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RESEARCH PAPER

Gene Expression Analysis of the Early Flowering 6 Homologues in Apricot Reveals Their Potential Role in Developmental Stages

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Abstract

In higher plants, regulation of gene expression and chromatin formation occurs by histone methylation and demethylation. Genes encoding JmjC-JmjN domains belong to the histone demethylase family and have an important role in the regulation of plant growth and development. *Early Flowering 6* (*AtELF6*), which encodes the JmjC-JmjN domain in *Arabidopsis thaliana*, is a demethylase that regulates growth and development as well as the transition to flowering, but it has not been identified in apricot so far. In this study, two genes homologous to *AtELF6* were identified for the first time in apricot. Gene expression analysis by RT-qPCR revealed that both *ELF6* homologs were expressed in 12 different developmental stages of three different tissues. The fact that both homologues were expressed, especially in the flower bud, suggested that they play a role in the transition to flowering, similar to *Arabidopsis thaliana*. In summary, the information obtained from this study will provide a unique resource for understanding the role of *ELF 6* in apricot growth and development, as well as for future functional characterization studies for the manipulation of the flowering transition.

1. Introduction

Epigenetic is change that represent enzymatically reversible modifications of gene expression without any changes in DNA sequences and can be passed on to future generations (Akimoto et al., 2007). Epigenetic mechanisms are classified as non-coding RNA editing, covalent modification of histones, nucleosome restructuring and DNA methylation (Allis and Jenuwein, 2016). The epigenetic mechanism activates or inhibits transcription by modifying histone proteins, regulating chromatin state, or directly recruiting specific effector proteins (Stricker et al., 2017). In plants, histone proteins undergo eight different epigenetic changes: methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deamination and proline isomerization (Berger, 2007). Plants control their growth and development by spatiotemporally regulating gene expression with these epigenetic mechanisms (Yamaguchi, 2022).

Histone demethylation is one of the most complex epigenetic mechanisms that regulate plant development from flowering to fruiting and resistance to biotic and abiotic stresses (Klose and Zhang, 2007). Histone demethylation is a reversible process organized by demethylase (He et al., 2021). There are two classes of histone lysine demethylases in eukaryotes, Lysine-specific demethylase 1 (LSD1) and Jumonji C domain-containing demethylases (JMJ-C), and they are highly conserved in plants, yeast, and humans (Lan et al., 2008). Histone lysine demethylases (“erasers”) dynamically regulate methylation levels at Lys4 (K4), Lys9 (K9), Lys27 (K27), and Lys36 (K36) of histone H3 in *Arabidopsis thaliana* (Liu et al., 2010). In general, histone H3K9 and H3K27

methylation is characterized by transcriptionally silenced regions, and H3K4 and H3K36 methylation is characterized by active genes (Berger, 2007). Different studies have shown that H3K27me3 represses flower development genes in *A. thaliana* seedlings and must be reactivated by the addition of active chromatin marks for mature *Arabidopsis* plants to switch to flowering (Wang et al., 2016; Pfluger and Wagner, 2007). Among the H3K27me3 demethylases identified to date, three genes that have been shown to regulate floral development stand out: *Early Flowering 6* (*ELF6*)/*JMJ11*, *Relative of ELF6* (*REF6*)/*JMJ12* and *JMJ13* (Keyzor et al., 2021; Yamaguchi, 2022). Although changes in flower morphology have been observed in *elf6 jmj13 ref6* triple mutants in *A. thaliana*, the function of each demethylase in the control of flower development and self-pollination is not yet known (Yan et al., 2018). *AtELF6* and its closest homolog *AtREF6* encode nuclear proteins with JmjC, JmjN and zinc finger (ZnF) domains and are critical regulators of flowering time in *A. thaliana* (Lee et al., 2005; Metzger et al., 2005). In *A. thaliana*, ELF6 and JMJ13 are in an antagonistic relationship during the flower development stage. Compared to the wild type, increased self-fertility was observed in *elf6* mutants, while self-fertility was decreased in *jmj13* mutants (Keyzor et al., 2021). In addition, JMJ13 promotes stamen growth by activating the expression of *SAUR26*, while jasmonic acid suppresses carpel growth by activating signaling. Based on transcription data in *A. thaliana*, *ELF6* has been shown to promote carpel elongation by activating expansin genes (Yamaguchi, 2022; Keyzor et al., 2021). In angiosperms, *FLOWERING LOCUS T* (*FT*) is the key gene for the transition to flowering (Kobayashi et al., 1999), and *ELF6* delays flowering by demethylation of histones in the region where *FT* initiates transcription (Jeong et al., 2009). *BraELF6*, an *AtELF6* homolog in *Brassica rapa*, was cloned and functionally characterized by transferring it into *Arabidopsis elf6-4* mutants. The expression of *BraELF6* in T1 plants was relatively higher in transgenic plants, and later flowering was observed than in *elf6-4* mutants (Li et al., 2019).

Apricot, a member of the *Prunus* genus within the highly diverse *Rosaceae* family, is one of the most delicious and commercially traded fruits in the world (Erdogan-Orhan and Kartal, 2011). Based on data from the Food and Agriculture Organization Institutional Statistics Database, global apricot production in 2022 was determined to be 3,863,180 metric tons, up from 3,622,553 tons in 2021, with an increase of 6.6% (FAOSTAT, 2024). In addition to being consumed as a fruit, this type of drupe is also widely used for edible, cosmetic and medicinal purposes (Shi et al., 2023). However, late spring frosts severely damage the plant due to its short dormancy period and early flowering. In recent years, in order to solve this problem, the strategy of investigating key genes related to flower bud dormancy, control of flowering time and cold tolerance and determining their expression patterns has come to the fore. To date, although *ELF6* has been identified in model plants, especially *Arabidopsis*, and its relationship with flowering has been demonstrated, it has not yet been identified in the apricot and its possible roles have not been revealed. In this study, apricot homologues of *AtELF6*, which were shown to be involved in the epigenetic control of flowering, were identified by bioinformatics methods, and their expression patterns in 12 different developmental stages were analyzed. In addition to understand the possible roles of *ELF6* in apricot, this study will be a unique resource for future functional characterization studies to create later and/or earlier flowering apricot lines with genome editing techniques.

2. Material and Methods

2.1. Identification of *ELF6* genes in apricot

To identify ELF6 homologs in apricot, JmjC (PFAM02373) and JmjN (PFAM02375) domains were downloaded from the InterPro database (https://www.ebi.ac.uk/interpro/). Then, using the amino acid sequences of the JmjC and JmjN domains as queries, putative PaELF6 proteins were identified from the *Prunus armeniaca* genome database (https://www.rosaceae.org/) (Jung et al., 2019) by BLAST (Camacho et al., 2009). Additionally, the obtained proteins were re-examined using InterPro (https://www.ebi.ac.uk/interpro/) and SMART (http://smart.embl-heidelberg.de/) with an E value cut-off level of 1.0 and 5.0, respectively. After eliminating low similarity sequences, PARG18718m01 and PARG18718m02 sequences were identified AtELF6 homologues. These identified homologs were named *ParELF6-1* and *ParELF6-2*, respectively.

2.2. Plant materials and sample collection

In this study, 12-year-old apricot trees (*Prunus armeniaca* L. cultivar Sekerpare) that were fully productive and grown within the scope of standard horticultural practices in the garden of Burdur Mehmet Akif Ersoy University (Burdur, Türkiye) (latitude: 37° 41′ 27.4″; longitude: 30° 20′ 35.42″) were used. Tissues used in gene expression studies were collected in the following order in 2023; flower bud, leaf bud, young leaf (2 cm diameter), mature leaf (5 cm diameter), flower organs such as sepals, petals, stamens, carpels, young fruit (30 DAB), large green fruit (45 DAB), breaker fruit (60 DAB), mature fruit (75 DAB) and separated and frozen immediately in liquid nitrogen, and stored at -80°C until used. Each sample consisted of three biological replicates from three different trees, and each biological replicate contained at least three samples collected from one tree.

2.3. RNA extraction and RT-qPCR analysis

In this study, total RNA of developmental stages was isolated by a Plant/Fungi Total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturers' instructions. Tissues from which total RNA was isolated were treated with RNase-Free DNase I (Norgen Biotek Corp., Thorold, ON, Canada) to eliminate DNA contamination. Total RNA quality was measured with microplate spectrophotometer (Epoch Microplate Spectrophotometer, Biotek Instruments, Inc.) for A260/A280 ratio and visualized by agarose gel electrophoresis. For cDNA synthesis, 1 µg RNA was reverse-transcribed using the VitaScript™ FirstStrand cDNA Synthesis Kit (Procomcure Biotech) according to the manufacturer's procedure. The RT-qPCR system contained 5 µl iTaq Universal SYBR Green Super Mix (Bio-Rad Laboratories, Hercules, CA, USA), 1 µl forward and reverse primers, 4.0 µl cDNA and 4.0 µl deionized water in a final volume of 10 µl.

The RT-qPCR procedure was devised as follows: 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 40 s. The apricot *Translation Elongation Factor 2* (*TEF2*) (Tong et al., 2009) and *Actin* (*ACT*) (Niu et al., 2014) genes were employed as internal controls for normalizing the transcript level of the target gene among different samples. Three biological replicates were performed for each gene, with each biological repeat having three technical replicates. The $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001) was employed to calculate the relative expression levels of *ParELF6* genes. The primers for RT-qPCR analysis were designed using Primer-BLAST and showed Table 1. (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

2.4. Statistical analysis

In this study, samples were taken at different developmental stages, following a randomized plot design involving three replications, and were examined using a general linear model. In significant cases, comparisons were made using the Fisher (LSD) test ($p < 0.05$). The error bars in the figures represents the standard error. Variance analysis and comparison tests were conducted using the statistical package program XLSTAT (v.2016.02.28451, Addinsoft, France). The graphs were created using the ggplot2 (Gómez-Rubio, 2017) and openxlsx packages in the R programming language, utilizing the RStudio platform (v.2023.12.1.402, Posit Software, PBC, Boston, MA) (Allaire, 2011).

3. Results and Discussion

RT-qPCR is a technique developed by combining PCR with fluorescence techniques and is widely used to analyze gene expression (Gibson et al., 1996; Tichopad et al., 2003). It is used to examine transcript levels in basic research due to its sensitivity, specificity, wide dynamic range and high throughput capacity (Karuppaiya et al., 2017). In this study, to reveal the possible functional roles of *ELF6* homologues in apricot, they were analyzed via RT-qPCR in 12 different developmental stages of leaves, flowers and fruits. As shown in Figure 1, it has been determined that the expression of *ParELF6* genes varies at different levels in leaves, flowers and fruits. Two of the three different leaves developmental stages showed similarities in *ParELF6* genes, but at the mature leaf stage, *ParELF6-2* showed higher expression. In flower bud and floral organs, both genes showed a decreasing pattern, except that *ParELF6-2* was higher in stamens. It was found that the expression of *ParELF6* genes gradually decreased in contrast to fruit ripening. Expression data analysis indicated that great differences in expression of *PaELF6* homologs in leaf, flower and fruit developmental stages, indicating that these genes participate in different developmental stages and may have important effects in adapting to physiological processes. To summarize, the high variance in the expression levels of these two *ELF6* homologs identified in apricot at 12 different developmental stages implied that these genes have more than one potential function in apricot development (Figure 1).

Li et al. (2019) measured the expression of the BraELF6 gene in four different organs at three different developmental stages (four-leaf seedling plant, bud stage, and flowering stage) in *Brassica rapa*. Accordingly, the expression of the BraELF6 gene gradually increased in the stem, leaf and flower during three developmental stages, while it remained constant in the roots. While BraELF6 was expressed three times higher in flowers than in roots

Table 1. Primer sequences specific to the *ParELF6* genes used in this study.

Primer name	Primer sequence (5'-3')
ParELF6-1-F	GAGGCTCAGAGTCCCCCATC
ParELF6-1-R	AGTGATCAGGGCTTTGGAGAGG
ParELF6-2-F	GACCGCCTAGGTCCAGTTTC
ParELF6-2-R	TGGCAGCTTCCCCACAGTTA
TRANSLATION ELONGATION FACTOR 2-F	GGTGTGACGATGAAGAGTGATG
TRANSLATION ELONGATION FACTOR 2-R	TGAAGGAGAGGGAAGGTGAAAG
ACTIN-F	GTTATTCTTCATCGGCGTCTTCG
ACTIN-R	CTTCACCATTCCAGTTCCATTGTC

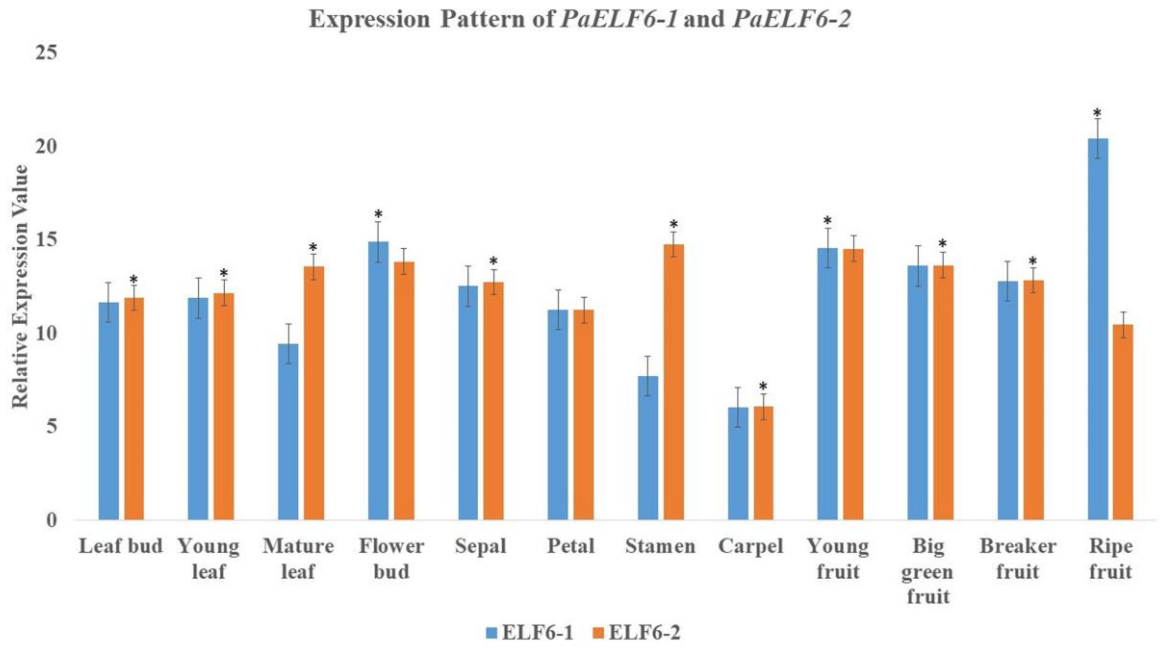

Figure 1. The expression profiles of *ParELF6* genes at different developmental stages analyzed by RT-qPCR.
Bars represent the mean of replicates ± standard error and indicate a significant difference at $P < 0.05$, determined by Fisher (LSD) test. Leaf, flower and fruit development stages are shown with green, red and orange bars, respectively.

at the flowering stage, its expression in stems was found to be similar to that in leaves. It was observed that the expression of BraELF6 was at its highest level in the floral tissue at three growth stages and increased gradually. There was an 11-fold higher expression of BraELF6 in floral tissue at the flowering stage than in roots, which suggested by the authors that the BraELF6 gene in *Brassica rapa* is associated with flowering. These differences in expression between apricot and *Brassica rapa* can be explained by the fact that both species belong to different families and have different growth patterns.

Transcriptomic is defined as a snapshot of gene expression in a specific tissue, provided by capturing the total RNA within that tissue at a specific moment in time, and is an important analysis for understanding plant growth and development (Ward et al., 2012). According to transcriptome data, ELF6 is expressed at 47 different developmental stages in *A. thaliana*, with the highest expression in the dry seed and the lowest in the vegetative rosette stages (Winter et al., 2007). In *Solanum lycopersicum* cv. Heinz, the expression of ELF6 peaked in the root and fully opened flower. Conversely, in *Solanum pimpinellifolium*, the expression is the highest on the fifth day of breaker fruits and in the leaf (Shi et al., 2012). In heterozygous diploid *Solanum tuberosum*, ELF6 is expressed in the root, tuber and shoot apex, from high to low, respectively (Massa et al., 2011). In *Oryza sativa*, ELF6 is expressed highest in the young flowering stage and lowest in the flowering stage (Jain et al., 2007). To summarize, information from both RT-qPCR and transcriptome data reveals that ELF6 is an important actor of growth and development in the plant kingdom. However, further functional characterization studies, such as mutant or gene editing, are needed to validate the information obtained from both RT-qPCR and transcriptome studies.

4. Conclusion

In this study, ELF6 gene homologues, which play a critical role in the epigenetic regulation of the flowering transition in *Arabidopsis thaliana*, were identified for the first time in apricot. Expression patterns were revealed by RT-qPCR in 12 different developmental stages of three different organs of apricot. According to the analysis, both ELF6 homologs, ParELF6-1 and ParELF6-2, showed strong expression at all developmental stages. The information obtained from this study will be a unique resource for understanding the epigenetic regulation of apricot flowering and for obtaining early or late flowering cultivars in the future by genome editing methods such as CRISPR.

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