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Molecular Landscape of Bolting in Spinach Explored Through Gene Expression Profiling

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Abstract

This study aims to investigate the molecular mechanisms governing bolting in spinach (*Spinacia oleracea*) by analyzing gene expression patterns in key regulatory pathways. Two cultivars, Kashan (early bolting) and Virofly (late-bolting), were analyzed using quantitative real-time polymerase chain reaction (qRT-PCR) at different developmental stages. The photoperiod pathway genes Cryptochrome2 (*CRY2*) and Flavin-binding kelch repeat F-box protein (*FKF1*) showed distinct expression dynamics, highlighting their roles in the vegetative-to-reproductive transition. Aging pathway genes Topless (*TPL*), Squamosa promoter binding protein5 (*SPL5*), *SPL15*, and Alpha-ketoglutarate-dependent dioxygenase AlkB-like (*AlkB*) revealed complex expression patterns, with *SPL5* and *SPL15* differing significantly in Virofly. Circadian pathway genes LATE ELONGATED HYPOCOTYL (*LHY*) and TIMING OF CAB EXPRESSION 1 (*TOC1*) showed distinct patterns, particularly *LHY* in Kashan. Autonomous pathway genes LUMINIDEPENDENS (*LD*) and FLOWERING LOCUS D (*FLD*) also varied, with *LD* higher in Kashan at the eight-leaf stage, while *FLD* was generally elevated. These findings provide insights into the interactions among photoperiod, aging, circadian, and autonomous pathways, suggesting regulatory mechanisms influencing bolting time. Further research into these pathways could enhance spinach breeding for improved yield and quality.

Keywords Spinach · Bolting · Gene expression · Photoperiod and circadian pathways · Autonomous and aging pathways

Introduction

Spinach is a leafy green vegetable belonging to the family Amaranthaceae, as classified by the Plant List database. Its taxonomy places it in the genus *Spinacia* and species *oleracea*, reflecting its botanical classification. This nutritious vegetable has its origins traced back to ancient Persia, with historical evidence dating to over 2000 years ago. It has since become widely distributed and cultivated across the globe, favored for its adaptability to different climates and its versatility in various culinary applications (Koh et al. 2012; Xu et al. 2017). Its importance lies in its abundant nutrient content, serving as an excellent source of vitamins, minerals, and antioxidants, making it a valuable component of a balanced diet (Koh et al. 2012). Moreover, its cultivation supports the livelihoods of many farmers and contributes significantly to the global vegetable market. Spinach boasts a high yield potential, with average production ranging from 10 to 14 tons per hectare (Safdar et al. 2022; Niu et al. 2023). Spinach yield is subjected to multifarious influencing factors necessitating careful consideration in agricultural practices. These factors encompass environmental variables including temperature, light exposure, water, humidity, nutrition, and stressors (van Treuren et al. 2017). However, a significant factor with substantial ramifications for spinach yield is the phenomenon known as bolting. Bolting signifies the premature initiation of flowering and subsequent seed development in spinach plants, resulting in undesirable alterations in leaf flavor profile characterized by bitterness. Furthermore, bolting leads to a shift in the plant's resource allocation away from leaf production, further diminishing its overall yield potential (Cho et al. 2017; Chen et al. 2019).

Bolting is controlled by a combination of environmental and genetic factors. Environmental factors such as temperature, photoperiod, and light quality play pivotal roles in regulating bolting. High temperatures and extended daylight hours often trigger the transition from vegetative

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growth to reproductive development. Additionally, exposure to stressors such as water scarcity or nutrient deficiencies can exacerbate the tendencies for bolting (Cho et al. 2017; Beharav and Hellier 2020). The regulation of bolting in spinach involves a multitude of molecular pathways, each comprising a distinct set of genes with specialized functions (Boss et al. 2004). These pathways include the photoperiod (Ito et al. 2012), vernalization (Sung and Amasino 2004; Searle et al. 2006), circadian clock (Yu et al. 2008; Dalchau et al. 2011; Zakhrabekova et al. 2012), autonomous (Qi et al. 2022), age-related (Hinckley and Brusslan 2020), and the gibberellin pathways (Chandler et al. 2000; Winter et al. 2015). Among molecular factors, gene expression plays a crucial role in bolting control, with key genes orchestrating this process. The upregulation or downregulation of these genes in response to environmental cues and developmental stages can either promote or inhibit the occurrence of bolting. Using a transcriptome analysis approach, Abolghasemi et al (2021) identified key genes and pathways associated with bolting regulation. The research elucidates the roles of the photoperiod, circadian clock, and vernalization pathways in modulating bolting timing (Abolghasemi et al. 2021). Notably, in the photoperiod pathway, central players include genes such as CONSTANS (*CO*) and FLOWERING LOCUS T (*FT*), with CO acting as a promoter of flowering under long-day conditions and FT serving as a mobile signal for flowering induction (Mouradov et al. 2002; Takada and Goto 2003). In the vernalization pathway, the VERNALIZATION INSENSITIVE 3 (*VIN3*) gene stands out for its role in epigenetic regulation and repression of bolting in response to prolonged cold exposure (Sung and Amasino 2004). Within the autonomous pathway, genes like *FLD* are involved in promoting flowering independently of environmental cues, and mutations in these genes can result in early bolting (Chou and Yang 1998; Singh et al. 2013). The age-related pathway encompasses genes like SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (*SPL*) genes, which are targeted by miR156 and miR172 to regulate the timing of flowering (Wu et al. 2009; Chen et al. 2010; Preston and Hileman 2013; Ma et al. 2021). The gibberellin pathway influences bolting through genes like GIBBERELLIN INSENSITIVE DWARF1 (*GID1*) and DELLA proteins. *GID1* perceives gibberellin signals, leading to DELLA protein degradation and the promotion of bolting (Griffiths et al. 2006; Du et al. 2017). Similarly, in the circadian clock pathway, genes like *LHY* and *TOC1* exhibit rhythmic expression patterns, synchronizing the bolting process with the day-night cycle (Fenske et al. 2015).

Despite previous research, significant knowledge gaps persist in comprehension of the intricate regulatory pathways underlying bolting in spinach. Hence, in this study, we aimed to deeply investigate molecular mechanisms that govern the critical developmental transition of bolting in spinach during different developmental stages. To achieve this, we focused on gene expression patterns within essential regulatory pathways, including the photoperiod, circadian clock, autonomous, and age-related pathways. These pathways have been implicated in the precise timing of bolting initiation under various environmental conditions. Indeed, our research sought to provide a comprehensive understanding of molecular mechanisms governing bolting in spinach. This knowledge has implications not only for improving spinach cultivation but also for gaining insights into the broader regulation of flowering and developmental transitions in other plant species.

Results

In the present study, we systematically assessed the expression profiles of two genes within the photoperiod pathway, four genes associated with the aging pathway, two genes in the circadian pathway, and two genes within autonomous pathway. This investigation was conducted across various developmental stages in two distinct spinach cultivars, namely the early bolting Kashan and the late-bolting Virofly, shedding light on the intricate molecular dynamics during spinach development.

Photoperiod Pathway Genes

In flowering plants, photoperiodic control represents the principal regulatory pathway governing flower formation, where the perception of light signals serves as the seminal and requisite incipient step in this intricate and multifaceted process. Extensive investigations, predominantly conducted on model plants like Arabidopsis, has unequivocally delineated the involvement of two blue light photoreceptors, *CRY2* and *FKF1*, in the promotion of flowering through distinct pathways. Our experimental findings show that the transcript levels of these aforementioned genes exhibit a conspicuous disparity between two contrasting genotypes, 'Kashan' and 'Virofly', with a pronounced increase in the expression of both genes observed during the early flowering phase in the former (Fig. 1). The progressive increment in CRY2 expression up to the eight-leaf stage in 'Kashan' is indicative of its pivotal role in orchestrating the transition from the vegetative phase to the reproductive phase within this genotype, whereas *FKF1* displays a marked downregulation at this specific developmental juncture. Conversely, 'Virofly' demonstrates a comparatively modest and relatively stable expression pattern of *CRY2* during the vegetative stage, while *FKF1* shows a remarkable sixfold increase at the eight-leaf stage. Notably, in both genotypes, the lowest expression levels of these genes is consistently observed during the reproductive phase, underscoring a convergence in

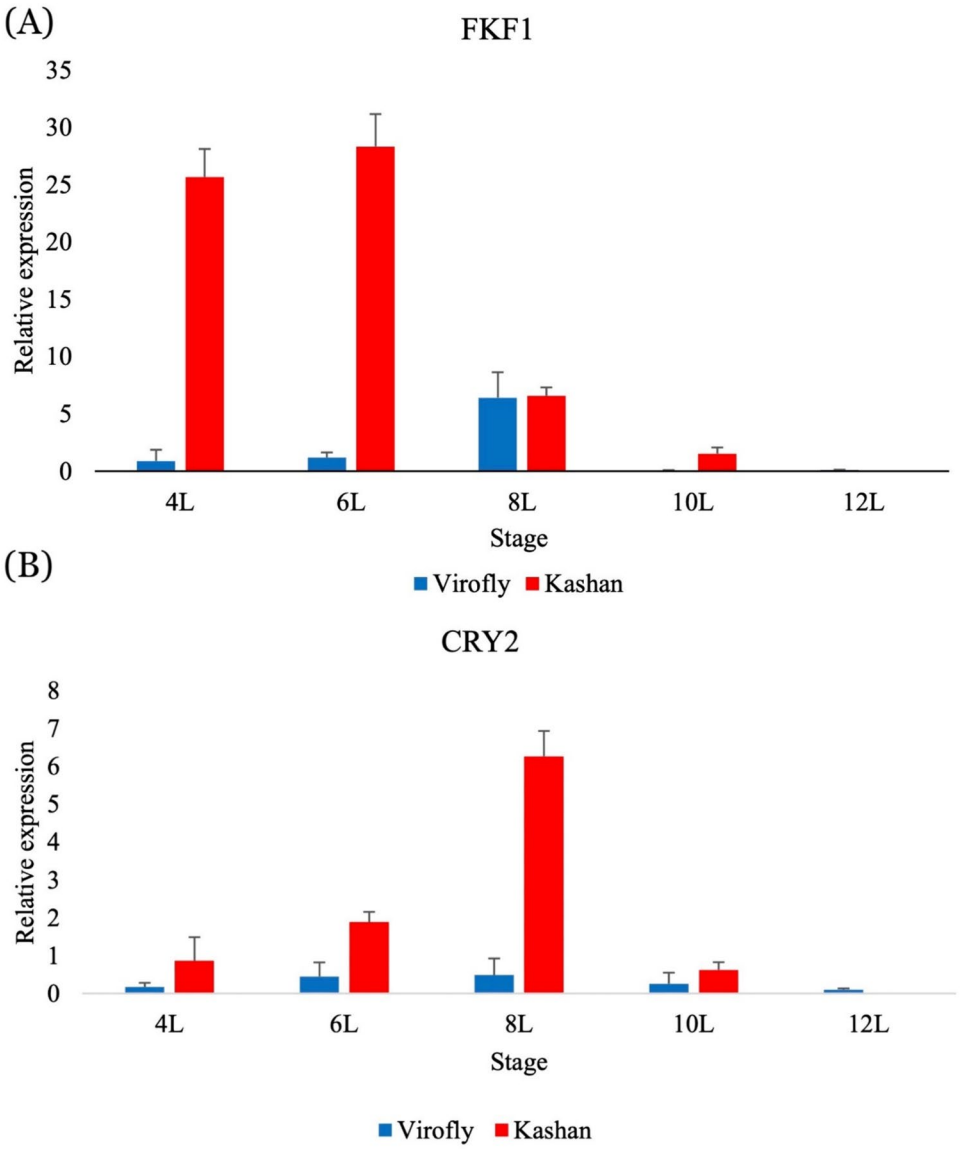

Fig. 1 Expression profiles of genes related to photoperiod pathway. **A** *FKF1* gene, and **B** *CRY2* gene. 4L, 6L, 8L, 10L, and 12L represent the four, six, eight, ten and twelve leaf stages of Kashan and Virofly, respectively

the photoperiodic regulatory mechanisms during this distinct phase of development."

Aging Pathway Genes

The age pathway in plants, a critical flowering-related pathway, encompasses genes such as *TPL* (Negative), *SPL5* (Positive), *AlkB* (Negative), and *SPL15* (Positive), each exerting distinct impacts on the regulation of flowering time. Previous research on the flowering and bolting time of plants has firmly established the pivotal role of *SPL* gene family in orchestrating diverse facets of plant development, with a pronounced emphasis on their involvement in the precise regulation of flowering time. The present investigation focused on two specific members of this gene family, namely, *SPL5* and *SPL15*, which are integral components of the aging-associated pathway. An in-depth examination of *SPL5* gene expression in spinach revealed a noticeable disparity between the two genotypic variants under scrutiny (Fig. 2). The transcript abundance of *SPL5* in "Kashan" was nearly undetectable, however, a marginal expression was observed during the six-leaf stage. In contrast, the "Virofly" genotype exhibits a considerably elevated *SPL5* expression during the vegetative phase, reaching an impressive 28-fold greater level than those in "Kashan" at the six-leaf developmental stage. Furthermore, a marked reduction in *SPL5* expression levels became apparent during the transition from the vegetative phase to the reproductive phase in the late-bolting genotype. The comprehensive examination of *SPL15* expression unveiled a consistent and stable expression pattern within each genotype during the vegetative stages, spanning from four to six leaves. Intriguingly,

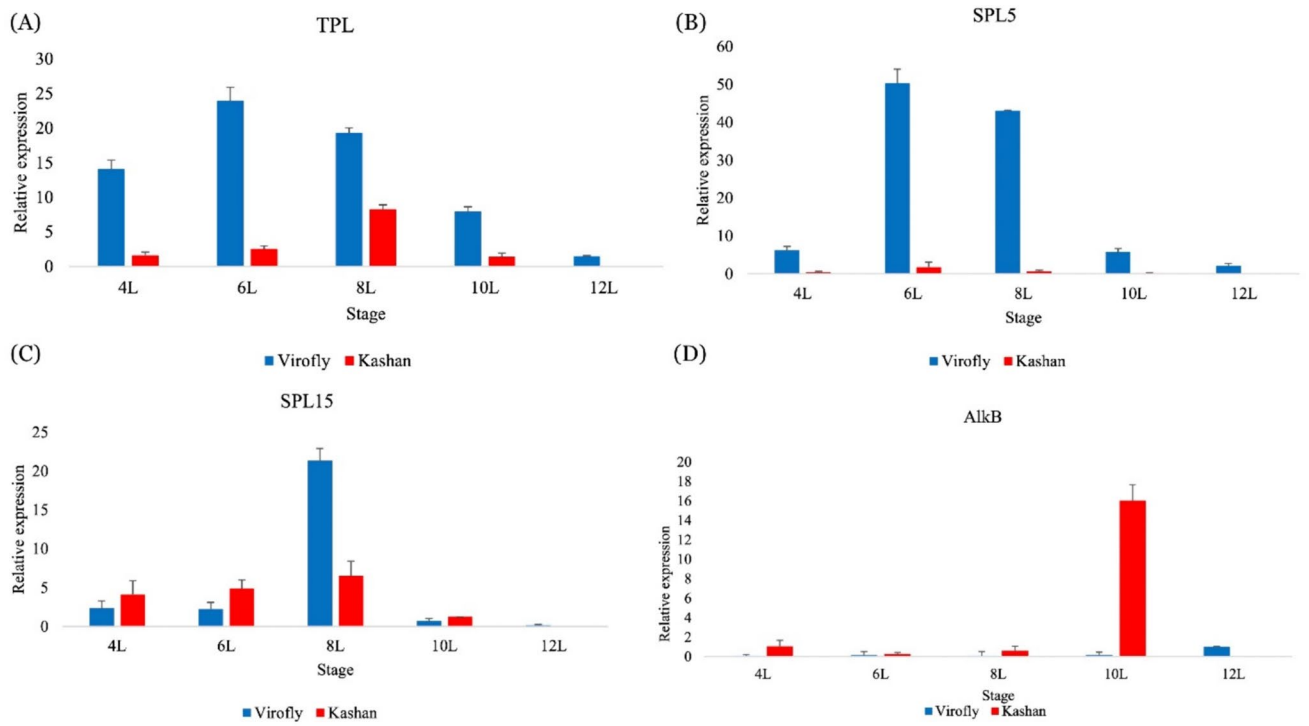

Fig. 2 Expression profiles of genes related to age pathway. **A** *TPL*, **B** *SPL5*, **C** *SPL15*, and **D** *AlkB*. 4L, 6L, 8L, 10L, and 12L represent the four, six, eight, ten and twelve leaf stages of Kashan and Virofly, respectively

at the critical juncture of the eight-leaf stage, a remarkable tenfold increase in *SPL15* expression was discerned in the late-flowering "Virofly" genotype. In contrast, the "Kashan" genotype displayed a modest and relatively marginal increment in gene expression at this specific stage. It is noteworthy that, irrespective of genotypic variations, both "Virofly" and "Kashan" exhibited the lowest levels of *SPL15* expression during the reproductive phase (Fig. 2). These results indicate the pivotal role of *SPL15* in the temporal regulation of the transition from the vegetative-to-reproductive phases, with a marked difference in expression dynamics between the late-flowering "Virofly" and "Kashan" genotypes. Given the recognized stimulatory effects of these genes on the flowering process, particularly in the context of their prominent expression in the late-flowering "Virofly" genotype, it is conceivable to postulate the existence of counteracting inhibitory factors that modulate the influence of these genes on the flowering process.

Alongside members of the *SPL* family within the age pathway, we also examined the expression patterns of two additional genes, *TPL* and *AlkB*, in this investigation (Fig. 2). *TPL* stands out as a pivotal flowering-related gene linked to the age pathway in plants, operating as a transcriptional corepressor. TPL's regulatory role spans various developmental processes, including floral patterning and organogenesis, achieved through interactions with diverse transcription factors, ultimately influencing gene expression. Exploring the genetic control of flowering in spinach has brought forth compelling insights into the role of the *TPL* gene. The present study unraveled a notable upregulation of the *TPL* gene in the "Virofly" genotype, showcasing prominent expression levels during vegetative growth, peaking at the six-leaf stage. In subsequent stages, there was a gradual decline, with minimal mRNA levels during reproduction, underscoring TPL's pivotal role in the intricate flowering process. Notably, the early flowering genotype exhibited comparatively lower TPL gene expression, aligning with its recognized role in flowering regulation. In the "Kashan" genotype, TPL maintained consistent expression during the four- and ten-leaf stages. However, a significant fourfold increase appeared at the eight-leaf stage, suggesting its likely role in the formation of flower. This underscores the sensitivity and significance of the eight-leaf stage as a critical juncture for transitioning to the reproductive stage in the "Kashan" genotype. This transition may be mediated by TPL gene expression and its interactions with key genes such as *TOE1* and *SPL*. *AlkB* is a functional RNA N6-methyladenosine demethylase. Reduction in AlkB levels affects the stability of flowering time genes including *FT*, *SPL3* and *SPL9*. Plants harboring mutations in the *ALKBH10B* gene exhibit early flowering. An in-deepth analysis of the *ALKBH10B* homolog in spinach (Spo25656:

Alpha-ketoglutarate-dependent dioxygenase AlkB-like) revealed intriguing insights into its pivotal involvement in developmental transitions. In both genotypes, the expression of *AlkB* gene was generally restricted during various developmental stages. Nonetheless, a significant 26-fold increase in *AlkB* gene expression was observed at the 10-leaf stage in the Kashan genotype compared to the 8-leaf stage. Additionally, a decrease in expression of *AlkB* was observed at the six and eight-leaf stages compared to the four-leaf stage. In contrast, the Virofly genotype exhibited elevated expression at the 12-leaf stage, suggesting a potential association with its involvement in flower morphogenesis.

Circadian Pathway Genes

Given the crucial role of the circadian clock in the regulation of flowering/bolting time in plants, we investigated the expression levels of two genes, *LHY* and *TOC1* (Fig. 3), within this important pathway to gain insight into the functioning of genes in developmental stages of spinach. Analysis of the expression data revealed that the *LHY* gene exhibited its lowest expression levels during the four-leaf (vegetative) stage in both cultivars, with notable upregulation observed at the 8-leaf and 12-leaf stages in the Kashan and Virofly, respectively. These stages are indicative of the plant transitioning into the reproductive phase or initiating flowering. Notably, in the early bolting Kashan cultivar, *LHY* displayed significantly higher expression at the 8-leaf stage compared to the late-flowering Virofly cultivar, while the opposite pattern observed at the six-leaf stage. This suggests that elevated *LHY* gene expression occurs at the onset of flowering initiation and entry into the reproductive phase. *TOC1* is a transcription factor that affects the period of plants' circadian rhythms and is involved in the clock's evening loop, which directly inhibits transcription of morning loop genes *LHY* and *CCA1*. *TOC1* acts as a

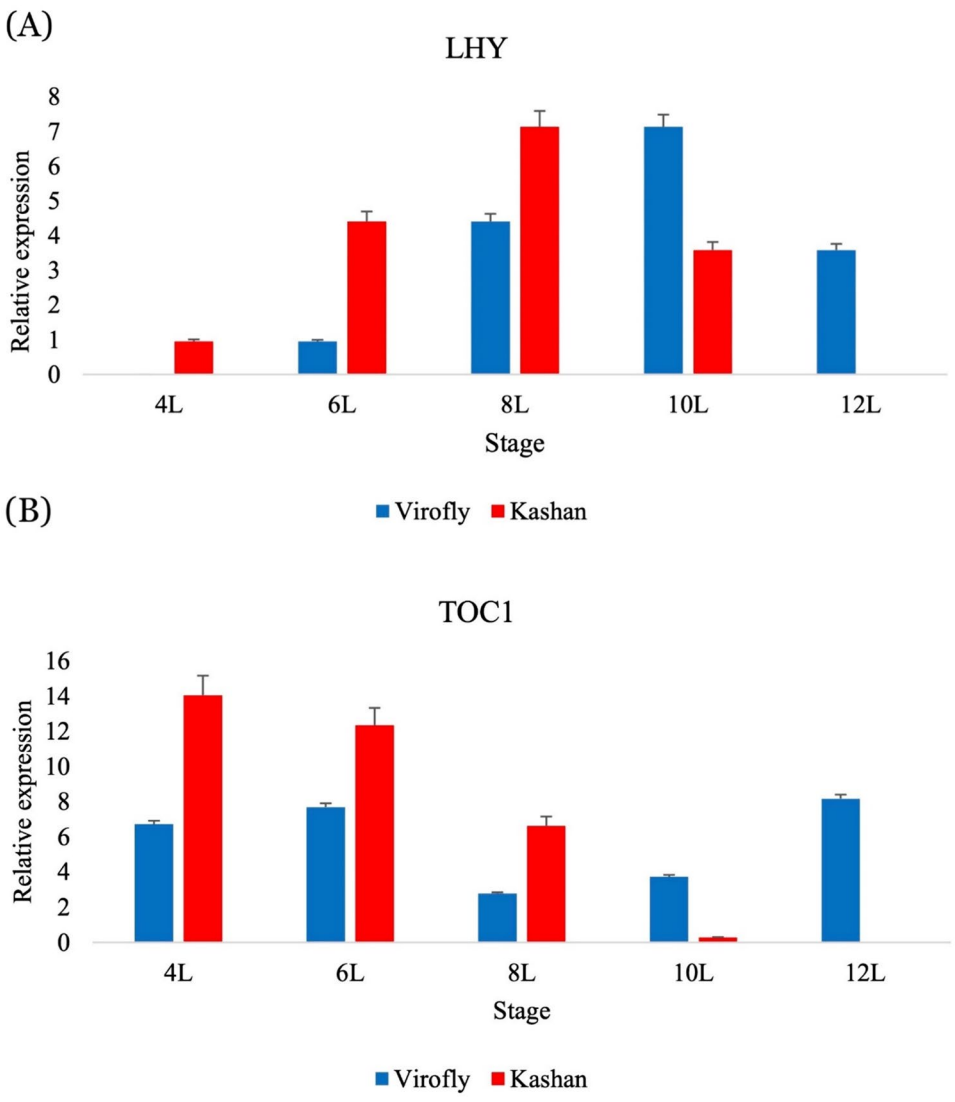

Fig. 3 Expression profiles of genes related to circadian pathway. **A** *LHY* gene, and **B** *TOC1* gene. 4L, 6L, 8L, 10L, and 12L represent the four, six, eight, ten and twelve leaf stages of Kashan and Virofly, respectively

general transcriptional repressor with expression peaking at the onset of the night. Our results showed that the expression of this gene in the Kashan cultivar during the stages of four, six, and eight leaves (spanning from the vegetative stage to the onset of the reproductive phase) exceeded that of the Virofly variety. Conversely, at the 10-leaf stage, corresponding to the onset of the reproductive phase, *TOC1* expression in Virofly surpassed that of Kashan. Furthermore, a notable decline in expression was observed at the 10-leaf stage in Kashan during flowering, contrasting with the expression patterns observed in other developmental stages.

Autonomous Pathway Genes

The autonomous pathway is one of the regulatory pathways that controls flowering time. This pathway operates independently of external signals such as light and temperature, and instead, it is influenced by internal factors such as age and endogenous hormones. Two key genes in the autonomous pathway are *FLD* and *LD* (Fig. 4). *LD*, in particular, is a zinc finger transcription factor that positively regulates flowering time. The analysis of *LD* gene expression in spinach revealed a high level of mRNA in the vegetative stage of the Kashan cultivar, reaching its maximum level at the 8-leaf stage. A sharp decrease in expression was observed at the 10-leaf stage. Conversely, the expression of this gene in the Virofly variety was significantly lower than that in the Kashan cultivar. In Virofly, a significant and relative increase in expression was observed from the four-leaf stage to the eight-leaf stage, followed by a decrease in subsequent stages. In general, the expression of this gene was higher in the early bolting Kashan cultivar compared to the late-bolting Virofly variety. *FLD* negatively regulates flowering time by repressing the expression of the floral promoter *FLC*. Examination of the findings revealed that the *FLD* gene exhibited a higher relative expression in the Kashan cultivar compared to the Virofly variety up to the 8-leaf stage, although this disparity lacked statistical significance. It is noteworthy that

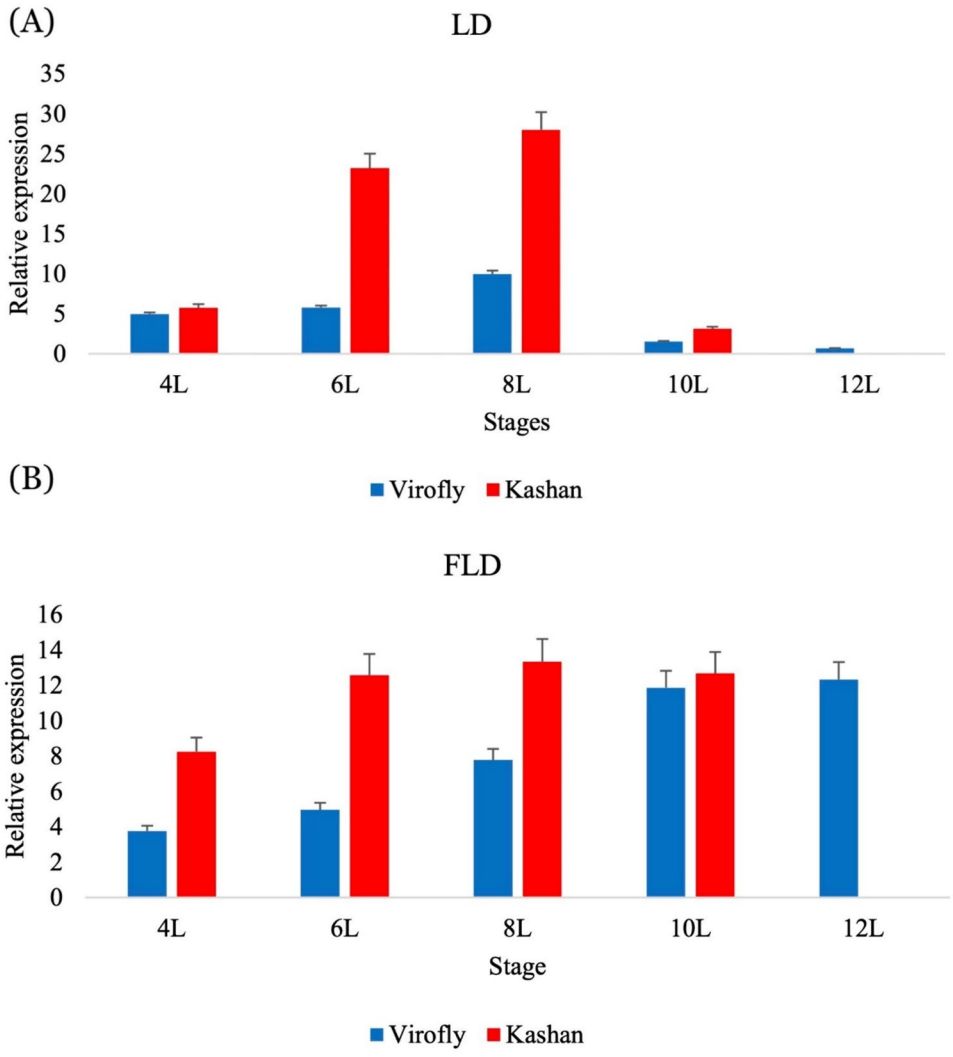

Fig. 4 Expression profiles of genes related to autonomous pathway. **A** *LD* gene, and **B** *FLD* gene. 4L, 6L, 8L, 10L, and 12L represent the four, six, eight, ten and twelve leaf stages of Kashan and Virofly, respectively

the expression of *FLD* was elevated during the vegetative stage in Kashan and in the reproductive stage in the Virofly variety. Furthermore, there was a gradual increase in gene expression in the Virofly variety, peaking at the reproductive stage. In Kashan, a slight reduction in expression was noted at the 10-leaf stage.

Discussion

In the present study, we systematically assessed the expression profiles of two genes within the photoperiod pathway, four genes associated with the aging pathway, two genes in the circadian pathway, and two genes within the autonomous pathway. This investigation was conducted across various developmental stages in two distinct spinach cultivars, namely the early bolting Kashan and the late-bolting Virofly, shedding light on the intricate molecular dynamics during spinach development. Our study investigates the expression patterns of *CRY2* and *FKF1*, two critical genes in the photoperiod pathway. The observed disparity in gene expression between 'Kashan' and 'Virofly' during the early flowering phase underscores the pivotal role of these genes in the transition from vegetative-to-reproductive phases. Notably, both genotypes exhibit reduced expression levels during the reproductive phase, suggesting a convergence in photoperiodic regulatory mechanisms during this developmental stage. *CRY2* is a blue light receptor that is widely expressed in plants, including in the leaves and flowers. It is involved in various light-dependent processes, such as phototropism, chloroplast movement, and stomatal opening. *FKF1*, on the other hand, is a gene that is specifically expressed in the afternoon and its protein product is involved in the regulation of *CO* protein stability, which is crucial for photoperiodic flowering. While *CRY2* is widely expressed in plants, *FKF1* has a more specific expression pattern and is only expressed in the afternoon. This temporal expression is crucial for the timing of *CO* stabilization, although the mechanisms by which *FKF1* stabilizes *CO* protein remain elusive. However, how *FKF1* stabilizes *CO* protein remains elusive. While *CRY2* and *FKF1* have different expression patterns and functions in plants, they may interact with each other to some extend to regulate *CO* protein stability and photoperiodic flowering (El-Din El-Assal et al. 2003; Liu et al. 2011; Yuan et al. 2019; Schiessl 2020; Jiang et al. 2022; Lee et al. 2023). However, further research is needed to comprehensively understand the relationship between *CRY2*, *FKF1*, and *CO* genes stability.

In the aging pathway, we selected and four key genes, namely *TPL*, *SPL5*, *SPL15*, and *AlkB* and investigated their intriguing dynamics expression profile at different growth stages. *SPL5* and *SPL15*, integral members of the *SPL* gene family, exhibit notable differences in expression between 'Kashan' and 'Virofly'. The late-bolting genotype, 'Virofly', displays significantly higher *SPL5* expression during the vegetative phase, highlighting its potential role in late-flowering. *SPL15*, on the other hand, exhibits a tenfold surge in expression at the eight-leaf stage in 'Virofly', further emphasizing its involvement in the transition to the reproductive phase. The expression dynamics of *TPL* indicate its critical role in flower formation, with 'Virofly' showcasing pronounced expression during vegetative growth. In contrast, 'Kashan' demonstrates a surge in *TPL* expression at the eight-leaf stage, underscoring its sensitivity during the critical transition to the reproductive phase. The *AlkB* gene, known for its influence on flowering time genes, exhibits nuanced expression patterns in both genotypes, emphasizing its role in developmental transitions. Considering the potent stimulatory effect of *SPL5* and *SPL15* genes on the flowering process, coupled with their prominent expression specifically in the late-flowering "Virofly" genotype, it is plausible to hypothesize that inhibitory factors are involved in counteracting the stimulating effect of these genes on flowering. This hypothesis aligns with previous findings reported in Arabidopsis (He et al. 2018), where they identified miR156-mediated post-transcriptional repression of ten members of the *SPL* transcription factor family. Moreover, investigations by Najaf Abadi et al. 2023 have demonstrated a significantly higher expression of miR156 in the vegetative and reproductive stages of the "Virofly" genotype, with levels 11.26- and 5.46-fold higher than those observed in the "Kashan" genotype, respectively. Thus, despite the elevated expression of *SPL15* genes in "Virofly", their impact on flowering appears to be suppressed, and the observed expression pattern may not directly correlate with flowering time. One notable aspect of this study is the evaluation of the *AlkB* gene, which has been implicated in inhibiting flowering-stimulating genes through epigenetic regulation in Arabidopsis (Duan et al. 2017). The involvement of *AlkB* in the regulation of flowering in spinach may be linked to the shared evolutionary origin of these plants. This gene, which is responsible for inhibiting flowering, is regulated by the interaction of its partners, the *ATF* (APETALA 2 (AP2)-domain-containing transcription factors) gene family, which is also known to have a crucial role in flowering control in plants (Shu et al. 2018).

The gene expression analysis of the *LHY* and *TOC1* genes revealed distinct patterns during plant development. *LHY* exhibited its lowest expression during the four-leaf vegetative stage in both cultivars but underwent significant upregulation at the 8-leaf and 12-leaf stages, coinciding with the transition to the reproductive phase. For *TOC1*, its expression in the Kashan cultivar exceeded that of Virofly during the stages of four, six, and eight leaves, but a shift occurred at the 10-leaf stage, where Virofly displayed higher *TOC1* expression during the onset of the reproductive phase.

Connecting these findings with the main points of previous research, which discusses the broader molecular context of *LHY* and *TOC1* regulation, several key insights emerge. Both *LHY* and *TOC1*, crucial components of the plant circadian clock, are subject to diverse regulatory pathways, including cellular signaling, molecular clock components, and environmental cues. The circadian clock itself influences the expression of these genes (Park et al. 2016; Yang et al. 2023). The observed variations in *LHY* and *TOC1* expression during different developmental stages align with their roles in influencing flowering time via the circadian clock. However, the molecular mechanisms differ between the two genes; *TOC1* modulates the clock function to regulate flowering timing, while *LHY* acts distinctly through the circadian clock. This is further supported by the early flowering exhibited in *LHY* mutants, which is linked to clock defects and reduced photoperiodic sensitivity (Park et al. 2016; Guo et al. 2022). The opposing expression patterns of *LHY* and *TOC1*, where *LHY* negatively regulates *TOC1*, corroborate the intricate balance required for proper circadian clock function. The involvement of *TOC1* in various processes, including abscisic acid signaling and thermoresponsive growth suppression, adds complexity to its role, while LHY's higher expression in the early morning and its participation in the morning loop of the circadian clock contributes to a comprehensive understanding (Bäurle and Dean 2006; Adams et al. 2018; Nakamichi 2020).

Investigation of the autonomous pathway's influence on flowering time revealed a notable discrepancy in *LD* gene expression between the early bolting Kashan and the late-bolting Virofly. Kashan exhibited higher *LD* gene expression, particularly at the 8-leaf stage. Our observation of increased *LD* expression aligns with the known influence of internal factors, such as age, on *LD* regulation. Moreover, the results from our study indicated elevated relative expression of the *FLD* gene in Kashan, especially up to the 8-leaf stage. This aligns with previous research insights into FLD's role as a negative regulator of flowering time, repressing the expression of *FLC*. The interconnected nature of *LD* and *FLD* in the regulation of flowering time adds another layer to the understanding of the observed differences between Kashan and Virofly (Liu et al. 2009; Kim et al. 2013; Bouché et al. 2016; Wu et al. 2020; Yi et al. 2021; Zheng et al. 2022).

The intricate molecular dynamics observed across pathways in different spinach cultivars hint at potential interactions and crosstalk, highlighting the complexity of flowering time regulation in plants. The differences in the expression profile of photoperiod genes in Kashan and Virofly may suggest an intersection with the circadian pathway. The heightened *CRY2* expression in Kashan during the eight-leaf stage implies a potential connection between photoperiodic responses and the circadian clock, influencing flowering timing. Contrasting *SPL5* and *SPL15* expression patterns in Kashan and Virofly hint at interactions between the aging and autonomous pathways. The fluctuating expression of *LD* in Virofly, peaking at the reproductive stage, might influence the expression of *SPL* genes, thereby contributing to differences in flowering time. The upregulation of *TPL* in Virofly during the vegetative phase aligns with circadian clock dynamics, suggesting potential crosstalk between age and circadian pathways, that may influence flowering. *LHY* and *TOC1* align with the autonomous pathway in Kashan and Virofly. The genes expression pattern during critical stages suggest potential crosstalk between circadian and autonomous pathways in regulating flowering time. The intricate relationship between the photoperiod and age pathways holds pivotal significance in the regulation of flowering in plants. In some plant species, the photoperiod pathway is dominant and the age pathway plays a minor role. In these species, the initiation of flowering is primarily controlled by day length and the plant will only start flowering when the critical day length is attained, regardless of its age. However, in other species, the age pathway is dominant and the photoperiod pathway plays a minor role. In these species, flowering is primarily controlled by plant age and the plant will flower at a specific age, regardless of the day length. In some cases, the photoperiod and age pathways can interact to fine-tune flowering time. For example, in some long-day plants, the aging pathway can accelerate flowering in response to extended daylight, allowing the plant to flower earlier than it would if it relied solely on the photoperiod pathway. Similarly, in some short-day plants, the aging pathway can delay flowering in response to short days, allowing the plant to accumulate sufficient resources before flowering (Golembeski et al. 2014; Ream et al. 2014; Fernández et al. 2016).

Conclusion

In conclusion, our study elucidates the intricate interplay of molecular pathways governing flowering in spinach cultivars Kashan and Virofly. Noteworthy variations in the photoperiod genes *CRY2* and *FKF1* during the early flowering phase unveil their pivotal roles, while expression pattern suggest a potential cross-influence on circadian dynamics. In the aging pathway, the contrasting expressions of *SPL5* and *SPL15* hint at unexplored regulatory factors, suggesting crosstalk with the autonomous pathway. This is underscored by expressions of *TPL* and *AlkB*, indicative of a potential interplay between age and circadian pathways. Circadian genes *LHY* and *TOC1* not only align with their expected roles but also suggest an intriguing influence on the autonomous pathway, hinting at a complex regulatory network. The autonomous pathway genes *LD* and *FLD*, with their significant differences, imply a deeper connection to circadian

and aging dynamics, contributing to the intricate molecular landscape of spinach flowering. These findings unravel hidden complexities and set the stage for further research to uncover the nuanced relationships among these pathways, offering unprecedented insights for future crop improvement endeavors.

Material and Methods

Plant Material

For genes expression analysis, we selected two spinach accessions, Kashan and Viroflay, representing early and late-bolting varieties, respectively, according to our previous investigation (Abolghasemi et al. 2019). Our findings categorized Viroflay and Kashan into late and early flowering groups, respectively, with the most significant variation observed in the "days to flowering" trait, spanning 87 days for Viroflay and 43 days for Kashan. To minimize environmental influences, seeds from each accession were sown in sterilized soil within 15 cm diameter, 25 cm high plastic pots, and cultivated in a growth chamber under controlled spring conditions (Temperature range of 24–46 and photoperiod of 12–14 h of light) for three months at Isfahan University of Technology, Iran. No specific permissions were required to obtain these samples. Formal plant material identification was carried out by the herbarium of the Agricultural and Natural Resources College at the University of Tehran, and no voucher specimens were collected, as there was no necessity given the absence of a novel species description. Additionally, our field studies were conducted in compliance with local Iranian legislation within the experimental greenhouse and growth chamber at Isfahan University of Technology, Isfahan, without the need for specific licenses.

Sampling, RNA Isolation, and cDNA Synthesis

Total RNA was extracted from leaf samples of two spinach accessions, Kashan and Viroflay, collected at the different stages (Fig. 5), from the four-leaf stage to the twelve-leaf stage (at the reproductive stage, when 50% of the plants had produced flower stalks). To minimize variability attributed to inter individual gene expression differences, each sample consisted of pooled material from a minimum of three plants. RNA extraction was performed in triplicate using the DENAzist column RNA isolation kit according to the manufacturer′s protocol. The concentration and purity of RNA were measured using a NanoDrop Spectrophotometer (NanoDrop Technologies) and agarose gel. Subsequently, the RNA samples were quantified, and cDNA library preparation was conducted. In the preparation of the cDNA library for reverse transcription quantitative polymerase chain reaction (RT-qPCR), we initiated the process by treating the isolated RNA samples with DNase I enzyme to ensure the removal of genomic DNA contamination. Briefly, 2 µg of total RNA was incubated with 1 U of DNase I (Thermo

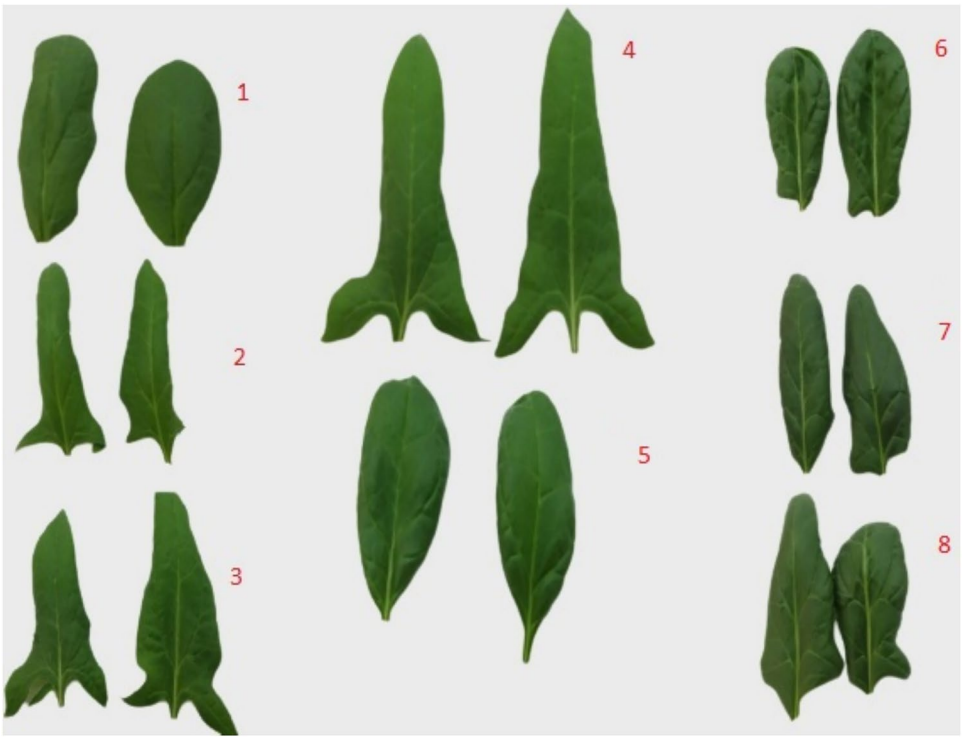

Fig. 5 Displaying the leaf samples from Kashan and Virofly cultivars at various developmental stages. The numbers 1–4 indicate the four, six, eight and ten-leaf stages of Kashan, respectively, and the numbers 5–8 indicate the four, six, eight and ten leaves of Virofla, respectively

Table 1 Gene names and primer sequences used for gene expression assays via qRT-PCR

Gene code	Gene name	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)
Spo25748	Flavin-binding kelch repeat F-box protein (FKF1)	F-GCGATCTGATGAGGCTTATACC R-GAGTCCTCCATGATGGTTTCTC	248	60
Spo03716	Cryptochrome2 (CRY2)	F-GGAAAACCTAGTTGCATTACCG R-CTTAGCGGATGAAGATTCTGCT	222	60
Spo16185	Topless (TPL)	F-AGAATAACTGGTTTGGCCTTCTC R-TGAGTCTCATGCACAACTAGGAA	212	55
Spo25656	Alpha-ketoglutarate-dependent dioxygenase AlkB-like (AlkB)	F-ATTGATCACCTCGTTCAGTGG R-TTGTAGTTGCCATCACCATCA	203	54.3
Spo16305	Squamosa promoter binding protein15 (SPL15)	F-GTCCATTATGTGGTCAGGTGATT R-CACTATGAGCTGCACTACACTGG	236	57.3
Spo26325	Squamosa promoter-binding-like protein5 (SPL5)	F-GCAGGTTTCATGAGCTATCAGAG R-ATGGGACTTTCTTGATCTTCTGG	169	55.5
Spo01117	LATE ELONGATED HYPOCOTYL (LHY)	F-TTTCTGCTCTTCTCCAGAATCC R-AGTACATAAGGGGAGCAATCCA	214	60
Spo08124	TIMING OF CAB EXPRESSION 1 (TOC1)	F- GTTGACGTTGAAGGTAATGCTG R- ACGACCTTTCCATAGAAGTGGA	184	60
Spo18440	FLOWERING LOCUS D (FLD)	F- TTCCTGCTGATTCTGTGACTG R- AACCCCGAAATTAACATACCC	239	60
Spo21974	LUMINIDEPENDENS (LD)	F- GTGAGGGAGTATTTTGCCAGTC R- CCTCGACATTAGAAGGACCAAC	181	60
Spo21495	Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	F-CGTGTCAGTTGATTTCAGGTGT R-GTTGTCCTTGCAGAAATCTTCC	223	60

Fisher Scientific) at 37 °C for 30 min, followed by heat inactivation at 75 °C for 10 min. Subsequently, the DNase I-treated RNA was subjected to reverse transcription using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Thermo Fisher Scientific). A reaction mixture containing 1 µg of DNase I-treated RNA, 200 U M-MLV RT, 500 µM dNTPs, 5 µM random hexamer primers, 10 mM DTT, and 20 U RNase inhibitor was incubated at 37 °C for 1 h, followed by enzyme inactivation at 70 °C for 15 min. The resulting cDNA was then employed as a template for quantitative PCR analysis.

Gene Selection and Primer Design

In genes expression evaluation, we specifically targeted on two genes within the photoperiod pathway (*CRY2* and *FKF1*), four genes associated with the aging pathway (*TPL*, *SPL15*, *AlkB*, and *SPL5*), two genes in the circadian pathway (*LHY* and *TOC1*), and two genes in autonomous pathways (*FLD* and *LD*). The selection of these genes was made based on their importance and documented influence on flowering/bolting time, as reported in previous literature (Nelson et al. 2000; Niwa et al. 2007; Park et al. 2016; Wu et al. 2021; Abolghasemi et al. 2021; Takagi et al. 2023). Finally, we designed primers for the selected genes (Table 1) using the Primer3 tool. Our primer design considered factors such as melting temperature, self-complementarity, hairpin potential, and primer product sizes. By utilizing these criteria, we designed specific and efficient primers for the amplification of the target genes in the study.

Quantitative Real-Time Polymerase Chain Reaction

To validate the expression patterns of bolting and flowering-related genes, we conducted qRT-PCR to quantitatively assess the expression of candidate genes in leaf tissues at various developmental stages. qRT-PCR experiments were carried out in triplicate using an StepOne Real-Time PCR system in a final volume of 15 µL. This volume included 7.5 µL of SYBR Green Master Mix (BioFACT, Korea), 2 µL of diluted cDNA, and 0.5 µL of each primer (10 pM), with the remainder filled with PCR-grade water. The qRT-PCR protocol involved an initial step of 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at the primer-specific annealing temperature, 20 s at 72 °C, and concluded with a melting curve program. The statistical analysis of gene expression followed the $2^{-\Delta\Delta Ct}$ method (Rao et al. 2013), utilizing *GADPH* as internal reference (housekeeping) gene.

Author Contributions Marziye Amini: investigation; methodology; formal analysis, writing-review and editing. Aboozar Soorni: Conceptualization; funding acquisition; investigation; project administration; methodology; formal analysis; validation; writing-original draft; writing-review and editing. Zahra hasanpour: investigation; methodology;

formal analysis, writing-review and editing. Rahim mehrabi: Investigation; methodology; funding acquisition; writing-review and editing.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

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