenson, D. B., Christensen, O. B., Ferro, C. A., Frei, C. et al. (2007). Future extreme events in European climate: An exploration of regional climate model projections. *Climatic Change, 81(1),* 71–95. DOI 10.1007/s10584-006-9226-z.
3. Jung, C., Müller, A. E. (2009). Flowering time control and applications in plant breeding. *Trends in Plant Science, 14(10),* 563–573. DOI 10.1016/j.tplants.2009.07.005.
4. Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T. et al. (2003). Positional cloning of the wheat vernalization gene *VRN1*. *PNAS, 100(10),* 6263–6268. DOI 10.1073/pnas.0937399100.
5. Yan, L. L., Loukoianov, A., Blechl, A., Tranquilli, G., Ramakrishna, W. et al. (2004). The wheat *VRN2* gene is a flowering repressor downregulated by vernalization. *Science, 303(5664),* 1640–1644. DOI 10.1126/science.1094305.
6. Yan, L., Fu, D., Li, C., Blechl, A., Tranquilli, G. et al. (2006). The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. *PNAS, 103(51),* 19581–19586. DOI 10.1073/pnas.0607142103.
7. Li, C., Dubcovsky, J. (2008). Wheat *FT* protein regulates *VRN1* transcription through interactions with *FDL2*. *Plant Journal, 55(4),* 543–554. DOI 10.1111/j.1365-313X.2008.03526.x.
8. Yoshida, T., Nishida, H., Zhu, J., Nitcher, R., Distelfeld, A. (2010). *Vrn-D4* is a vernalization gene located on the centromeric region of chromosome 5D in hexaploid wheat. *Theoretical and Applied Genetics, 120(3),* 543–552. DOI 10.1007/s00122-009-1174-3.
9. Kippes, N., Debernardi, J. M., Vasquez-Gross, H. A., Akpinar, B. A., Budak, H. et al. (2015). Identification of the *VERNALIZATION 4* gene reveals the origin of spring growth habit in ancient wheats from South Asia. *PNAS, 112(39),* E5401–E5410. DOI 10.1073/pnas.1514883112.
10. Beales, J., Turner, A., Griffiths, S., Snape, J. W., Laurie, D. A. (2007). A pseudo-response regulator is misexpressed in the photoperiod insensitive *Ppd-D1a* mutant of wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics, 115(5),* 721–733. DOI 10.1007/s00122-007-0603-4.
11. Distelfeld, A., Li, C., Dubcovsky, J. (2009). Regulation of flowering in temperate cereals. *Current Opinion in Plant Biology, 12(2),* 178–184. DOI 10.1016/j.pbi.2008.12.010.
12. Dhillon, T., Pearce, S. P., Stockinger, E. J., Distelfeld, A., Li, C. X. (2010). Regulation of freezing tolerance and flowering in temperate cereals: The *VRN-1* connection. *Plant Physiology, 153(4),* 1846–1858. DOI 10.1104/pp.110.159079.
13. Nishida, H., Yoshida, T., Kawakami, K., Fujita, M., Long, B. (2013). Structural variation in the 5′ upstream region of photoperiod-insensitive alleles *Ppd-A1a* and *Ppd-B1a* identified in hexaploid wheat (*Triticum aestivum* L.), and their effect on heading time. *Molecular Breeding, 31(1),* 27–37. DOI 10.1007/s11032-012-9765-0.
14. Sun, H., Guo, Z. A., Gao, L. F., Zhao, G. Y., Zhang, W. P. et al. (2014). DNA methylation pattern of *Photoperiod-B1* is associated with photoperiod insensitivity in wheat (*Triticum aestivum*). *New Phytologist, 204(3),* 682–692. DOI 10.1111/nph.12948.
15. Worland, A. J. (1996). The influence of flowering time genes on environmental adaptability in European wheats. *Euphytica, 89(1),* 49–57. DOI 10.1007/BF00015718.

16. Slafer, G. A. (1996). Differences in phasic development rate amongst wheat cultivars independent of responses to photoperiod and vernalization. A viewpoint of the intrinsic earliness hypothesis. *The Journal of Agricultural Science, 126(4),* 403–419. DOI 10.1017/S0021859600075493.
17. Reif, J. C., Maurer, H. P., Korzun, V., Ebmeyer, E., Miedaner, T. et al. (2011). Mapping QTLs with main and epistatic effects underlying grain yield and heading time in soft winter wheat. *Theoretical and Applied Genetics, 123(2),* 283–292. DOI 10.1007/s00122-011-1583-y.
18. Rousset, M., Bonnin, I., Remoue, C., Falque, M., Rhone, B. et al. (2011). Deciphering the genetics of flowering time by an association study on candidate genes in bread wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics, 123(6),* 907–926. DOI 10.1007/s00122-011-1636-2.
19. Bennett, D., Izanloo, A., Edwards, J., Kuchel, H., Chalmers, K. et al. (2012). Identification of novel quantitative trait loci for days to ear emergence and flag leaf glaucousness in a bread wheat (*Triticum aestivum* L.) population adapted to Southern Australian conditions. *Theoretical and Applied Genetics, 124(4),* 697–711. DOI 10.1007/s00122-011-1740-3.
20. Le Gouis, J., Bordes, J., Ravel, C., Heumez, E., Faure, S. et al. (2012). Genome-wide association analysis to identify chromosomal regions determining components of earliness in wheat. *Theoretical and Applied Genetics, 124(3),* 597–611. DOI 10.1007/s00122-011-1732-3.
21. Gawroński, P., Ariyadasa, R., Himmelbach, A., Poursarebani, N., Kilian, B. et al. (2014). A distorted circadian clock causes early flowering and temperature-dependent variation in spike development in the *Eps-3A$^m$* mutant of einkorn wheat. *Genetics, 196(4),* 1253–1261. DOI 10.1534/genetics.113.158444.
22. Zikhali, M., Wingen, L. U., Griffiths, S. (2016). Delimitation of the *Earliness per se D1* (*Eps-D1*) flowering gene to a subtelomeric chromosomal deletion in bread wheat (*Triticum aestivum*). *Journal of Experimental Botany, 67(1),* 287–299. DOI 10.1093/jxb/erv458.
23. Fan, M., Miao, F., Jia, H. Y., Li, G. Q., Powers, C. et al. (2021). O-Linked N-acetylglucosamine transferase is involved in fine regulation of flowering time in winter wheat. *Nature Communications, 12(1),* 2303. DOI 10.1038/s41467-021-22564-8.
24. Zhao, X. Y., Hong, P., Wu, J. Y., Chen, X. B., Ye, X. G. et al. (2016). The tae-miR408-mediated control of *TaTOC1* genes transcription is required for the regulation of heading time in wheat. *Plant Physiology, 170(3),* 1578–1594. DOI 10.1104/pp.15.01216.
25. Liu, H., Li, T., Wang, Y. M., Zheng, J., Li, H. F. et al. (2019). *TaZIM-A1* negatively regulates flowering time in common wheat (*Triticum aestivum* L.). *Journal of Integrative Plant Biology, 61(3),* 359–376. DOI 10.1111/jipb.12720.
26. Zhang, L., Zhang, H., Qiao, L. Y., Miao, L. F., Yan, D. et al. (2021). Wheat MADS-box gene *TaSEP3-D1* negatively regulates heading date. *The Crop Journal, 9(5),* 1115–1123. DOI 10.1016/j.cj.2020.12.007.
27. Xie, L., Zhang, Y., Wang, K., Luo, X. M., Xu, D. A. et al. (2021). TaVrt2, an SVP-like gene, cooperates with TaVrn1 to regulate vernalization-induced flowering in wheat. *New Phytologist, 231(2),* 834–848. DOI 10.1111/nph.16339.
28. Hicks, K. A., Millar, A. J., Carre, I. A., Somers, D. E., Straume, M. et al. (1996). Conditional circadian dysfunction of the Arabidopsis early-flowering 3 mutant. *Science, 274(5288),* 790–792. DOI 10.1126/science.274.5288.790.
29. McWatters, H. G., Bastow, R. M., Hall, A., Millar, A. J. (2000). The *ELF3* zeitnehmer regulates light signaling to the circadian clock. *Nature, 408(6813),* 716–720. DOI 10.1038/35047079.
30. Covington, M. F., Panda, S., Liu, X. L., Strayer, C. A., Wagner, D. R. et al. (2001). *ELF3* modulates resetting of the circadian clock in Arabidopsis. *Plant Cell, 13(6),* 1305–1315. DOI 10.1105/TPC.000561.
31. Kim, W. Y., Hicks, K. A., Somers, D. E. (2005). Independent roles for *EARLY FLOWERING 3* and *ZEITLUPE* in the control of circadian timing, hypocotyl length, and flowering time. *Plant Physiology, 139(3),* 1557–1569. DOI 10.1104/pp.105.067173.
32. Yoshida, R., Fekih, R., Fujiwara, S., Oda, A., Miyata, K. et al. (2009). Possible role of *EARLY FLOWERING 3* (*ELF3*) in clock-dependent floral regulation by *SHORT VEGETATIVE PHASE* (*SVP*) in *Arabidopsis thaliana*. *New Phytologist, 182(4),* 838–850. DOI 10.1111/j.1469-8137.2009.02809.x.

33. Thines, B., Harmon, F. G. (2010). Ambient temperature response establishes *ELF3* as a required component of the core Arabidopsis circadian clock. *PNAS, 107(7),* 3257–3262. DOI 10.1073/pnas.0911006107.
34. Boden, S. A., Weiss, D., Ross, J. J., Davies, N. W., Trevaskis, B. et al. (2014). *EARLY FLOWERING3* regulates flowering in spring barley by mediating gibberellin production and *FLOWERING LOCUS T* expression. *Plant Cell, 26(4),* 1557–1569. DOI 10.1105/tpc.114.123794.
35. Zhu, C. M., Peng, Q., Fu, D. B., Zhuang, D. X., Yu, Y. M. et al. (2018). The E3 ubiquitin ligase HAF1 modulates circadian accumulation of *EARLY FLOWERING 3* to control heading date in rice under long-day conditions. *Plant Cell, 30(10),* 2352–2367. DOI 10.1105/tpc.18.00653.
36. Zhao, J. M., Huang, X., Ouyang, X. H., Chen, W. L., Du, A. P. et al. (2012). *OsELF3-1*, an ortholog of *Arabidopsis EARLY FLOWERING* 3, regulates rice circadian rhythm and photoperiodic flowering. *PLoS One, 7(8),* e43705. DOI 10.1371/journal.pone.0043705.
37. Faure, S., Turner, A. S., Gruszka, D., Christodoulou, V., Davis, S. J. et al. (2012). Mutation at the circadian clock gene *EARLY MATURITY* 8 adapts domesticated barley (*Hordeum vulgare*) to short growing seasons. *PNAS, 109(21),* 8328–8333. DOI 10.1073/pnas.1120496109.
38. Zakhrabekova, S., Gough, S. P., Braumann, I., Muller, A. H., Lundqvist, J. et al. (2012). Induced mutations in circadian clock regulator Mat-a facilitated short-season adaptation and range extension in cultivated barley. *PNAS, 109(11),* 4326–4331. DOI 10.1073/pnas.1113009109.
39. Wang, J. P., Wen, W., Hanif, M., Xia, X. C., Wang, H. G. et al. (2016). *TaELF3-1DL*, a homolog of *ELF3*, is associated with heading date in bread wheat. *Molecular Breeding, 36(12),* 161. DOI 10.1007/s11032-016-0585-5.
40. Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25(4),* 402–408. DOI 10.1006/meth.2001.1262.
41. Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A., Allard, R. W. (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. *PNAS, 81(24),* 8014–8018. DOI 10.1073/pnas.81.24.8014.
42. Hicks, K. A., Albertson, T. M., Wagner, D. R. (2001). *EARLY FLOWERING 3* encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. *Plant Cell, 13(6),* 1281–1292. DOI 10.1105/tpc.13.6.1281.
43. Kardailsky, I., Shukla, V. K., Ahn, J. H., Dagenais, N., Christensen, S. K. et al. (1999). Activation tagging of the floral inducer *FT*. *Science, 286(5446),* 1962–1965. DOI 10.1126/science.286.5446.1962.
44. Samach, A., Onouchi, H., Gold, S. E., Ditta, G. S., Schwarz-Sommer, Z. et al. (2000). Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. *Science, 288(5471),* 1613–1616. DOI 10.1126/science.288.5471.1613.
45. Weller, J. L., Liew, L. C., Hecht, V. F., Rajandran, V., Laurie, R. E. et al. (2012). A conserved molecular basis for photoperiod adaptation in two temperate legumes. *PNAS, 109(51),* 21158–21163. DOI 10.1073/pnas.1207943110.