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ARTICLE

Photoperiod and ambient temperature control flowering time of cultivated oat genotypes

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Abstract: *Flowering time is a crucial step in the adaptation of annual crops such as oat. The main objectives of this work were to characterize flowering time in oat genotypes of different origins in field experiments, to isolate oat sequences of the flowering regulator gene ELF3, and to analyze the level of ELF3 expression in different oat genotypes. Field data showed that oat genotypes perceived and responded differently to changes in photoperiod and ambient temperature. Oat sequences with high identity to the circadian clock gene ELF3 were successfully cloned and sequenced. The level of ELF3 expression allowed differentiation between early and late flowering oat genotypes. These results support the involvement of ELF3 in the regulation of flowering time in hexaploid oat. Understanding the mechanisms that control flowering time in response to seasonal photoperiod and temperature cues will help to improve oat varieties with superior performance and resilience to climate change.*

Keywords: *Flowering time, hexaploid oat, day length, ELF3, gene expression*

INTRODUCTION

Oat (*Avena sativa* L.) is an important crop species, used both for grain and fodder. Oat has a large and complex allohexaploid genome (2*n* = 6x = 42, AACCDD) and belongs to the Poaceae family. Flowering time is a critical agronomic trait for the adaptation of oat genotypes to new growing areas worldwide and is a key factor in oat crop success. Flowering time must occur under optimal conditions to maximize fertilization, seed development and productivity. In subtropical regions, farmers typically grow spring oat varieties, and flowering time is mainly influenced by genotype-specific responses to seasonal photoperiod and ambient growth temperature (Mazurkievicz et al. 2019). In these regions, oat varieties with vernalization requirements may flower too late because natural vernalization temperatures may not meet these requirements (Ubert and Nava 2024).

Oat is a long day crop species and earlier flowering is observed in most genotypes grown under longer photoperiod conditions (Sorrells and Simmons 1992). Photoperiod defines the length of the light phase in a 24-hour period. The photoperiod pathway in annual plants is regulated by a similar network of genes that respond to changes in the length of the day (light phase) and night (dark phase) cycles (Osnato et al. 2022). Among the many genes that have been studied and characterized in *Arabidopsis thaliana*, *CONSTANS* (*CO*) is one of the most important regulators of flowering time in response to photoperiod (Wang et al. 2024). Under short days, *CO* expression peaks at night, leading to protein degradation and later flowering. Under long days, *CO* expression peaks

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during the day and light-mediated stabilization of the CO protein promotes earlier flowering (Valverde et al. 2004). The *CO* gene encodes a transcription factor and its activity is regulated at the transcriptional and post-transcriptional levels by several proteins associated with the circadian clock. In addition, CO also induces the expression of the *FLOWERING LOCUS T* (*FT*) gene in leaves. *FT* is a floral promoter and an orthologue of the *VERNALIZATION 3* (*VRN3*) gene in wheat and barley (Osnato et al. 2022).

Flowering time is also regulated by many other clock genes, including *EARLY FLOWERING 3* (*ELF3*), *PSEUDO RESPONSE REGULATORS* (*PRRs*) and *GIGANTEA* (*GI*). *ELF3*, *EARLY FLOWERING 4* (*ELF4*) and *LUX ARRHYTHMO* (*LUX*) are components of the Evening Complex (EC) in the plant circadian clock (Maple et al. 2024). In wheat, loss-of-function mutations in the *ELF3* gene result in early flowering under both long and short days, suggesting that the EC is a critical transcriptional repressor of flowering time (Alvarez et al. 2023). *ELF3* also links temperature and light signaling pathways, allowing plants to synchronize their physiological activities with daily and seasonal cycles (Wang et al. 2024). Recently, *ELF3* was found to repress *PHOTOPERIOD 1* (*PPD1*, also known as *PRR37*), a key gene involved in photoperiodic responses in wheat. PPD1 promotes flowering by increasing the *VRN3* gene expression under long days (Distelfeld et al. 2009, Alvarez et al. 2023). In addition to seasonal changes in day length, ambient growth temperatures also influence flowering time. However, the genetic and molecular mechanisms by which plants sense and respond to changes in ambient temperature for optimal flowering time regulation remain unclear. Further research is needed to understand how the circadian clock and ambient temperature pathways control flowering time, which is essential for the development of new oat varieties adapted to an ever-changing environment. The main objectives of this work were to characterize flowering time in oat genotypes of different origins in field experiments, to isolate oat sequences of the flowering regulator gene *ELF3*, and to analyze the level of *ELF3* expression in different oat genotypes.

MATERIALS AND METHODS

Plant materials

Oat genotypes developed in different breeding programs were evaluated in field experiments for flowering time. The oat genotypes included in this study were FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura. The FL0206B-S-B-S1 and Leggett genotypes are from North America and were developed at the University of Florida, USA, and Agriculture and Agri-Food Canada, Canada, respectively. The UFRGS 078030-1 and URS Taura genotypes are both from Brazil and were developed at the Federal University of Rio Grande do Sul (UFRGS). The genealogy of each genotype is shown in parentheses as follows: FL0206B-S-B-S1 (UFRGS 995088-3/LA9535D118-4), Leggett (OT294/Pc94), UFRGS 078030-1 (UPF 93203-4/FL98061F_1(IL90-1847/LA90122-BBB-7-1-1)) and URS Taura (UFRGS 970216-2/UFRGS 970461).

Evaluation of flowering time under field conditions

The oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura were evaluated for flowering time in field experiments conducted in 2016, 2017 and 2018 at early, intermediate and late planting dates at the Agronomy Experimental Station of UFRGS, Brazil (lat 30º 05' 47.50" S, long 51º 40' 26" W, alt 46 m asl). In 2016, the early, intermediate and late planting dates were 28 June, 15 July and 27 July, respectively. Fifteen plants of each oat genotype within each planting date were grown in hill plots. In 2017 and 2018, the early planting dates were 27 and 29 June, the intermediate planting date was 13 July in both years, and the late planting dates were 28 and 27 July. Fifteen plants of each oat genotype within each planting date were sown in one meter rows. The experiments were conducted in a randomized complete block design with four replications in each year and planting date. Flowering time was estimated at stage 55 of the Zadoks scale. Response to late planting was estimated by the difference in the number of days to flowering in late planting versus early planting for the oat genotypes and years. Temperature sum from emergence to flowering was estimated for each genotype and planting date. Analysis of variance and comparison of means using the Tukey test ($p < 0.05$) were performed using the R software (www.r-project.org). Mean daily temperatures and photoperiod data were obtained from a weather station at the UFRGS Agronomy Experimental Station and from Time and Date (https://www.timeanddate.com/sun/@6317055), respectively.

DNA extraction

Seeds from a single panicle of oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura were

germinated using a biochemical oxygen demand chamber. Coleoptile tissues were harvested, frozen in liquid nitrogen, ground and stored at -20 °C. DNA extraction was performed following the protocol reported by Pellizaro et al. (2016). The concentration and quality of extracted DNA were analyzed using 1% agarose gels, a Genesys™ spectrophotometer and a Qubit 3.0 fluorometer (Thermo Fisher Scientific).

Cloning of *ELF3* in cultivated oat

Genomic sequences of the *ELF3* gene from different grass species were first obtained from the National Center for Biotechnology Information (NCBI) website. Sequences from wheat, barley and *Brachypodium distachyon* were aligned to identify conserved regions of the *ELF3* gene. Primer pairs were designed and were then used to amplify the *ELF3* gene in oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1, and URS Taura. Three primer pairs were used: ELF3-3 (F: 5'-CCACCCAGCTTAAGAGCAAG-3' & R: 5'-TTAGGCCTTTCTCCGACAGA-3'), ELF3-5 (F: 5'-TTGCTGTCCAAGTGTTCGAG-3' & R: 5'-AACAACTGGTGTGCTCCTCC-3') and ELF3-6 (F: 5'-CCTCAGAATCAGTGGCTCGT-3' & R: 5'-GTTCTGCTGCCTCTCCATC-3'). Polymerase chain reactions (PCR) were performed using a C1000 Touch™ thermal cycler (Bio-Rad Laboratories). The PCR conditions used in this study included an initial denaturation step at 94 °C for 3 minutes; followed by 40 cycles of 30 seconds at 94 °C, 45 seconds varying from 55 °C to 65 °C depending on the best annealing temperature of each primer pair, and 2 minutes at 72 °C; followed by 10 minutes at 72 °C and then holding at 4 °C. PCRs were performed using 15 µL containing 30 ng of DNA, 120 nM of the forward and reverse primers, 0.9 mM of $MgCl_2$, 120 µM dNTPs, 1.5 U of Taq DNA polymerase (Invitrogen) and 1X PCR buffer. The PCR products from ELF3-3, ELF3-5 and ELF3-6 primers were visualized on 2% agarose gels stained with ethidium bromide. The amplified DNA fragments were ligated into the PCR™ 2.1-TOPO vector (Invitrogen) and transformed into *Escherichia coli* cells by the heat shock method. Sequencing reactions were carried out according to Zimmer et al. (2017). Oat fragments were sequenced on an ABI 3500 Genetic Analyzer (Applied Biosystems).

In silico comparative analysis

The cloned oat sequences obtained in this study were compared with known genes using a Basic Local Alignment Search Tool (BLAST) from NCBI and GrainGenes, a database for Triticeae and Avena. Oat sequences were searched by BLAST against the NCBI core nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Oat sequences were also searched by BLAST against GrainGenes using the oat reference genomes PepsiCo OT3098 Hexaploid Oat v2 pseudomolecules and the *Avena sativa* Oat Sang v1 pseudomolecules (Kamal et al. 2022, https://wheat.pw.usda.gov/blast). The Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0, developed by Kumar et al. (2016), was used for sequence alignment, comparison, and translation of nucleotide sequences in the gene exons to amino acid sequences.

Analysis of relative gene expression

The level of *ELF3* expression was analyzed in the oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura. Plants of each genotype were grown in a BDW 40 growth chamber (Conviron) under a 12-hour photoperiod at 22 ºC during the day and 17 ºC at night. Leaf samples were collected 14 days after emergence over a 24-hour period, with a four-hour interval between each collection. Time of day (TD) was used as a standardized 24-hour notation of the circadian cycle, with TD zero as the beginning of the light phase and TD 12 as the beginning of the dark phase. TD zero was used as a reference for analyzing the *ELF3* gene expression compared to other times of day in the light (TD 4 and TD 8) and dark (TD 12, TD 8, and TD 4) phases. Total RNA was isolated using the TRIzol method (Thermo Fisher Scientific) and purified with DNase I (Invitrogen). The complementary DNA strand (cDNA) was synthesized using Moloney murine leukaemia virus (M-MuLV, Invitrogen) reverse transcriptase. Real-time quantitative PCR (qPCR) was performed to determine the relative expression of the *ELF3* gene. The primer pair ELF3X (F: 5'-AGGAAGTGGGGATCCAAGAG-3' and R: 5'-ACTTTGGGAGTCCCTTGACC-3') was used. This primer pair was designed from the *in silico* analysis of the results obtained in the cloning and sequencing phase of this study. Fluorescence was read at each amplification cycle using the Applied Biosystems 7300 Fast Real-Time PCR System (Life Technologies). SYBR® Green (Life Technologies) was used as a reporter fluorophore and the 18S subunit of the ribosomal RNA gene was used as an endogenous control in the experiments. All reactions were subjected to the same analysis conditions and normalized to the signal of the ROX reference dye (Invitrogen). The relative expression of the *ELF3* gene was analyzed by interpreting the dissociation curve

according to the method described by Schmittgen and Livak (2008). Differences in the relative expression level of the *ELF3* gene among oat genotypes were analyzed by the standard error using the t-test in the R software, with $p < 0.05$.

RESULTS AND DISCUSSION

Phenotypic data

In this study, flowering time was evaluated in oat genotypes developed in different oat breeding programs in Brazil, Canada and the United States. Oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura were evaluated in field experiments conducted in 2016, 2017 and 2018 at early, intermediate and late planting dates. Small differences in photoperiod were found among years and planting dates, while significant differences in ambient growth temperature were found among years at early, intermediate and late planting dates (data not shown). The highest average number of days to flowering and temperature sum among oat genotypes was observed in 2018, which coincided with the lowest mean daily temperatures recorded among years and planting dates. The Leggett oat genotype consistently had the highest average number of days to flower across years and planting dates. The FL0206B-S-B-S1 genotype flowered earlier than Leggett in all years and planting dates, but later than the Brazilian oat genotypes UFRGS 078030-1 and URS Taura, except for the early 2018 planting, where no significant difference was found between FL0206B-S-B-S1 and URS Taura, and for the late 2018 planting, where no significant difference was found between FL0206B-S-B-S1, UFRGS 078030-1 and URS Taura. UFRGS 078030-1 and URS Taura showed an early phenotype and were very similar in days to flowering, differing significantly only for the early 2016 and 2018 plantings. In addition, Leggett showed no significant difference between early and intermediate planting in 2017. FL0206B-S-B-S1, UFRGS 078030-1 and URS Taura showed no differences between intermediate and late planting in 2018 (Table 1).

Table 1. Phenotypic data for flowering time in oat genotypes evaluated in field experiments in different years and planting dates

Genotype	2016 field trials					
	Early		Intermediate		Late	
	NDFT[a]	TS[b]	NDFT[a]	TS[b]	NDFT[a]	TS[b]
FL0206B-S-B-S1	82 Ba	1279 Ba	71 Bb	1156 Bb	68 Bc	1132 Bb
Leggett	107 Aa	1746 Aa	101 Ab	1730 Ab	97 Ac	1712 Ab
UFRGS 078030-1	74 Da	1146 Da	68 Cb	1111 Cb	61 Cc	1018 Cc
URS Taura	76 Ca	1185 Ca	67 Cb	1096 Cb	61 Cc	1018 Cc
	2017 field trials					
FL0206B-S-B-S1	76 Ba	1378 Ba	72 Bb	1332 Ba	65 Bc	1257 Bb
Leggett	104 Aa	1936 Aa	101 Aa	1913 Aa	89 Ab	1741 Ab
UFRGS 078030-1	65 Ca	1157 Ca	62 Cb	1130 Ca	53 Cc	1013 Cb
URS Taura	67 Ca	1192 Ca	62 Cb	1129 Cb	54 Cc	1034 Cc
	2018 field trials					
FL0206B-S-B-S1	89 Ba	1399 Ba	80 Bb	1300 Ba	75 Bb	1279 Ba
Leggett	122 Aa	2045 Aa	114 Ab	1993 Aa	106 Ac	1924 Aa
UFRGS 078030-1	82 Ca	1288 Ba	72 Cb	1157 Cab	70 Bb	1186 Bb
URS Taura	87 BCa	1365 Ba	73 Cb	1180 BCb	70 Bb	1175 Bb
	Response to late planting[c]					
	2016 (%)		2017 (%)		2018 (%)	
FL0206B-S-B-S1	-14 (17.07) Ba		-11 (14.47) Bb		-14 (15.73) Bab	
Leggett	-10 (09.34) Cb		-15 (14.42) Ba		-16 (13.11) BCa	
UFRGS 078030-1	-13 (15.57) Bb		-12 (18.46) Aa		-12 (14.63) Bb	
URS Taura	-15 (19.74) Aa		-13 (19.40) Aa		-17 (19.54) Aa	

[a] NDFT is the average number of days from emergence to flowering time. [b] TS is the temperature sum from emergence to flowering time, calculated as the difference between the sum of the mean daily temperature and the basal temperature, assumed to be 0 °C for hexaploid oat based on previous studies. Capital letters denote significant differences in flowering time and temperature sum among oat genotypes evaluated in different years and planting dates. Lowercase letters on the same line indicate significant differences in flowering time and temperature sum within the same oat genotype evaluated in different years and planting dates. Means were compared using the Tukey test with $p < 0.05$. [c] Response to late planting indicates variation in flowering time between late and early planting for each oat genotype and year. Capital letters denote significant differences in the percentage response to late planting among oat genotypes evaluated in different years. Lowercase letters on the same line indicate significant differences in response to late planting within the same oat genotype evaluated in different years. Means were compared using the Tukey test with $p < 0.05$.

Response to late planting varied among oat genotypes and years. Late planting resulted in a significant reduction in the number of days to flowering compared to early planting in all oat genotypes and years. Late-planted oat genotypes were exposed to longer photoperiods and higher temperatures, which accelerated the onset of flowering. This response to late planting has been observed in different oat populations in previous studies (Nava et al. 2012, Sunstrum et al. 2019, Ubert and Nava 2024). In our study, the most significant response to late planting was observed for URS Taura in 2016, with an approximately 20% reduction in flowering time. Despite the differences in photoperiod and temperature between early and late planting, URS Taura responded similarly to late planting in all years, demonstrating its high adaptability and stability to environmental conditions in southern Brazil. In contrast, Leggett showed the lowest response to late planting in all years, with an average reduction in flowering time of 12.3%. FL0206B-S-B-S1 and UFRGS 078030-1 exhibited an intermediate response to late planting in all years, with an average reduction in flowering time of 15.76% and 16.22%, respectively (Table 1).

The field data indicated that FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura perceived and responded differently to changes in photoperiod and ambient growth temperature. The Brazilian genotypes UFRGS 078030-1 and URS Taura were less sensitive to photoperiod and more dependent on higher temperatures than the North American genotypes Leggett and FL0206B-S-B-S1. In addition, the late flowering phenotypes observed in Leggett and FL0206B-S-B-S1 suggest that these genotypes have a vernalization requirement that was not fully met in our field experiments. In particular, Leggett is derived from a single cross, containing lines highly adapted to the western Canadian environment. FL0206B-S-B-S1 is also derived from a single cross and although it has a Brazilian line in its genealogy, it is well adapted to the southern United States. The differences in adaptation of the oat genotypes evaluated in this study resulted in a wide range of phenotypes observed for flowering time, indicating genetic variation for key genes regulating this important trait. We propose that one of these genes, *ELF3*, plays a critical role in regulating flowering time in hexaploid oat by linking responses to seasonal photoperiod and ambient growth temperature. Furthermore, because of its important function in regulating flowering time, the *ELF3* gene has been conserved throughout oat evolution.

Oat sequences targeting *ELF3*

DNA sequences associated with the circadian clock gene *ELF3* were isolated in oat genotypes by orthology with other grass species. A total of 17 amplicons targeting the *ELF3* gene in oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1 and URS Taura were generated with primer pairs ELF3-3, ELF3-5 and ELF3-6, then cloned and sequenced. Complete coding sequences of 625 nucleotides were amplified in the oat genotypes using the ELF3-3 primer pair (GenBank accessions PQ241503 to PQ241509). Partial coding sequences of 498 nucleotides were amplified in the oat genotypes using the ELF3-5 primer pair (GenBank accessions PQ241510 to PQ241514). Complete coding sequences of 681 nucleotides were amplified with the ELF3-6 primer pair (GenBank accessions PQ241515 to PQ241519). Based on multiple alignments performed for primer design, ELF3-3 amplified oat sequences from the second exon of the *ELF3* gene. ELF3-5 amplified sequences including a small portion of the third exon of 36 nucleotides, the third intron of 116 nucleotides, and 344 nucleotides at the beginning of the fourth exon of the *ELF3* gene. ELF3-6 amplified sequences from the fourth exon of the *ELF3* gene which do not overlap with the sequences amplified by ELF3-5. Combining the oat sequences amplified with the different primer pairs, approximately 50% of the length of the *ELF3* gene was cloned and sequenced in this study. In cultivated wheat, comparative genomic analysis isolated the *TaELF3* gene located on the long arms of the wheat chromosome group 1, designated *TaELF3-1AL*, *TaELF3-1BL*, and *TaELF3-1DL*, with lengths of 3703, 3721, and 4038 nucleotides, respectively. The larger size of the *TaELF3-1DL* gene is related to the presence of a transposable element of 307 nucleotides in the second intron (Wang et al. 2016).

The oat sequences amplified with primer pairs ELF3-3, ELF3-5 and ELF3-6 were searched by BLAST against the NCBI core nucleotide database. These sequences showed 79-99% similarity to the *ELF3* genes of *Aegilops tauschii*, *T. aestivum*, *Triticum dicoccoides*, *Triticum monococcum*, *Triticum urartu*, *B. distachyon*, *Hordeum vulgare*, *Lolium perenne*, *Lolium rigidum* and *A. sativa*. For *A. sativa*, only one sequence related to the *ELF3* gene was found at NCBI under GenBank accession MK211562.1. Cloned oat sequences were also searched by BLAST against the GrainGenes OT3098 and Sang oat reference genomes. For the OT3098 reference genome, oat sequences amplified with primer pairs ELF3-3, ELF3-5 and ELF3-6 showed 96-99.5% similarity to the ELF3-like protein 2, which is located on oat chromosome 1A. Interestingly, the position of the oat sequences amplified by the ELF3-3 primer pair was also found close to the position of a major QTL

(QHD.TxH-Mrg28.3) affecting flowering time, which was previously mapped in the TX07CS-1948 × Hidalgo population on oat chromosome 1A (Sunstrum et al. 2019). For the Sang reference genome, oat sequences amplified with primer pairs ELF3-3, ELF3-5 and ELF3-6 showed 96-99% similarity to the candidate gene *EARLY FLOWERING 3*, which is located on oat chromosomes 1A and 5D. The identification of oat sequences related to the *ELF3* gene sequences of wheat, barley, *Brachypodium* and other grasses in GenBank, and two oat reference genomes in GrainGenes, confirms the presence of *ELF3* in the hexaploid oat genome. In addition, the close position of the oat sequences to the flowering date QTL in the OT3098 reference genome supports our proposal that *ELF3* is a flowering regulator in hexaploid oat. In wheat and barley, the genomic sequences of the *ELF3* gene are very similar, suggesting a similar function (Wang et al. 2016). *Elf3* mutants were associated with early flowering in wheat, barley and *Brachypodium* under short and long days, suggesting that the function of the *ELF3* gene is conserved in these species and acts as a flowering inhibitor (Alvarez et al. 2023).

The translation of the cloned oat sequences corresponding to the *ELF3* gene exons into amino acid sequences is shown in Figure 1. Single amino acid changes were identified among the oat sequences generated with different primer pairs.

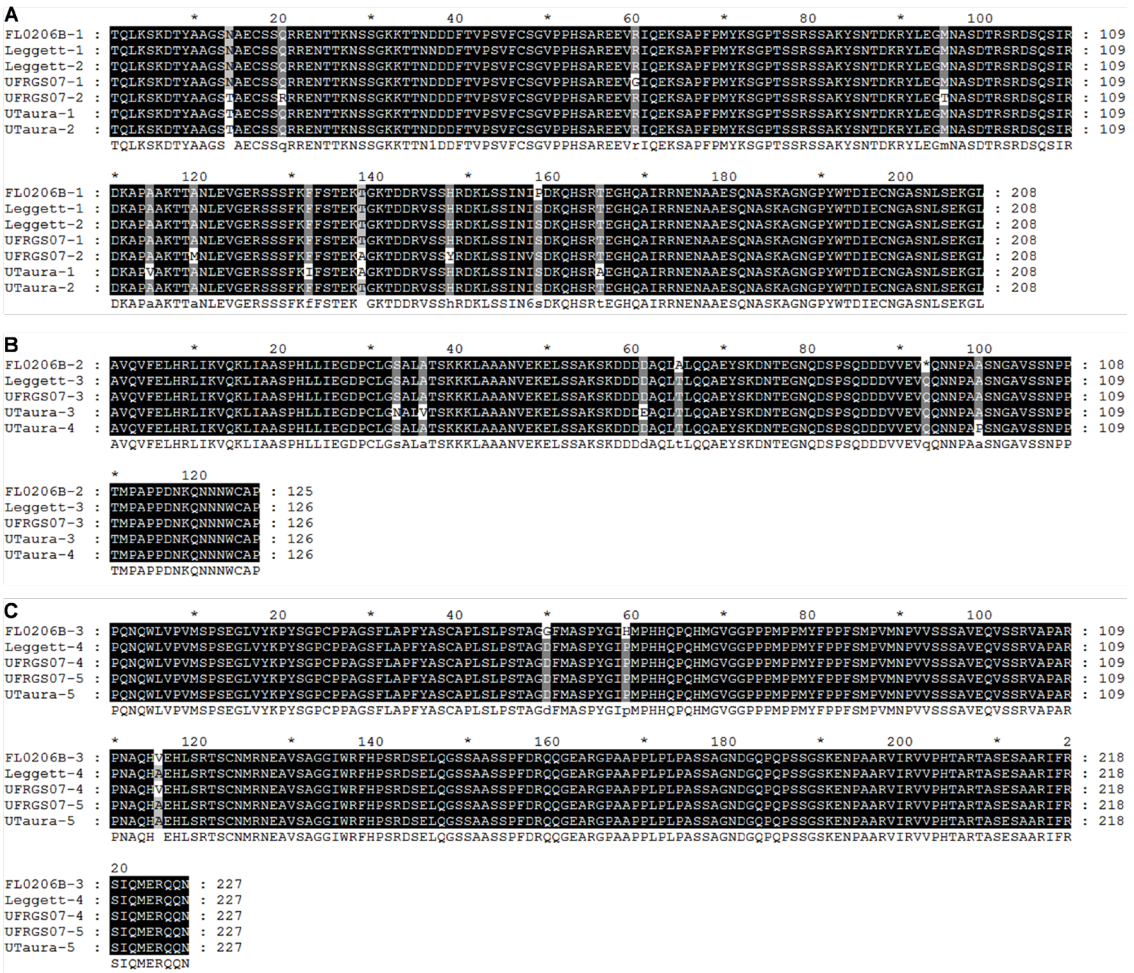

Figure 1. Amino acid sequences corresponding to the *ELF3* gene in oat genotypes of different origins. Single amino acid changes among the genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1, and URS Taura isolated with the primer pair ELF3-3 (A), ELF3-5 (B) and ELF3-6 (C). The names of FL0206B-S-B-S1, UFRGS 078030-1, and URS Taura are given in their abbreviated form as FL0206B, UFRGS07 and UTaura, respectively, followed by the corresponding amino acid sequence number. In the FL0206B-2 sequence isolated with the ELF3-5 primer pair, the asterisk at position 93 represents a premature termination codon with no biological explanation, which may indicate a sequencing error. This sequence corresponds to GenBank accession PQ241510 and has been published on NCBI with the annotation 'Similar to early flowering 3'.

Most of these changes occur in the amino acid sequences of the same oat genotype, probably due to the sequencing of segments of different alleles of the *ELF3* gene located in different oat genomes or different alleles that are duplicated in the same oat genome. From the amino acid sequences, it was not possible to establish a direct relationship with the variation in flowering time observed among the oat genotypes evaluated in this study. However, the amino acid change identified at position 14 in the oat sequences amplified with the ELF3-3 primer pair may contribute to the differences in flowering time observed between URS Taura and the North American genotypes Leggett and FL0206B-S-B-S1. At this position, URS Taura has a threonine in both sequences UTaura-1 and UTaura-2, whereas FL0206B-1, Leggett-1, and Leggett-2 have an asparagine. Although threonine and asparagine belong to the same hydrophilic or polar side group, they can alter protein folding and function. Another single amino acid change was found at position 158, where the FL0206B-1 sequence contains a proline, whereas all the other sequences have a serine (Figure 1A). For ELF3-5, the most notable change was identified at position 65, where the FL0206B-2 sequence has an alanine and the other sequences have a threonine (Figure 1B). Two single amino acid changes were identified in the ELF3-6 amplified sequences at positions 50 and 59, where FL0206B-3 has a glycine and a histidine and the other sequences have an aspartic acid and a proline, respectively, at these positions (Figure 1C). Further studies could validate whether these amino acid changes are associated with the variation in flowering time in hexaploid oat. In addition, considerable research is still needed to clone the entire DNA segment of *ELF3* in hexaploid oat, to analyze a larger number of oat sequences corresponding to *ELF3* to gain a better understanding of the allelic variation of this gene and how it affects heading date in hexaploid oat, and to develop new molecular markers based on PCR reactions to assist oat breeding programs in the selection of diverse germplasm for flowering time.

Level of *ELF3* expression in oat

The level of *ELF3* gene expression was analyzed in the oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1, and URS Taura. Time of day (TD) zero, corresponding to the onset of the light phase, was used as the reference time to compare the *ELF3* gene expression at different times of the day. No differences in *ELF3* expression were found in the oat genotypes at TD zero. When analyzing TD 4 in the light phase, the highest abundance of *ELF3* transcripts was detected in Leggett, while the lowest abundance was detected in UFRGS 078030-1. Significant differences were measured between the early flowering UFRGS 078030-1 and the late flowering FL0206B-S-B-S1. At TD 8 in the light phase, the highest abundance of *ELF3* transcripts was also detected in Leggett, while the lowest abundance was detected in the FL0206B-S-B-S1 genotype. Significant differences in gene expression were measured between FL0206B-S-B-S1 and the early flowering URS Taura. No significant difference in *ELF3* expression was found in the oat genotypes at the dark phases TD 12, TD 8 and TD 4. The amount of *ELF3* transcripts decreased significantly in all oat genotypes in the dark phase at TD 4 (Figure 2).

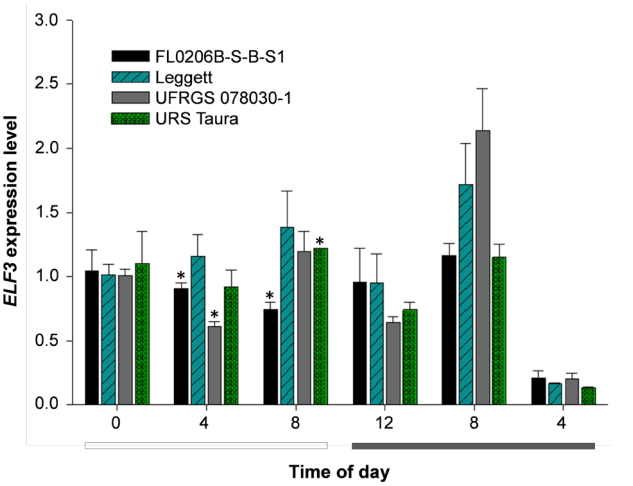

Figure 2. *ELF3* expression level in oat genotypes FL0206B-S-B-S1, Leggett, UFRGS 078030-1, and URS Taura. Time of day (TD) zero represents the beginning of the light phase and TD 12 represents the beginning of the dark phase. TD zero is the reference time for the analysis of *ELF3* gene expression compared to other times of the day in the light and dark phases. The asterisks above the vertical bars indicate the differences in the relative expression of the *ELF3* gene among oat genotypes using the t-test, with $p < 0.05$. The vertical bars above the columns indicate the standard error. The white and black bars on the x-axis represent the different times of day corresponding to the light and dark phases of the gene expression analysis.

Analysis of the level of *ELF3* expression revealed significant differences among the oat genotypes evaluated in this study. The most significant differences in the gene expression distinguished early and late oat genotypes during the light phase of plants grown under an induced photoperiod of 12 hours and at varying light/dark temperatures. These results suggest that differences in *ELF3* expression over a 24-hour period are related to temperature changes during the day. Furthermore, our results support the proposal that the transcription factor ELF3 plays an important role in the circadian rhythms of oat plants and,

consequently, in the phenotypic expression of flowering time, as has been demonstrated in numerous other crop species (Alvarez et al. 2023, Zhu and Wang 2024). A similar circadian expression pattern of the *ELF3* gene has been identified in wheat. The peak level of *ELF3* transcripts was observed six hours after dawn in early wheat lines grown at 12 °C, while the lowest expression level occurred three hours after the onset of darkness and remained constant during this period (Ochagavía et al. 2019). To the best of our knowledge, this is the first time that the level of *ELF3* expression has been analyzed and reported in hexaploid oat. While the results of this study represent a significant advance in oat genetics and genomics, future research on *ELF3* expression should include a larger set of oat genotypes with different flowering time phenotypes, analyze *ELF3* expression at different stages of flowering transition and development, and assess gene expression under different photoperiods and temperatures, both under field conditions and in controlled environments.

DATA AVAILABILITY

The datasets generated and/or analyzed in this study are available from the corresponding author upon reasonable request.

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