



RESEARCH PAPER

KHZ1 and KHZ2, novel members of the autonomous pathway, repress the splicing efficiency of *FLC* pre-mRNA in *Arabidopsis*

Zongyun Yan^{*}, Huiying Shi^{*}, Yanan Liu, Meng Jing and Yuzhen Han[†]

State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China

* These authors contributed equally to this work.

† Correspondence: hanyuzhen@cau.edu.cn

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Abstract

As one of the most important events during the life cycle of flowering plants, the floral transition is of crucial importance for plant propagation and requires the precise coordination of multiple endogenous and external signals. There have been at least four flowering pathways (i.e. photoperiod, vernalization, gibberellin, and autonomous) identified in *Arabidopsis*. We previously reported that two *Arabidopsis* RNA-binding proteins, KHZ1 and KHZ2, redundantly promote flowering. However, the underlying mechanism was unclear. Here, we found that the double mutant *khz1 khz2* flowered late under both long-day and short-day conditions, but responded to vernalization and gibberellin treatments. The late-flowering phenotype was almost completely rescued by mutating *FLOWERING LOCUS C (FLC)* and fully rescued by overexpressing *FLOWERING LOCUS T (FT)*. Additional experiments demonstrated that the KHZs could form homodimers or interact to form heterodimers, localized to nuclear dots, and repressed the splicing efficiency of *FLC* pre-mRNA. Together, these data indicate that the KHZs could promote flowering via the autonomous pathway by repressing the splicing efficiency of *FLC* pre-mRNA.

Keywords: *Arabidopsis*, autonomous, flowering, *FLOWERING LOCUS C*, KHZ1, KHZ2.

Introduction

The transition from vegetative to reproductive development is a vital process in the life cycle of a flowering plant, and this process is coordinately regulated by various endogenous and exogenous factors (Mouradov *et al.*, 2002; Yuan *et al.*, 2016). Numerous molecular genetic studies on the model dicotyledonous plant *Arabidopsis* have identified at least four major flowering pathways: photoperiod, vernalization, gibberellin (GA), and autonomous (Srikanth and Schmid, 2011). Studies have demonstrated that these pathways are interconnected and ultimately converge to a set of floral integrators, such as *FLOWERING LOCUS T (FT)* and *SUPPRESSOR*

OF OVEREXPRESSION OF CONSTANS1 (SOC1). The floral integrators in turn regulate the expression of floral meristem identity (FMI) genes, such as *APETALA1 (AP1)* and *FRUITFULL (FUL)*, to promote the floral transition (Yamaguchi *et al.*, 2005; Corbesier *et al.*, 2007; Jang *et al.*, 2009; Yu *et al.*, 2016).

Plants can perceive environmental light signals and use the circadian clock as a time-keeping mechanism to monitor day length and ensure flowering at the proper time (Samach and Coupland, 2000; Hayama and Coupland, 2003; Yanovsky and Kay, 2003). As a model plant, *Arabidopsis* flowers early under

long-day conditions and flowers late under short-day conditions (Yuan *et al.*, 2016). CONSTANS (CO) and FT are vital regulators in response to photoperiod in Arabidopsis. The circadian clock regulates the expression of CO, which further activates the expression of FT (Wigge *et al.*, 2005; Yuan *et al.*, 2016). The FT protein, which is synthesized by specialized companion cells in Arabidopsis, induces the expression of the FMI genes and initiates flowering (Abe *et al.*, 2005; Corbesier *et al.*, 2007; Chen *et al.*, 2018).

Many plants, such as winter cereals (Sharma *et al.*, 2017), require a prolonged period of cold treatment before flowering, which is a process known as vernalization (Kim *et al.*, 2009). Genetic analyses of the winter-annual varieties of Arabidopsis revealed that *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) are two key genes that respond to vernalization (Michaels and Amasino, 1999; Johanson *et al.*, 2000). *FRI* functions by activating the expression of *FLC* (Geraldo *et al.*, 2009), which encodes a MADS-box transcription factor and acts as a repressor of flowering by suppressing the expression of flower-promoting genes (e.g. *FT* and *SOC1*) (Searle *et al.*, 2006; Michaels, 2009). Vernalization promotes flowering primarily via the epigenetic repression of *FLC* (Michaels, 2009). Studies have revealed three regulators, *VERNALIZATION INSENSITIVE 3* (*VIN3*), *VERNALIZATION 1* (*VRN1*), and *VERNALIZATION 2* (*VRN2*), that are needed for initiation and maintenance of *FLC* silencing after vernalization (Gendall *et al.*, 2001; Levy *et al.*, 2002; Sung and Amasino, 2004; Srikanth and Schmid, 2011). The inhibition of *FLC* is mainly achieved by the modification of *FLC* chromatin by histone deacetylation and methylation (He, 2009).

GA is a class of plant hormone that promotes flowering in Arabidopsis. Mutations in the GA biosynthesis and signaling pathways delay the flowering time, whereas exogenous application of GA accelerates flowering (Mouradov *et al.*, 2002; Srikanth and Schmid, 2011). The *GA1* gene, which encodes an *ent*-kaurene synthase, is needed for the first step of GA biosynthesis (Sun *et al.*, 1992). Flowering was delayed for the *ga1-3* mutant under continuous light and could not be induced under short-day conditions (Wilson *et al.*, 1992). *SPINDLY* (*SPY*), which encodes an *O*-linked *N*-acetylglucosamine transferase, negatively regulates GA signaling (Jacobsen and Olszewski, 1993). GA signaling was constitutively active in the *spy* mutant. Mutation of *SPY* could overcome the late flowering of *ga1* as demonstrated by the finding that the flowering time of the *ga1 spy* double mutant was similar to that of the wild-type (WT) plant (Jacobsen *et al.*, 1996; Swain *et al.*, 2001). Previous studies have demonstrated that there are several points of convergence between the GA pathway and other pathways. For instance, the promoter activity of the floral integrator gene *LEAFY* (*LFY*) was increased by GA treatment, but *LFY* expression was reduced 10-fold in the *ga1-3* mutant (Blázquez *et al.*, 1997, 1998). GA also plays an important role in the regulation of another floral integrator gene *SOC1*. *SOC1* expression is increased by exogenous treatment with GA but is nearly undetectable in the *ga1-3* mutant under short-day conditions (Moon *et al.*, 2003).

In the absence of exogenous inducers, plants will flower after a particular stage of vegetative growth. This flowering pathway is known as the autonomous pathway. Many proteins have been

identified in the autonomous pathway, such as *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS PA* (*FPA*), *FLOWERING LOCUS VE* (*FVE*), *FLOWERING LOCUS D* (*FLD*), *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS Y* (*FY*), *RELATIVE OF EARLY FLOWERING 6* (*REF6*), *FLOWERING LOCUS KH DOMAIN* (*FLK*) and *TBP-ASSOCIATED FACTOR 15b* (*TAF15b*); these factors accelerate flowering by inhibiting *FLC* (Lim *et al.*, 2004; Mockler *et al.*, 2004; Simpson, 2004; Srikanth and Schmid, 2011; Eom *et al.*, 2018). Mutants of the autonomous pathway have a late-flowering phenotype under both long-day and short-day conditions; however, the late-flowering phenotype of these mutants can be overcome by vernalization or exogenous GA (Mouradov *et al.*, 2002; Moon *et al.*, 2003). Generally, the factors involved in the autonomous pathway are divided into three functional categories: RNA processing, and transcriptional and epigenetic regulation (Eom *et al.*, 2018). In the RNA processing category, *FCA*, *FY*, *FPA*, cleavage stimulation factor 64 (*CstF64*), *CstF77*, and *Pcf11p*-similar protein 4 (*PCFS4*) are likely to participate in the cleavage and polyadenylation of mRNA (Macknight *et al.*, 1997; Schomburg *et al.*, 2001; Simpson *et al.*, 2003; Xing *et al.*, 2008; Liu *et al.*, 2010), while glycine-rich RNA-binding protein 7 (*GRP7*), *GRP8*, pre-mRNA processing protein 8 (*PRP8*), *PRP39-1*, and serine/arginine-rich 45 (*SR45*) are involved in RNA splicing (Ali *et al.*, 2007; Wang *et al.*, 2007; Streitner *et al.*, 2008; Marquardt *et al.*, 2014). For instance, *FCA* can interact with *FY* to regulate RNA 3' end-processing (Simpson *et al.*, 2003). *LD* is part of the transcriptional regulation category. It is localized to the nucleus and contains a glutamine-rich region at its C-terminus that is similar to the glutamine-rich domains found in several other transcription factors (Lee *et al.*, 1994; Aukerman *et al.*, 1999). The last category is involved in the epigenetic process. One of the repressors of *FLC* (*FLD*) has an important role in the histone deacetylation of *FLC* chromatin. Mutation of *FLD* results in the hyperacetylation of histone in the *FLC* chromatin (He *et al.*, 2003).

We previously reported that two RNA-binding proteins, *CCCH* zinc-finger and *K* homology (*KH*) domain proteins 1 and 2 (*KHZ1* and *KHZ2*), are involved in the regulation of flowering time in Arabidopsis (Yan *et al.*, 2017). In this study, we investigated the molecular mechanisms by which these two proteins regulated flowering. Our results indicate that *KHZ1* and *KHZ2* are novel members of the autonomous pathway that may function as homodimers or heterodimers to repress *FLC* expression primarily through the inhibition of its splicing efficiency. Taken together, our study demonstrates that *KHZ1* and *KHZ2* play important roles in regulating flowering via the autonomous pathway.

Materials and methods

Plant materials and growth conditions

All Arabidopsis (*Arabidopsis thaliana*) used in this study were of ecotype Columbia-0. The transgenic and mutants *KHZ1* and *KHZ2* lines were described previously (Yan *et al.*, 2017). Plants were cultivated at 22 °C with a relative humidity of 60% under long-day (16 h light/8 h dark) or short-day (8 h light/16 h dark) conditions.

Flowering time measurements

Flowering time was measured by recording the number of rosettes and cauline leaves, and the days when the first flower became visible. The plants were cultivated under long-day or short-day conditions. For GA treatment, 20 μ M GA was administered by spraying plants growing under long days twice a week until the first flower appeared. For vernalization treatment, the Arabidopsis seeds were held at 4 °C for 22 d or 6 weeks in darkness and subsequently transferred to long-day conditions (Koornneef *et al.*, 1991). At least 20 plants were measured and averaged for each measurement and statistical analysis. One-way ANOVA was used for statistical analysis of flowering time (* P <0.05; ** P <0.01).

Generation of *flc* single mutants and *khz1 khz2 flc* triple mutants

Two short guide (sgRNA) targets (Target1₃₆₈₃, TCAAGATCCTTGATCGATA-TGG; and Target2₄₀₅₅, AATATATGCAGCAAGCTTG-TGG) in the *FLC* gene were selected and cloned into the pHEE2A-TRI vector (Wang *et al.*, 2015). The CRISPR (clustered regularly interspaced short palindromic repeat) constructs were transformed into the WT and *khz1 khz2* mutants to generate the *flc* mutants and *khz1 khz2 flc* triple mutants, respectively. To obtain the homozygous mutants, we amplified the fragment surrounding the target by PCR using gene-specific primers (FLC-cri-LP/RP), and the product was analyzed by restriction enzyme digestion (*Cla*I for Target1 and *Hind*III for Target2). Fragments that could not be digested were sequenced. The specific primer pair zCas9-IDF3-2/rbcS_E9t-IDR was used to identify non-transgenic lines. The primers used in this study are presented in Supplementary Table S1 at JXB online.

Plasmid constructions

To generate the *FT* overexpression plasmid, the full-length coding sequence (CDS) was cloned into the binary vector pSuper 1300, which contains the *Cauliflower mosaic virus* (CaMV) 35S promoter. The resulting construct is referred to as 35S::*FT*. For the yeast two-hybrid assay, the full-length CDSs for *KHZ1* and *KHZ2* were cloned into the pGBKT7 and pGADT7 vectors (Clontech) to generate the *KHZ1-BD*, *KHZ2-BD*, *KHZ1-AD*, and *KHZ2-AD* constructs. For the bimolecular fluorescence complementation (BiFC) assay in Arabidopsis protoplasts, the full-length CDSs for *KHZ1* and *KHZ2* were cloned into the pSPYNE173 and pSPYCE(M) vectors to produce *YN-KHZ1*, *YN-KHZ2*, *YC-KHZ1*, and *YC-KHZ2* constructs (Waadt *et al.*, 2008). PCR amplification, enzyme digestion, and plasmid transformation were performed using standard protocols. Primers used for cloning are presented in Supplementary Table S2.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated as previously reported (Oñate-Sánchez and Vicente-Carbajosa, 2008). First-strand cDNA was synthesized from 1 μ g of total RNA using the M-MLV reverse transcription system (Takara, Beijing, China) according to the manufacturer's instructions (Promega, China). qRT-PCR was performed in a volume of 20 μ l using the ABI7500 Fast Real-time PCR system (Applied Biosystems, USA) and SYBR Green I Master Mix (Takara). *AT4G34270* served as the internal control. All qRT-PCR primers are listed in Supplementary Table S3.

Subcellular localization assay

Transgenic plants roots of 7-day-old seedlings grown on Murashige and Skoog medium were used for subcellular localization assay. According to the method previously described (Kim and Somers, 2010), the plasmids were transformed into Arabidopsis protoplasts to observe the transient expression of the green fluorescent protein (GFP) fusion proteins with a Zeiss 710 Meta laser scanning confocal microscope (Zeiss LSM710, Germany).

Splicing efficiency analysis of *FLC*

Splicing efficiency was measured as previously described (Marquardt *et al.*, 2014; Wu *et al.*, 2016). Unspliced primers were designed to specifically span the intron–exon junction, while the spliced primers were designed to cross the exon–exon junction. Total RNA (2.5 μ g) was reverse transcribed using specific primers and oligo d(T)₁₈ to generate the spliced and unspliced forms of *FLC*. qRT-PCR was performed to determine the levels of spliced and unspliced *FLC*. *AT4G34270* was used as an internal control. The spliced/unspliced ratio was calculated as the splicing efficiency. Data are presented as the means of three biological replicates. All the specific primers used for this analysis are presented in Supplementary Table S3.

Yeast two-hybrid assay

Yeast two-hybrid analysis was performed as previously described (Zhu *et al.*, 2011). *KHZ1-BD* and *KHZ2-BD* served as the bait constructs, and *KHZ1-AD* and *KHZ2-AD* were the prey constructs. The empty vectors (pGBKT7 and pGADT7) were used as negative controls. Bait and prey constructs were co-transformed into yeast strain AH109. Transformants were picked from SD–Leu–Trp (SD–LW) medium, gradient-diluted, and plated on SD–Leu–Trp–His–Ade (SD–LWHA) medium. Pictures were taken after 4 d of growth at 30 °C. The experiment was performed with three independent biological replicates.

BiFC assay

BiFC assay was performed as described previously (Ho *et al.*, 2009). Arabidopsis protoplasts were isolated from 4-week-old WT plants and transformed with different construct combinations. The protoplasts were incubated at 22 °C for 16–18 h, and then the BiFC signal was imaged using the channel for yellow fluorescent protein (YFP) fluorescence (Zeiss LSM710). Three independent biological replicates were performed.

Results

KHZ1 and *KHZ2* are not components of the photoperiod pathway

Our previous study revealed that *KHZ1* (AT3G12130) and *KHZ2* (AT5G06770) positively regulate flowering time redundantly in Arabidopsis (Yan *et al.*, 2017). To explore the underlying mechanisms of the KHZ proteins in regulating flowering, we analyzed the flowering phenotype of *khz1 khz2* double mutant plants under different photoperiod conditions. The flowering times of WT and *khz1 khz2* plants were 80 d and 102 d, respectively, under short-day conditions, and 35 d and 43 d, respectively, under long-day conditions. Thus, the double mutant flowered later than the WT plants under both conditions. The late-flowering phenotype of the *khz1 khz2* plants was confirmed by analyzing the number of leaves (Fig. 1A, B). These results demonstrated that the *khz1 khz2* plants could respond to the photoperiod, suggesting that the KHZs might not be components of the photoperiod pathway.

Expression of the key genes of the photoperiod pathway were also examined, including *FLAVINBINDING*, *KELCHREPEAT*, *F-BOX1* (*FKF1*), *TIMING OF CAB EXPRESSION1* (*TOC1*), *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *ZEITLUPE* (*ZTL*), *LATE ELONGATED HYPOCOTYL* (*LHY*), and *EARLY FLOWERING3* (*ELF3*). As shown in Fig. 1C, expression of the tested photoperiod genes did not significantly differ

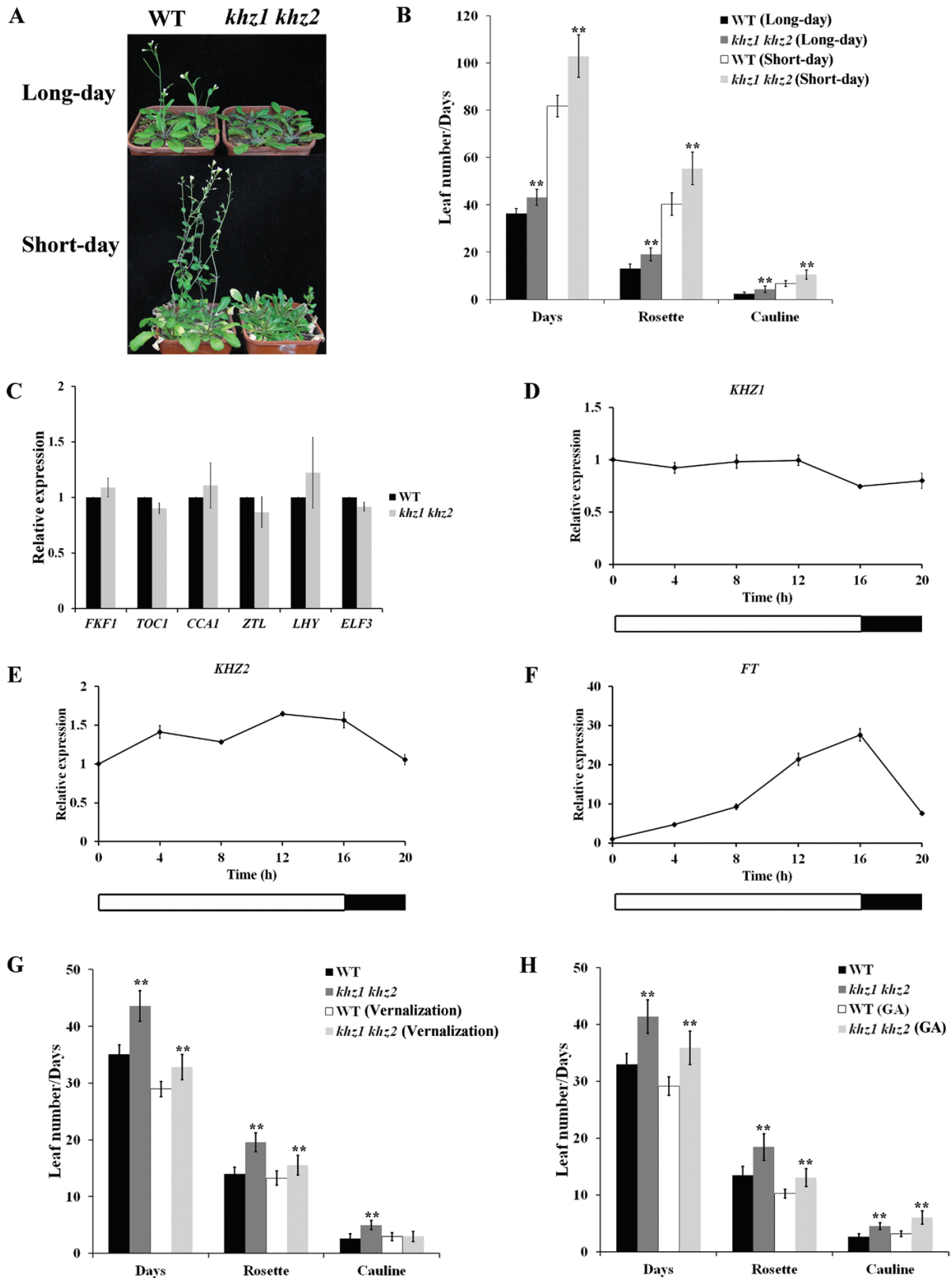


Fig. 1. *khz1 khz2* flowering time analysis in response to different conditions. (A) The flowering phenotype of *khz1 khz2* plants under long-day and short-day conditions. The pictures were taken on days 35 and 80 under long-day and short-day conditions, respectively. (B) The flowering time and leaf number of WT and *khz1 khz2* plants grown under long-day and short-day conditions. (C) *FKF1*, *TOC1*, *CCA1*, *ZTL*, *LHY*, and *ELF3* transcript levels in 10-day-old WT and *khz1 khz2* plants. (D–F) Relative gene expression of *KHZ1*, *KHZ2*, and *FT*. Ten-day-old WT seedlings under long-day conditions were harvested every 4 h. Data are presented as the mean \pm SD ($n=3$). (G) The flowering time and leaf number of WT and *khz1 khz2* plants after vernalization treatment for 22 d. (H) The flowering time and leaf number of WT and *khz1 khz2* plants following GA treatment. The flowering time and leaf number were recorded for at least 20 individual plants and are presented as the mean \pm SD (one-way ANOVA, $**P<0.01$). (This figure is available in color at *JXB* online.)

between the *khz1 khz2* and WT plants. The expression patterns of *KHZ1*, *KHZ2*, and *FT* were also assessed in WT plants under long-day conditions using qRT-PCR. The results showed that the expression of *KHZ1* and *KHZ2* was maintained at a constant level, whereas *FT* was rhythmic with a peak expression level in the late afternoon (Fig. 1D–F). These results further indicated that *KHZ1* and *KHZ2* are not components of the photoperiod pathway.

khz1 khz2 is responsive to vernalization and GA treatments

To examine the response of the *khz1 khz2* plants to vernalization treatment, the seeds were incubated in a cold room at 4 °C for 22 d in darkness, and then the seedlings were transferred to long-day conditions. Vernalization-treated *khz1 khz2* plants flowered earlier than untreated plants (32 d versus 43 d, respectively), albeit slightly later than the vernalization-treated WT plants (29 d) (Fig. 1G). The leaf numbers were also decreased by vernalization treatment, although the *khz1 khz2* plants had more leaves than the WT plants (Fig. 1G). Vernalization treatment for 6 weeks produced similar results (Supplementary Fig. S1A, C). Consistent with the late-flowering phenotype of the *khz1 khz2* double mutant, the *FLC* mRNA level was higher in untreated *khz1 khz2* compared with WT plants, but the *FLC* mRNA level was drastically reduced after vernalization (Supplementary Fig. S1B). These results showed that the *khz1 khz2* plants were sensitive to vernalization.

To analyze the effects of GA, a solution containing 20 μM GA₃ was sprayed twice a week on the growing plants until they flowered. Similar to vernalization treatment, GA significantly promoted the flowering of *khz1 khz2* plants. Flowering of GA-treated *khz1 khz2* plants was initiated at 35 d with a rosette leaf number of ~13, while the untreated plants flowered at 41 d with a rosette leaf number of ~18. However, the GA-treated *khz1 khz2* plants still flowered later than the GA-treated WT plants (Fig. 1H). These results demonstrated that the *khz1 khz2* plants could respond to GA.

Loss of *FLC* function eliminates the late-flowering phenotype of *khz1 khz2* plants

It is known that the mutants of the autonomous pathway genes flower late under both long-day and short-day conditions and are highly sensitive to vernalization and GA (Martinez-Zapater and Somerville, 1990; Koornneef *et al.*, 1991; Mouradov *et al.*, 2002; Moon *et al.*, 2003; Lim *et al.*, 2004). Because *KHZ1* and *KHZ2* share all these properties, we speculate that they may function in the autonomous pathway. The autonomous pathway genes promote flowering by repressing the central flowering repressor gene *FLC* (Cheng *et al.*, 2017). Mutations in autonomous pathway genes combined with *flc* can rescue the late-flowering phenotype (Rouse *et al.*, 2002; Eom *et al.*, 2018). Thus, we investigated whether flowering time could be restored in *khz1 khz2* mutants after combination with a mutation in *FLC*. We generated *khz1 khz2 flc-cri* triple mutants and *flc-cri* single mutants using CRISPR/Cas9 technology. Two sgRNA targets for *FLC* were selected and cloned into the

pHEE2A-TRI vector and then transformed into *khz1 khz2* and WT plants. We identified homozygous *khz1 khz2 flc-cri* and *flc-cri* lines among T₂ progeny by sequencing. From this process, we obtained two *khz1 khz2 flc-cri* triple mutants and two *flc-cri* single mutants, of which *khz1 khz2 flc-cri1* and *flc-cri1* resulted from sgRNA Target1, *khz1 khz2 flc-cri2* and *flc-cri2* resulted from sgRNA Target2 (Supplementary Fig. S2A–D). Among these mutants, *flc-cri1*, *flc-cri2*, and *khz1 khz2 flc-cri2* were non-transgene mutants (Supplementary Fig. S2E, F). The detection of *FLC* expression showed that the abundance of *FLC* mRNA was increased in *khz1 khz2*, and decreased in *flc-cri* and *khz1 khz2 flc-cri* mutants compared with the WT (Fig. 2B). Evaluation of the flowering phenotype of the mutants under long-day conditions revealed that the *khz1 khz2 flc-cri* triple mutants flowered much earlier than the *khz1 khz2* double mutants, albeit slightly later than WT and *flc-cri* plants (Fig. 2A, C). We also examined the effect of *flc* mutation on the number of rosette leaves. The *khz1 khz2 flc-cri* triple mutants had a few more rosette leaves than WT and *flc-cri* plants, but far fewer rosette leaves than *khz1 khz2* plants (Fig. 2D). The flowering phenotype of *khz1 khz2 flc-cri* triple mutants resembled the phenotype of *flc* plants, which flowered slightly earlier than WT plants. These results demonstrated that *FLC* loss of function largely rescued the late-flowering phenotype displayed by *khz1 khz2* plants, which revealed that *KHZ1* and *KHZ2* are novel members of the autonomous pathway. Because mutation of *FLC* could not fully rescue the late-flowering phenotype of *khz1 khz2*, indicating that *FLC* is not the sole target of *KHZs*, there might be other factors involved in the pathway.

FT can mediate signals from the photoperiod, vernalization, and autonomous pathways (Han *et al.*, 2008; Blümel *et al.*, 2015), and *FT* expression is repressed by *FLC* (Lee *et al.*, 2000; Michaels *et al.*, 2005; Helliwell *et al.*, 2006). In addition, *FT* expression is significantly decreased in *khz1 khz2* mutant plants (Yan *et al.*, 2017). Therefore, we investigated whether *FT* overexpression could rescue the late-flowering phenotype observed for the *khz1 khz2* plants. We introduced the 35S::*FT* plasmid into WT and *khz1 khz2* plants to obtain the transgenic lines *FT-OE* and *khz1 khz2*×*FT-OE*, respectively. The *FT-OE* lines had two types of expression, low (1.5- to 3-fold) and high (1500- to 30 000-fold) (Supplementary Fig. S3A–D). *FT* expression in the *khz1 khz2*×*FT-OE* lines was similar to that in the low expression *FT-OE* lines (Fig. 2F; Supplementary Fig. S3B). The *FT-OE* high expression lines flowered much earlier than the WT (19 d versus 34 d), while the flowering time and rosette leaves of the *FT-OE* low expression lines and *khz1 khz2*×*FT-OE* lines were similar to those of the WT plants (Fig. 2E, G, H; Supplementary Fig. S3E, F).

The splicing of *FLC* pre-mRNA is altered in *khz1 khz2* mutants

The subcellular localization of an RNA-binding protein and its biochemical function are closely related (Heintzen *et al.*, 1997; Lorkovic and Barta, 2002). If the protein localized to the cytoplasm, it may regulate mRNA stability or translation. Alternatively, if the protein localized to the nucleus, it is more likely to modulate the pre-mRNA processing (e.g. pre-mRNA

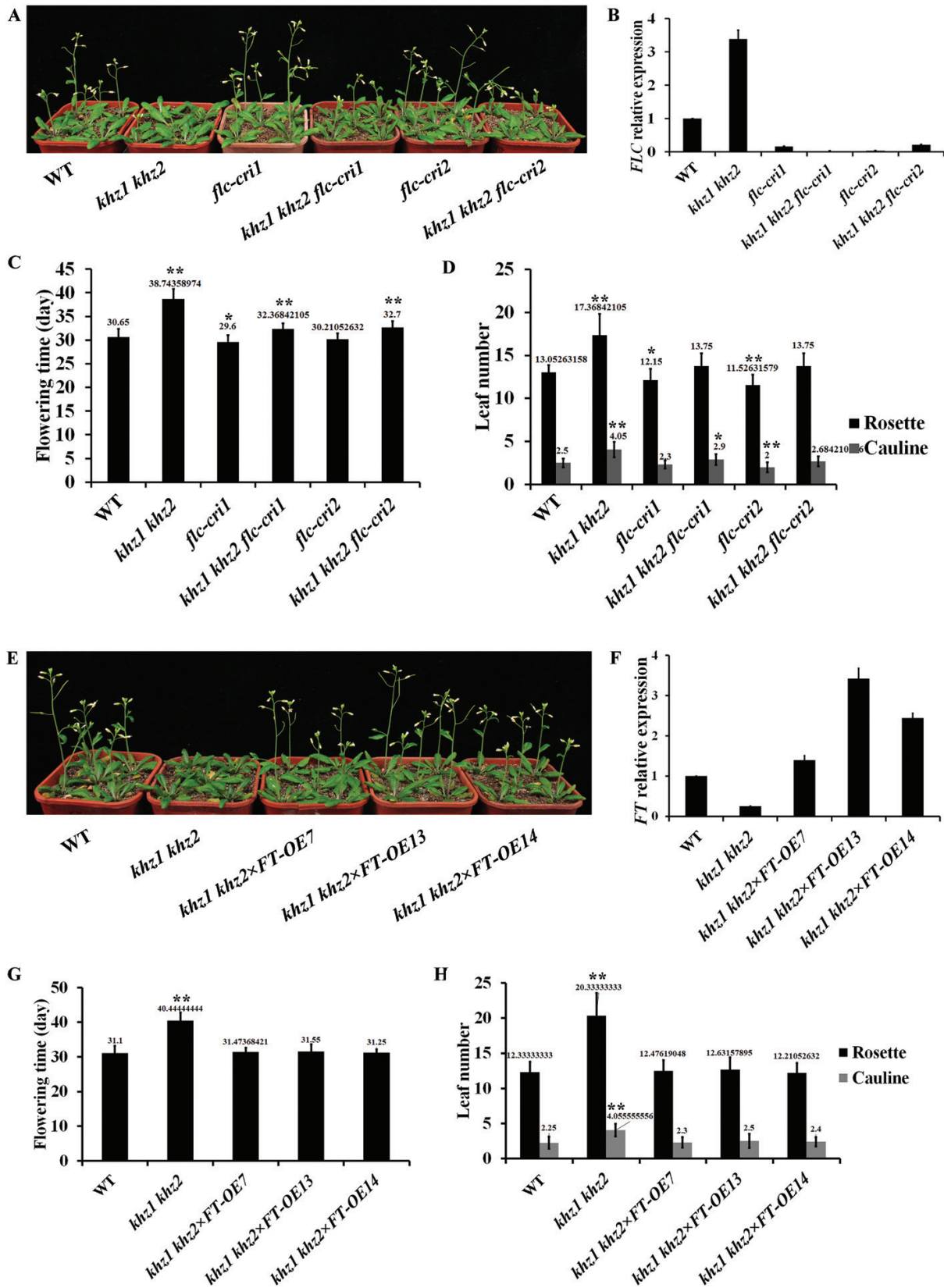


Fig. 2. Mutation of *FLC* or overexpression of *FT* rescued the *khz1 khz2* late-flowering phenotype. (A) The flowering phenotype of WT, *khz1 khz2*, *flc-cri1*, *khz1 khz2 flc-cri1*, *flc-cri2*, and *khz1 khz2 flc-cri2* plants under long-day conditions. Pictures were taken on day 32. (B) *FLC* transcript levels in 10-day-old WT, *khz1 khz2*, *flc-cri1*, *khz1 khz2 flc-cri1*, *flc-cri2*, and *khz1 khz2 flc-cri2* seedlings. (C) The flowering time and (D) leaf number of plants grown under long-day conditions. (E) The flowering phenotype of WT and *khz1 khz2 × FT-OE* plants under long-day conditions. Pictures were taken on day 32. (F) *FT* transcript levels in 10-day-old WT, *khz1 khz2*, and *khz1 khz2 × FT-OE* seedlings. (G) The flowering time and (H) leaf number of WT and *khz1 khz2 × FT-OE* plants grown under long-day conditions. In (B) and (F), data are presented as the mean \pm SD ($n=3$). The flowering time and leaf number were recorded for at least 20 individual plants and are presented as the mean \pm SD (one-way ANOVA, $*P<0.05$, $**P<0.01$). (This figure is available in color at JXB online.)

splicing) or mRNA transport (Lim *et al.*, 2004). Here, we found that the GFP protein was evenly distributed in the cytoplasm and nucleus (Fig. 3A), while KHZ1::GFP and KHZ2::GFP were localized to the nucleus (Yan *et al.*, 2017). In particular, KHZ1::GFP and KHZ2::GFP were localized to nuclear dots (Fig. 3B, C) that look like the nuclear speckles where many pre-mRNA splicing factors are localized (Wu *et al.*, 2016; Xin *et al.*, 2017). The nuclear dots of KHZ1::GFP were much brighter than those of KHZ2::GFP (Fig. 3B, C). Because KHZ1 and KHZ2 promote flowering mainly through the repression of *FLC* expression, we examined whether the splicing of *FLC* pre-mRNA was altered in *khz1 khz2* mutants. We evaluated the splicing efficiency of specific introns of four different *FLC* isoforms (Fig. 4A). The splicing efficiency of intron 6 in isoform 1 (*FLC.1*) was significantly increased in the double mutant plants (Fig. 4B, D), while the splicing efficiency of *FLC.1* in the *KHZ1* overexpression lines was significantly decreased (Fig. 4D). The splicing efficiency of intron 6 in isoform 2 (*FLC.2*) was also significantly increased in the double mutant plants (Fig. 4B).

A previous report demonstrated that *FLC.1* is the most abundantly expressed *FLC* isoform that encodes a functional FLC protein, while the other isoforms are expressed at much lower levels (Wu *et al.*, 2016). Therefore, we examined the expression of *FLC* and the splicing efficiency of *FLC.1* in WT, *khz1*, *khz1 khz2*, *flc-cri2*, *khz1 khz2 flc-cri2*, and *KHZ1-OE4* plants. We found that the expression of *FLC* and the splicing efficiency of *FLC.1* had a linear correlation (Fig. 4C–E) with a correlation coefficient as high as 0.99. These results further suggested that KHZ1 and KHZ2 down-regulated the *FLC* transcript level primarily by repressing the splicing efficiency of *FLC.1*.

KHZ1 and KHZ2 interact with each other or themselves in vitro and in vivo

The autonomous pathway accelerates flowering primarily by inhibiting the central flowering repressor *FLC* (Sang *et al.*,

2017). Several autonomous pathway genes, such as *FCA*, *FLD*, *FLK*, *FPA*, *FVE*, *FY*, and *LD*, have been widely studied in this pathway (Simpson, 2004; Marquardt *et al.*, 2006; Srikanth and Schmid, 2011; Cheng *et al.*, 2017). However, none of these factors regulated one another at the mRNA level (Simpson, 2004). In the present study, we examined the relationship between the *KHZ* genes and other autonomous pathway genes in *khz1 khz2* plants. No significant differences in the expression of autonomous pathway genes were observed between the *khz1 khz2* and WT plants (Supplementary Fig. S4), indicating that *KHZ1* and *KHZ2* are not the regulators of the other autonomous pathway genes.

Previous studies had reported that some autonomous pathway proteins (e.g. *FCA*/*FY*) can physically interact and regulate RNA processing (Simpson *et al.*, 2003). Thus, we determined whether KHZ1 and KHZ2 could interact with other autonomous pathway proteins using the yeast two-hybrid assay. The results showed that none of the tested proteins could interact with KHZ1 or KHZ2 (Supplementary Fig. S5). Interestingly, KHZ1 could interact with itself and KHZ2 in yeast (Fig. 5A). Using the BiFC assay, we also demonstrated that KHZ1 could interact with KHZ2 and itself in Arabidopsis protoplasts (Fig. 5B). In addition, KHZ2 could also interact with itself and KHZ1 in yeast and Arabidopsis protoplasts (Fig. 5). These results suggested that KHZ proteins could interact with each other to form heterodimers or with themselves to form homodimers.

Discussion

We previously reported that the RNA-binding proteins KHZ1 and KHZ2 regulate flowering (Yan *et al.*, 2017). However, to date, the underlying mechanisms have not been clear. Here, we provide an in-depth characterization of the mechanisms by which the KHZ proteins modulate flowering.

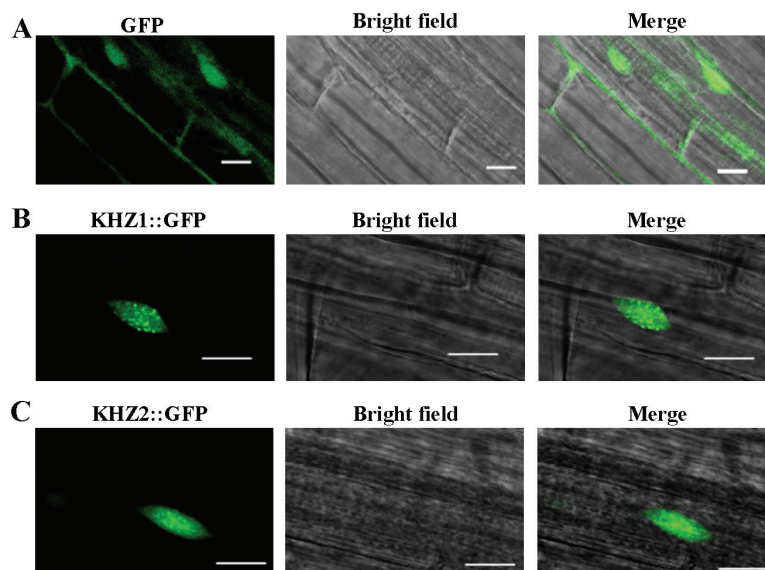


Fig. 3. KHZ1::GFP and KHZ2::GFP are localized to nuclear dots in 7-day-old Arabidopsis root tips. (A) The GFP protein was evenly distributed in the cytoplasm and nucleus. The KHZ1::GFP (B) and KHZ2::GFP (C) proteins were distributed to nuclear dots. Scale bar=10 μ m. (This figure is available in color at JXB online.)

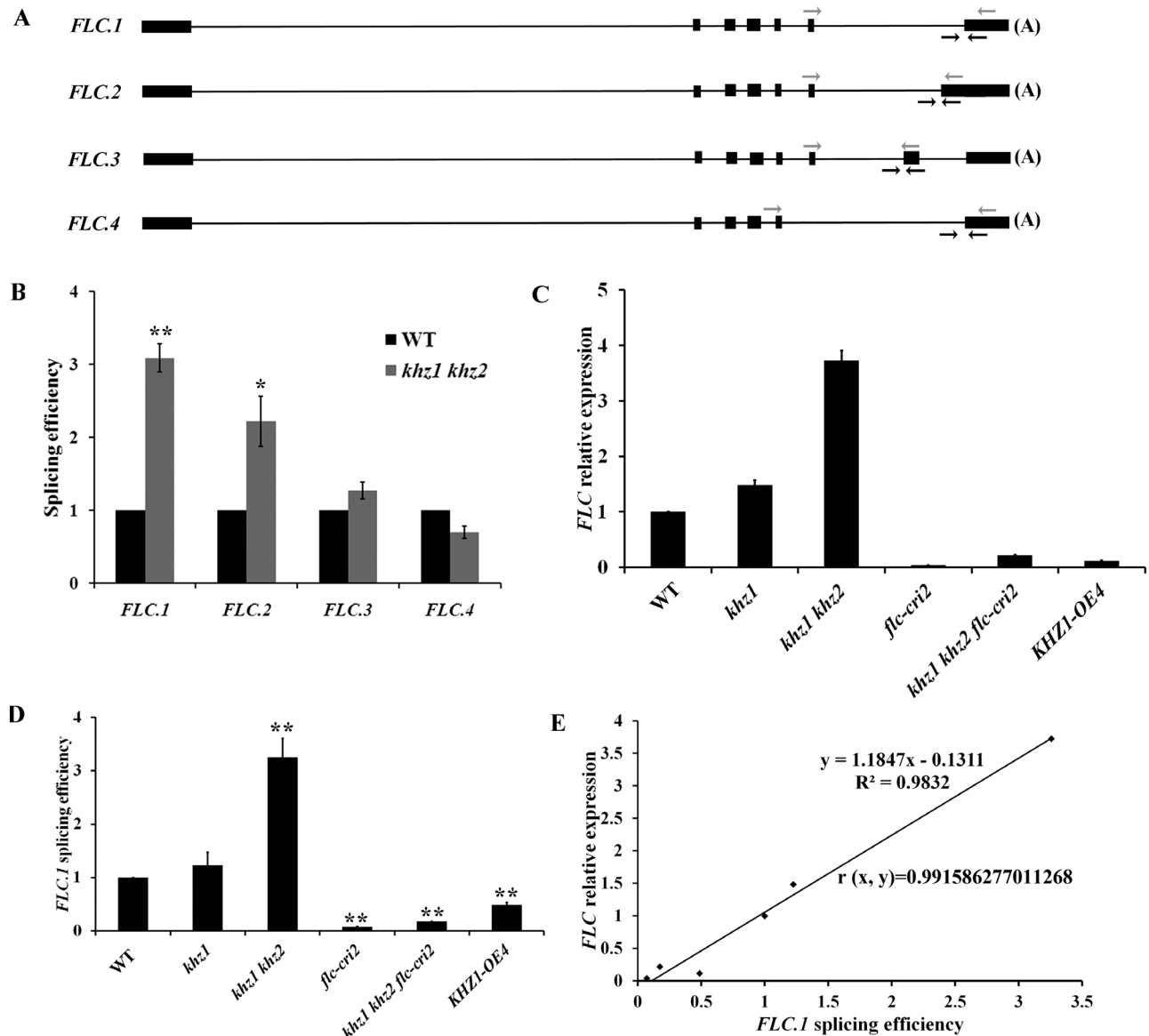


Fig. 4. Analysis of *FLC* splicing efficiency. (A) Schematic of *FLC* isoforms. The locations of the primers are indicated by arrows. The primers with gray arrows detected the spliced pre-mRNA, while the primers with black arrows detected the unspliced pre-mRNA. The spliced/unspliced ratio was calculated as the splicing efficiency. (B) Examination of splicing efficiency of select introns associated with the different *FLC* isoforms in WT and *khz1 khz2* mutant plants. (C) *FLC* transcript levels in 10-day-old WT, *khz1*, *khz1 khz2*, *flc-cri2*, *khz1 khz2 flc-cri2*, and *KHZ1-OE4* seedlings. (D) The splicing efficiency of *FLC.1* in 10-day-old WT, *khz1*, *khz1 khz2*, *flc-cri2*, *khz1 khz2 flc-cri2*, and *KHZ1-OE4* seedlings. (E) The relationship between *FLC* relative expression and *FLC.1* splicing efficiency. The correlation coefficient was calculated by the Excel formula: =CORREL (array-x, array-y). * $P < 0.05$, ** $P < 0.01$, data are presented as the mean \pm SD for three replicates.

KHZ1 and *KHZ2* are autonomous pathway factors

Autonomous pathway genes (e.g. *FCA*, *FLD*, *FLK*, *FPA*, *FVE*, *FY*, and *LD*) are thought to function largely in parallel with the vernalization pathway upstream of *FLC* and the photoperiod pathway (Boss *et al.*, 2004; Simpson, 2004; Quesada *et al.*, 2005). They repress *FLC* and thus act as promoters of the floral transition (Simpson, 2004). Mutations in these genes are generally recessive and delay flowering under both long-day and short-day conditions, and the inhibitory effect of the mutations can be overcome by vernalization or exogenous GA treatments (Abou-Elwafa *et al.*, 2011). The regulation of flowering time by the autonomous pathway is mediated by *FLC* and the floral integrators downstream of *FLC*, such as *FT*. Mutations of *FLC*

can eliminate the late-flowering phenotype caused by mutations in autonomous pathway genes (Koornneef *et al.*, 1994; Lee *et al.*, 1994; Sanda and Amasino, 1996; Michaels and Amasino, 2001; Abou-Elwafa *et al.*, 2011). Similar to these autonomous pathway factors, the *khz1 khz2* double mutant plants displayed a late-flowering phenotype under both long-day and short-day conditions when compared with the WT plants, and they were sensitive to vernalization and GA treatment (Fig. 1). Mutation of *FLC* in the *khz1 khz2* double mutants significantly accelerated flowering. Moreover, the combination of *khz1 khz2* with *FT-OE* lines fully rescued the late-flowering phenotype of *khz1 khz2* double mutants (Fig. 2). Taken together, it can be concluded that Arabidopsis *KHZ1* and *KHZ2* are novel factors of the autonomous pathway.

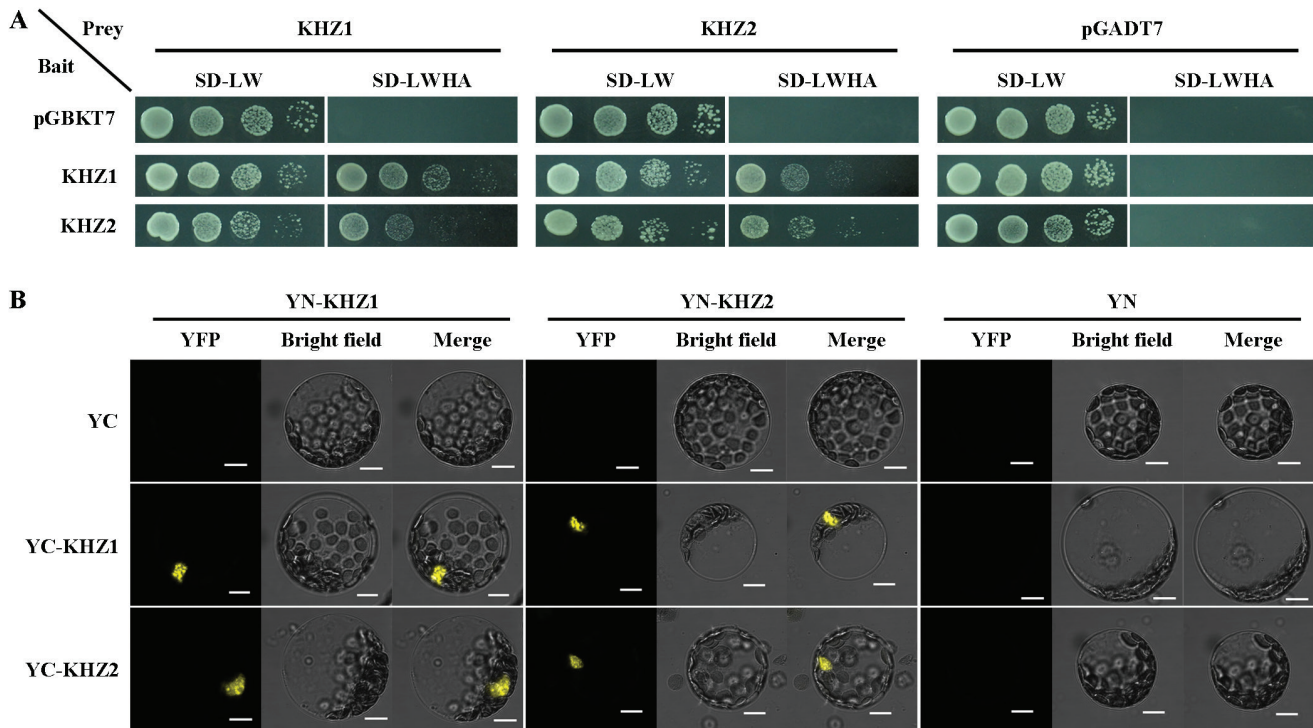


Fig. 5. Interactions of KHZ1 and KHZ2. (A) The interactions of KHZ1 and KHZ2 in yeast. Three independent experiments were performed with similar results. (B) KHZ1 and KHZ2 interactions in Arabidopsis protoplasts detected by BiFC assay. Scale bar=10 μ m. Three independent experiments were performed with similar results. (This figure is available in color at JXB online.)

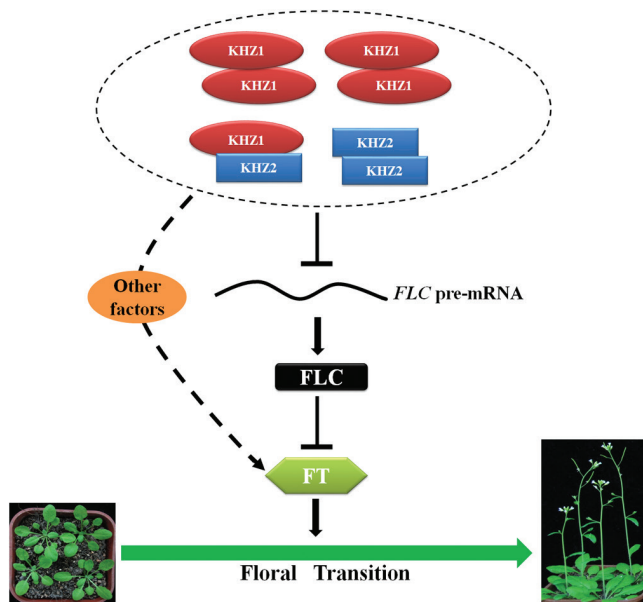


Fig. 6. A working model of KHZ proteins in the regulation of the floral transition. KHZ proteins may function as homodimers or heterodimers (most prominently KHZ1–KHZ1 homodimers) to inhibit the splicing efficiency of *FLC*, which in turn relieves the inhibition of *FT*. These events promote the transition from vegetative to reproductive growth. Mutation of *FLC* cannot fully rescue the late-flowering phenotype of *khz1 khz2*, but *FT* overexpression can completely rescue the late-flowering phenotype of *khz1 khz2*, which indicates that KHZ proteins may also regulate other factors which then modulate FT to promote flowering. (This figure is available in color at JXB online.)

Although vernalization promotes the flowering time significantly, it cannot fully rescue the late-flowering phenotype of *khz1 khz2* mutants. Consistent with this effect, the *khz1 khz2*

flc-cri triple mutants flowered slightly later than the WT and the *flc-cri* mutants. We hypothesized that *FLC* is the main, but not sole, target of the KHZs; there may be other factors that participate in KHZ-mediated flowering regulation. This situation is similar to that of other autonomous pathway genes, such as *FLK* (Lim *et al.*, 2004), *LD* (Lee *et al.*, 1994), *FLD* (Sanda and Amasino, 1996), and *PCFS4* (Xing *et al.*, 2008). Furthermore, the autonomous pathway is known to mediate the effects of environmental cues (e.g. ambient growth temperature) (Blázquez *et al.*, 2003; Halliday *et al.*, 2003) and internal signals by ultimately modulating *FT* expression (Lim *et al.*, 2004). Thus, we speculated that KHZs might also affect these factors to promote flowering.

KHZ1 and *KHZ2* regulate the splicing efficiency of *FLC*

RNA-binding proteins play key roles in RNA metabolism (i.e. splicing, polyadenylation, mRNA stability, mRNA localization, and translation) (Lee and Schedl, 2006). The CCCH domain and the KH domain are two critical RNA-binding elements in RNA-binding proteins. Proteins that contain these domains have multiple functions in regulating plant development and hormone and stress responses. For instance, HUA1, which contains six CCCH-type zinc finger motifs, is a regulator of stamen and carpel identities (Li *et al.*, 2001). The KH domain RNA-binding protein, HOS5, is important for pre-mRNA splicing in Arabidopsis and contributes to the tolerance against various stresses and the responses to the hormones abscisic acid (ABA) and jasmonic acid (JA) (Xiong *et al.*, 1999; Chen *et al.*, 2013; Thatcher *et al.*, 2015).

RNA-binding proteins are also involved in the autonomous pathway. *FLC* levels are primarily regulated by autonomous pathway proteins through RNA processing, post-transcriptional regulation, and chromatin modification (Cheng *et al.*, 2017; Eom *et al.*, 2018). For example, FCA and FPA, two RNA recognition motif-containing RNA-binding proteins, regulate alternative polyadenylation of antisense RNAs and 3' end formation of *FLC* (Macknight *et al.*, 1997; Schomburg *et al.*, 2001; Liu *et al.*, 2007; Hornyik *et al.*, 2010; Liu and Mara, 2010). *FLK*, an RNA-binding protein with three KH domains, is part of the autonomous pathway and represses *FLC* at the transcriptional level or through RNA-mediated chromatin regulation (Veley and Michaels, 2008; Ripoll *et al.*, 2009). To date, the precise molecular mechanisms by which the majority of autonomous pathway proteins regulate *FLC* expression are unknown.

KHZ1 and KHZ2 are two RNA-binding proteins that contain both the CCCH and KH domains, and they are both localized to the nucleus (Yan *et al.*, 2017). In the current study, we found that KHZ1 and KHZ2 are specifically localized to nuclear dots (Fig. 3), which are very like nuclear speckles, where pre-mRNA splicing factors are localized (Spector and Lamond, 2011). This result indicated that the KHZ proteins might be involved in the regulation of the pre-mRNA process, similar to other autonomous pathway proteins.

In Arabidopsis, *FLC* is the key repressor of flowering and the integrator of the autonomous pathway. It has four isoforms, and the most abundantly expressed isoform is *FLC.1*, which encodes the functional *FLC* protein (Wu *et al.*, 2016; Cheng *et al.*, 2017). Our study revealed that the KHZ proteins repressed the splicing efficiency of *FLC* pre-mRNA, especially *FLC.1*, and there was a positive linear correlation between splicing efficiency and *FLC* levels (Fig. 4). These results suggest that the KHZ proteins promote flowering mainly by suppressing the production of mature *FLC* mRNA.

KHZ proteins may function as dimers

The flowering time of plants is affected by well-delineated factors that are involved in complex genetic networks in *A. thaliana*. Eom *et al.* (2018) demonstrated that the factors in the autonomous pathway were independent of each other at the transcriptional level. For example, mutation in *FLK* does not affect the expression of *FCA* or *FPA*, and the expression of *FLK* is normal in *fca* or *fpa* mutants (Lim *et al.*, 2004). These findings are consistent with our own results that the *khz1 khz2* double mutants did not alter the expression of other autonomous pathway genes. We believed that KHZ proteins might act in parallel to other autonomous pathway proteins to promote *FLC* expression.

At the translation level, Simpson *et al.* (2003) revealed that a physical interaction between FCA and FY is important for RNA processing. In this study, KHZ1 and KHZ2 did not physically interact with any of the other autonomous pathway proteins tested (Supplementary Fig. S5). They did, however, interact with each other and themselves (Fig. 5), suggesting that KHZ1 and KHZ2 form hetero- or homodimers. In our previous work, we showed that KHZ1 and KHZ2 are redundant for promoting flowering, but KHZ1 activity is more predominant than that of KHZ2 (Yan *et al.*, 2017). Therefore, we proposed that KHZ1–KHZ1

is the primary dimer, with dimers utilizing KHZ2 used as an alternative.

In our work, we identified KHZ1 and KHZ2 as novel members of the autonomous pathway. They may act as homo- or heterodimers to repress *FLC* mRNA levels through the suppression of the splicing efficiency of *FLC.1*, which subsequently leads to the up-regulation of *FT* expression (Fig. 6). Based on the fact that the late-flowering phenotype of *khz1 khz2* was almost completely rescued by mutation of *FLC* or completely rescued by *FT* overexpression, the KHZ proteins might also promote flowering by modulating *FT* through other factors in parallel. Our results suggest that KHZ1 and KHZ2 are involved in the regulation of the floral transition through the autonomous pathway. This study sheds more light on the molecular mechanisms of the autonomous pathway in Arabidopsis and provides a better understanding of KH and CCCH domain-containing proteins. However, further investigation is still needed to fully understand the exact mechanism.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Flowering time analysis after vernalization treatment for 6 weeks.

Fig. S2. The generation of *fca-cris* and *khz1 khz2 fca-cris* mutants by CRISPR/Cas9.

Fig. S3. The analysis of *FT*-overexpressing lines.

Fig. S4. Expression levels of autonomous pathway genes in 10-day-old WT and *khz1 khz2* seedlings.

Fig. S5. Interaction analysis of KHZ1 and KHZ2 with autonomous pathway proteins in yeast.

Table S1. Primers used for genotyping.

Table S2. Primers used for plasmid construction.

Table S3. Primers used for qRT-PCR.

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Author contributions

YH conceived the research, and supervised the experiments; ZY designed the experiments and prepared the figures; ZY and HS performed the experiments; YL and MJ provided technical assistance; and ZY, HS, and YH wrote the manuscript.

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