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RESEARCH ARTICLE

Analysis of temperature effects on the protein accumulation of the FT–FD module using newly generated Arabidopsis transgenic plants

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Abstract

Arabidopsis flowering is dependent on interactions between a component of the florigens FLOWERING LOCUS T (FT) and the basic leucine zipper (bZIP) transcription factor FD. These proteins form a complex that activates the genes required for flowering competence and integrates environmental cues, such as photoperiod and temperature. However, it remains largely unknown how FT and FD are regulated at the protein level. To address this, we created *FT* transgenic plants that express the N-terminal FLAG-tagged FT fusion protein under the control of its own promoter in *ft* mutant backgrounds. *FT* transgenic plants complemented the delayed flowering of the *ft* mutant and exhibited similar *FT* expression patterns to wild-type Col-0 plants in response to changes in photoperiod and temperature. Similarly, we generated *FD* transgenic plants in *fd* mutant backgrounds that express the N-terminal MYC-tagged FD fusion protein under the *FD* promoter, rescuing the late flowering phenotypes in the *fd* mutant. Using these transgenic plants, we investigated how temperature regulates the expression of FT and FD proteins. Temperature-dependent changes in FT and FD protein levels are primarily regulated at the transcript level, but protein-level temperature effects have also been observed to some extent. In addition, our examination of the expression patterns of FT and FD in different tissues revealed that similar to the spatial expression pattern of *FT*, *FD* mRNA was expressed in both the leaf and shoot apex, but FD protein was only detected in the apex, suggesting a regulatory mechanism that restricts FD protein expression in the leaf during the vegetative growth phase. These transgenic plants provided a valuable platform for investigating the role of the FT–FD module in flowering time regulation.

KEYWORDS
Arabidopsis, FD, flowering, FLOWERING LOCUS T, temperature

1 | INTRODUCTION

Flowering, also known as floral transition, is a crucial developmental process in the life cycle of plants, through which they acquire

Kyung-Ho Park and Sol-Bi Kim contributed equally to this study.

flowering competence to produce reproductive organs (Jin & Ahn, 2021; Kinoshita & Richter, 2020; Osnato et al., 2022). Given that flowering must occur under optimal conditions to ensure maximum seed production and progeny survival, the timing of flowering is critical for plants to synchronize flower development with environmental conditions. Plants have evolved sophisticated signaling networks that integrate endogenous and environmental signals, including nutrition, water availability, temperature, and photoperiod, in order to determine the correct time for flowering (Li et al., 2021; Romera-Branchat et al., 2014; Song et al., 2015).

Photoperiod, which refers to the period of time in a day that plants are exposed to light, is one of the major environmental cues influencing flowering time (Osnato et al., 2022; Song et al., 2015). Flowering is induced by a critical day length, which varies depending on the genetic background. Arabidopsis flowering is accelerated under long-day (LD) conditions and delayed under short-day (SD) conditions (Andrés & Coupland, 2012; Hayama & Coupland, 2004; Wolabu et al., 2016; Xu et al., 2021). Photoperiodic flowering in Arabidopsis is highly dependent on the expression of the *FLOWERING LOCUS T* (*FT*) gene (Andrés & Coupland, 2012; Osnato et al., 2022; Song et al., 2015). *FT* is substantially expressed in the leaf under LDs, whereas under SDs, it is strongly suppressed, resulting in delayed flowering. CONSTANS (CO) transcription factor, along with its upstream regulator GIGANTEA (GI) and FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1), activates the *FT* transcription under LDs, whereas FLOWERING LOCUS C (FLC) functions as a floral repressor, preventing the transcription of *FT* and other genes required for flowering under non-inductive SDs (Crevillen & Dean, 2011; Shim et al., 2017; Wang et al., 2021).

Temperature is another key environmental cue influencing flowering time in plants. In Arabidopsis, exposure to low temperatures causes a delay in flowering, whereas high temperatures accelerate it (Balasubramanian et al., 2006; Jin & Ahn, 2021; Mcclung et al., 2016). The floral integrator *FT*, previously known for its role in photoperiodic flowering pathways, is also critical for thermoresponsive flowering regulation. As temperature increases, the transcription of the *FT* gene is strongly induced, promoting flowering, even under non-inductive SD conditions (Jin & Ahn, 2021; Mcclung et al., 2016). The transcriptional control of the *FT* gene in response to temperature involves several transcription factors. Among them, two MADS-box transcription factors, FLOWERING LOCUS M (FLM) and SHORT VEGETATIVE PHASE (SVP) play a pivotal role in thermoresponsive flowering by repressing the *FT* gene transcription across a wide range of ambient temperatures (Hwan Lee et al., 2014; Jin & Ahn, 2021; Lee et al., 2007). Conversely, PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) acts with its interacting partner CO to positively regulate thermoresponsive flowering by stimulating *FT* gene transcription at high temperatures (Fernández et al., 2016; Kumar et al., 2012).

FT protein is a key component of florigen that functions as a mobile signal translocated from the leaf to the shoot apical meristem (SAM) (Collani et al., 2019; Corbesier et al., 2007; Jaeger & Wigge, 2007; Romera-Branchat et al., 2020; Taoka et al., 2013; Turck et al., 2008). Under flowering-inductive conditions, the FT protein is expressed in phloem companion cells of leaves and then transported to the SAM through the phloem sieve elements (Corbesier et al., 2007; Jackson & Hong, 2012; Jaeger & Wigge, 2007; Mathieu et al., 2007). At the SAM, FT interacts with the basic leucine zipper (bZIP) transcription factor FD to form a protein complex that activates multiple floral meristem identity genes and floral homeotic genes such as *APETALA1* (*AP1*), *FRUITFUL* (*FUL*), and *LEAFY* (*LFY*), which are essential for the acquisition of flowering competence (Abe et al., 2005; Jung et al., 2016; Liu et al., 2021; Wigge et al., 2005). Although FD may bind to target gene promoters without the interaction with FT, the presence of FT and its paralog TWIN SISTER OF FT (TSF) enhances the enrichment of FD on a subset of target loci, including *AP1* and *FUL* genes (Abe et al., 2005; Jang et al., 2009; Yamaguchi et al., 2005).

The FT–FD module is highly responsive to environmental stimuli that are primarily incorporated by modulating *FT* transcription (Jung et al., 2016; Yamaguchi, 2021). However, the post-transcriptional regulation of the FT–FD module in response to environmental changes is not well understood. Recent studies have shown that FT protein interacts with phosphatidylglycerol (PG), a negatively charged phospholipid on cellular membranes in phloem companion cells and that this interaction is temperature-sensitive (Jaillais & Parcy, 2021; Nakamura et al., 2014; Susila et al., 2021). At low temperatures, FT is sequestered in membrane compartments of phloem companion cells in a PG-dependent manner, limiting the loading of FT into the phloem sieve elements and ultimately delaying flowering. Conversely, at high temperatures, FT is released into the phloem sieve element, transported to the SAM, and promotes flowering (Susila et al., 2021). Furthermore, it has been observed that FT protein undergoes protease-dependent cleavage and FD protein is phosphorylated by calcium-dependent kinases, suggesting the possibility of these processes being involved in environmental responses (Abe et al., 2005; Kawamoto et al., 2015; Kim et al., 2016; Taoka et al., 2011). However, additional research is required to determine the exact mechanisms and their significance in the context of environmental regulation of the FT–FD module.

Using transgenic plants in which FT and FD proteins are expressed as forms tagged with a specific peptide is the most common approach for examining how the FT–FD module is regulated by environmental factors at the protein level. However, in previously reported transgenic plants, both genes were expressed under the control of 35S or tissue-specific promoters rather than their native promoters (Abe et al., 2005; Chen et al., 2021; Chen & Penfield, 2018; Mathieu et al., 2007; Wigge et al., 2005), which do not accurately reflect gene expression patterns *in planta*. Particularly, the *FT* gene transcription is controlled by a long promoter containing multiple evolutionarily conserved regulatory regions, as well as the first intron and the 3′ downstream region of the *FT* gene (Adrian et al., 2010; Helliwell et al., 2006; Takada & Goto, 2003; Zicola et al., 2019), so it is difficult to clone a very large area of genomic sequence for the generation of *FT* transgenic plants. Making antibodies specific to FT and FD would be the easiest way to detect two proteins, but it is also challenging because there are many FT and FD paralogs in Arabidopsis (D'Aloia et al., 2011; Yamaguchi et al., 2005).

In this study, we generated *FT* transgenic plants expressing the N-terminal FLAG-tagged FT fusion protein under the *FT* native promoter in the *ft-10* mutant background. The *FT* transgenic lines successfully recovered the delayed flowering of the *ft-10* mutant, validating the functionality of the FLAG-FT fusion proteins *in planta*.

Similarly, we created *FD* transgenic lines in *fd-2* mutant backgrounds expressing the N-terminal MYC-tagged FD fusion protein under the *FD* native promoter, which also recovered the delayed flowering phenotype of the *fd-2* mutant. Using these transgenic plants, we examined the expression patterns of FT and FD proteins under different temperature conditions. Changes in both *FT* and *FD* gene expression at different temperatures were primarily controlled at the transcriptional level, whereas the accumulation of FT and FD proteins tended to be partially governed at the protein level. Furthermore, we observed that *FD* mRNA levels, like *FT*, were higher in the leaf compared with the shoot apex, but FD protein was not detected at all in the leaf, suggesting that there is an unknown mechanism that strongly inhibits the FD protein expression in the leaf at the vegetative growth stage.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

All *Arabidopsis thaliana* lines used in this study were in Columbia (Col-0) background. Seeds were sterilized with 75% ethanol with .1% Triton X-100 and washed in 70% ethanol. Sterilized seeds were cold-

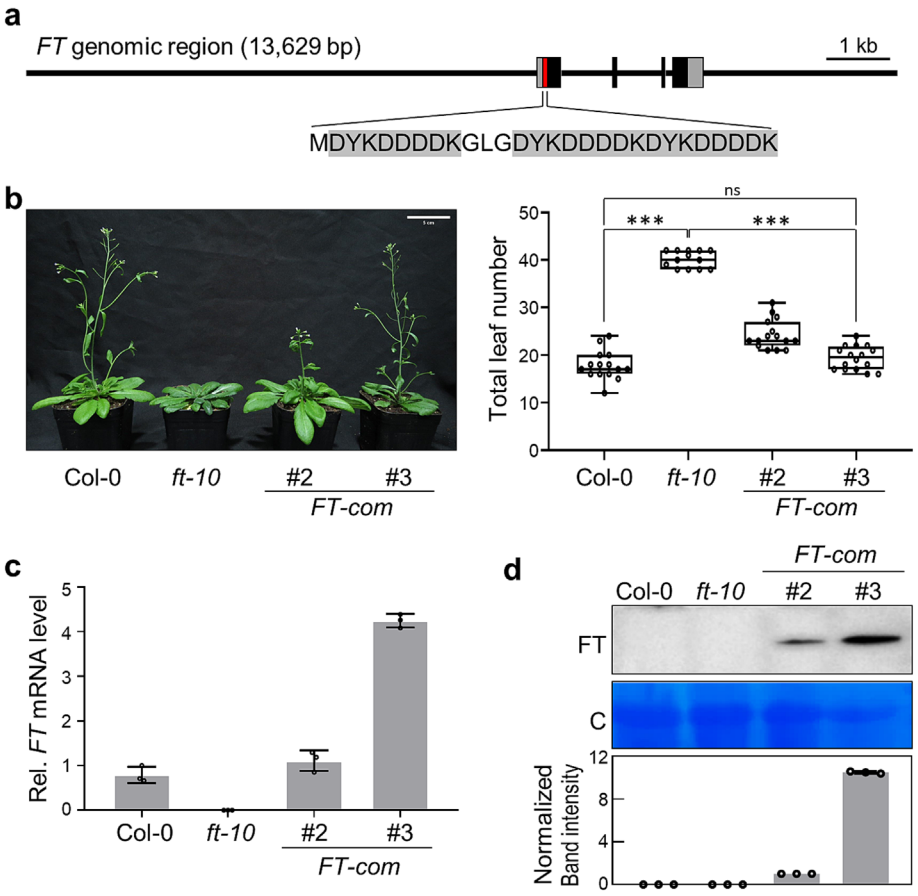

FIGURE 1 Generation of *FT* transgenic plants (*FT*-com) with *ft-10* mutant phenotypes complemented. In *FT*-com transgenic plants, the *FT* gene was expressed in the *ft-10* mutant background as an N-terminal FLAG-tagged fusion form under the control of its own promoter. (a) *FT* genomic region of 13.6 kb was used for the preparation of *FT-com* transgenic plants. Black boxes represent exons, and gray boxes represent untranslated regions (UTRs). The DNA fragment encoding three copies of the FLAG-tag sequence, denoted by the red box, is located to the right of the 5′-UTR. The shaded amino acid sequences at the bottom represent the FLAG-tag sequences of the FLAG-FT fusion protein. Scale bar, 1 kb. (b) Flowering time of *FT*-com transgenic plants. Seven-week-old plants grown in soil under long-day (LD) conditions were photographed (left panel). Col-0 and *ft-10* were added as control. The total leaf numbers of at least 15 plants were measured for each plant genotype (right panel). Each box is located between the lower and upper quartiles in the box plots, and the whiskers indicate the minimum and maximum values. The central bar in each box represents the median, and individual circles represent the total leaf number on each plant. Asterisks indicate a significant difference (***$P \leq .001$; ns, not significant; one-way ANOVA and Tukey's multiple comparisons tests). (c) RT-qPCR analysis of *FT* expression in *FT*-com transgenic plants. Whole seedlings grown at 22°C for 10 days under LD conditions were harvested at the zeitgeber time (ZT) 16 time point for total RNA isolation. RT-qPCR was performed to measure the *FT* mRNA levels. RT-qPCR was conducted in biological triplicates, and data for quantification were presented as mean ± standard error of mean (SEM). (d) Immunoblot analysis of FT protein abundance in *FT*-com transgenic plants. Whole seedlings grown at 22°C for 10 days under LD conditions were harvested at the ZT16 time point for protein extraction. A part of the membrane stained with Coomassie brilliant blue (C) was added as a loading control. The immunoblot analysis was conducted in biological triplicates, and data for quantification were presented as mean ± SEM.

stratified at 4°C in darkness for 3 days and allowed to germinate on 1/2 × Murashige and Skoog (MS) agar or in soil under SD (8-h light/16-h dark cycles) or LD (16-h light/8-h dark cycles) conditions with cool white light illumination at a light intensity of 120 μmol photons m^{-2} s^{-1}. Plants were grown in a controlled culture room set at either 22 or 27°C with a relative humidity of 60%. The *fd-2* (SALK_013288) and *ft-10* (GABI_290E08) mutants and transgenic lines have been reported previously (Collani et al., 2019; Jung et al., 2016).

For tissue-specific expression analysis of FT and FD proteins, we initially generated transgenic plants expressing both FT and FD proteins by crossing *FT* transgenic line #3 with *FD* transgenic line #8 (Figures 1 and 3). The *ft-10* and *fd-2* mutations were verified by PCR genotyping assays using specific primer sets (LP: 5′- GGTGGAGAAGACCTCAGGAAC -3′, RP: 5′- TTTTGGGAGACAAATTGATGC-3′, and LB: 5′- ATATTGACCATCATACTCATTGC-3′ for the *ft-10* mutant; LP: 5′- TTAAGTGCAAATGACCCGTTC -3′, RP: 5′- TTCCAAACTTCTTCCATGGTG -3′, and LB: 5′-ATTTTGCCGATTTCGGAAC-3′ for the *fd-2* mutant) (O'Malley et al., 2015). To sample AP, RL, and SA, we used 21-day-old plants grown under long-day conditions (16L/8D). The aerial parts (AP) exhibited floral bud formation at the center of the plant, but the inflorescence stem did not elongate. The AP were divided into rosette leaves (RL) and shoot apices (SA) and sampled them at the zeitgeber time (ZT) 16 time point. To sample SA, all the leaves including petioles were dissected from the AP of the plants using forceps and scissors.

2.2 | Generation of transgenic plants

To generate the FT construct, 13.6-kb genomic fragments of *FT* along with their respective promoters and downstream regions of the three prime untranslated regions (3′-UTRs) were amplified with a pair of primers, 5′-CACCAAAGACAAGATTCAGAATCATCCTCC-3′ and 5′-CGCCCATACGATATTAACTTTG-3′. In a similar way, 7.8 kb of *FD* genomic region was also amplified with a pair of primers, 5′-CACCTACAGAAGCTCTTTCAGATGATCAGCAACAGTC-3′ and 5′-TCAGCGATACTATTGGTGTCTCTTTTCC-3′. Both *FT* and *FD* genomic fragments were cloned into pENTR-D-TOPO vector (ThermoFisher Scientific) according to the manufacturer's procedure. To incorporate FLAG- and MYC-tag coding sequences, NEBuilder HiFi assembly (E2621X, New England Biolabs) strategy was

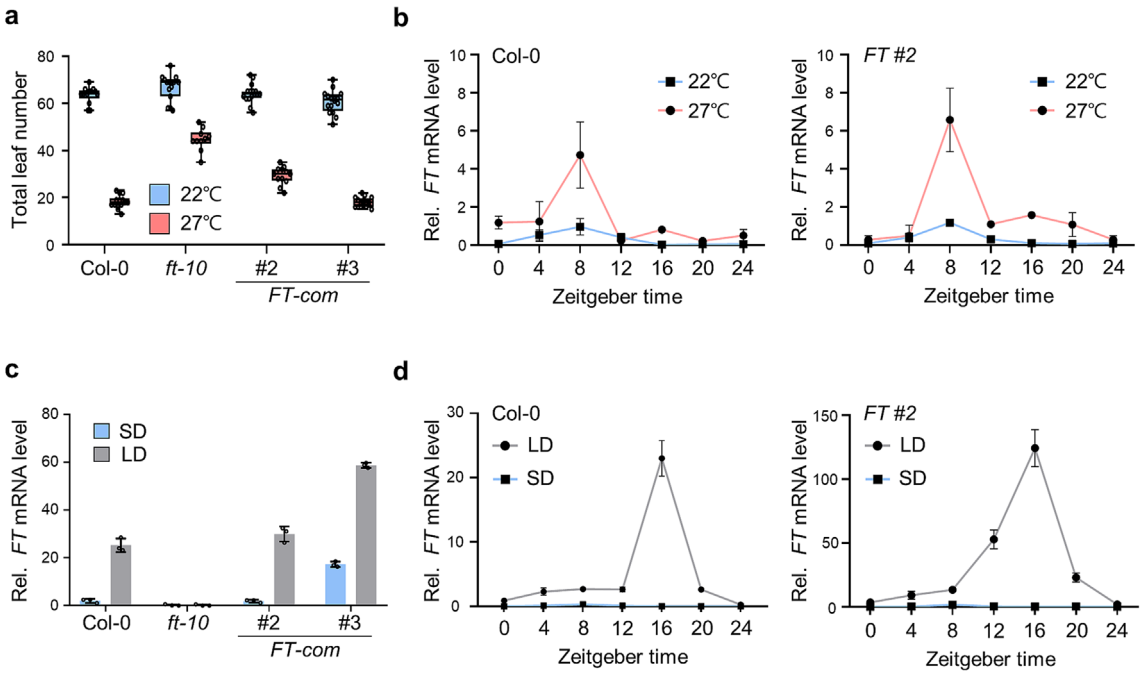

FIGURE 2 Thermal and photoperiodic responses of *FT*-com transgenic plants. In (b)–(d), levels of the *FT* mRNA were analyzed by RT-qPCR. The RT-qPCR reactions were conducted in biological triplicates and data for quantification were presented as mean ± SEM. In (b) and (d), the *FT-com* line #2 plants were used to examine the diurnal expression patterns of *FT* mRNA under different growth conditions. (a) Flowering time of *FT-com* transgenic plants at different temperatures. Plants were grown at 22 and 27°C under short-day (SD) conditions. Flowering time was measured by counting the total leaf numbers of at least 15 plants for each plant genotype. Each box is located between the lower and upper quartiles in the box plots, and the whiskers indicate the minimum and maximum values. The central bar in each box represents the median, and individual circles represent the total leaf number on each plant. (b) Diurnal expression patterns of *FT* gene at different temperatures. Seven-day-old seedlings grown at 22°C were incubated further for 7 days at either 22 or 27°C. Whole seedlings were harvested at the indicated zeitgeber time points for total RNA extraction. (c) Photoperiodic effect on *FT* expression in *FT-com* transgenic plants. Whole seedlings grown under SD and LD conditions for 14 days were harvested for total RNA extraction at the ZT16 and ZT8 time points, respectively. (d) Diurnal expression patterns of *FT* gene under different photoperiodic conditions. Seedlings grown at 22°C for 14 days were harvested at the indicated zeitgeber time points for total RNA extraction.

used to insert them into the entry plasmids containing *FT* and *FD* genomic regions, respectively. The resulting *FT*- and *FD*-containing entry plasmids were then recombined using LR clonase (11791019, Invitrogen) into the gateway binary vectors pJHA212K and pJHA212B, respectively (Yoo et al., 2005). The binary constructs were introduced into *A. tumefaciens* strain GV3101 and transformed into *ft-10* and *fd-2* mutants using the floral dipping method (Zhang et al., 2006). The *FT-com* and *FD-com* transgenic plants were isolated by selecting for kanamycin and basta resistance, respectively, and propagated to obtain single insertion lines that rescued the delayed flowering phenotype of *ft-10* and *fd-2* mutants.

2.3 | Flowering time measurements

For flowering time measurements, seeds were stratified in .1% agar solution for 3 days at 4°C in the dark and then incubated in soil under LD or SD conditions after germination. To examine the temperature effect on flowering, 10-day-old plants grown at 22°C under SDs were transferred to either 22 or 27°C under SD conditions and grown until flowering. Flowering time was determined by counting the number of rosette and cauline leaves when the main inflorescence stem reached 1 cm in length. At least 15 plants were used to measure the flowering time of each genotype.

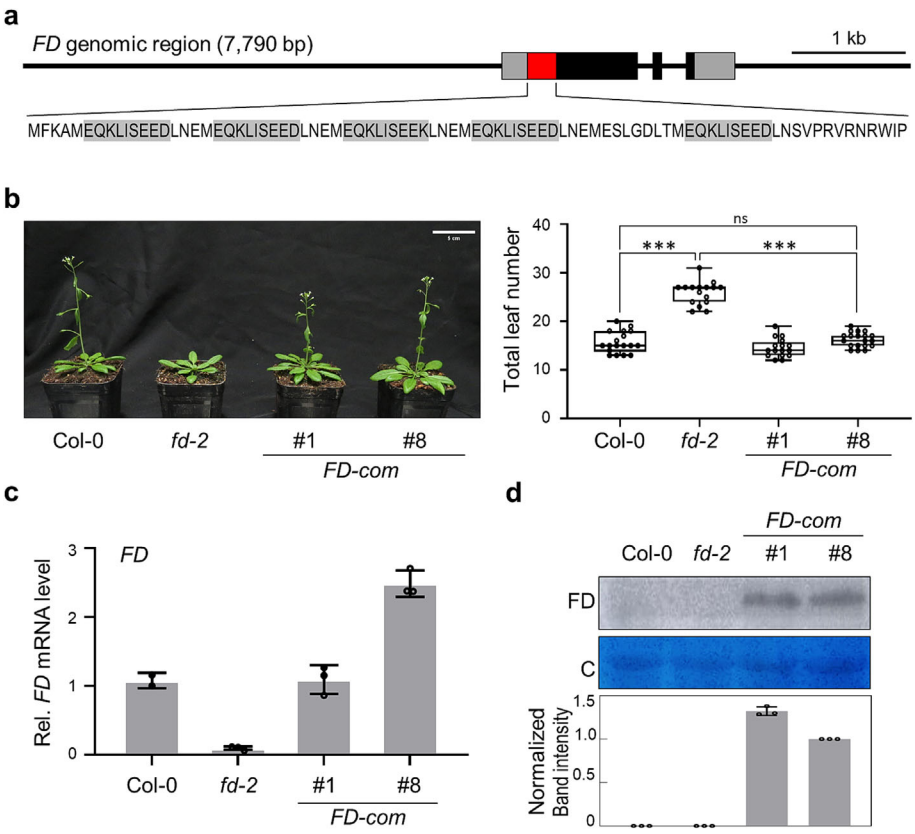

FIGURE 3 Generation of *FD* transgenic plants (*FD*-com) with *fd-2* mutant phenotypes complemented. In *FD*-com transgenic plants, the *FD* gene was expressed in the *fd-2* mutant background as an N-terminal MYC-tagged fusion form under the control of its own promoter. (a) *FD* genomic region of 7.79 kb was used for the preparation of *FD-com* transgenic plants. Black boxes represent exons, and gray boxes represent untranslated regions (UTRs). The DNA fragment encoding three copies of the MYC-tag sequence, denoted by the red box, is located to the right of the 5′-UTR. The shaded amino acid sequences at the bottom represent the MYC-tag sequences of the MYC-FD fusion protein. Scale bar, 1 kb. (b) Flowering time of *FD*-com transgenic plants. Seven-week-old plants grown in soil under long-day (LD) conditions were photographed (left panel). Col-0 and *fd-2* were added as control. The total leaf numbers of at least 15 plants were measured for each plant genotype (right panel). Each box is located between the lower and upper quartiles in the box plots, and the whiskers indicate the minimum and maximum values. The central bar in each box represents the median, and individual circles represent the total leaf number on each plant. Asterisks indicate a significant difference (***$P \leq .001$; ns, not significant; one-way ANOVA and Tukey's multiple comparisons tests). (c) RT-qPCR analysis of *FD* expression in *FD*-com transgenic plants. Whole seedlings grown at 22°C for 10 days under LD conditions were harvested at the ZT16 time point for total RNA isolation. RT-qPCR was performed to measure the *FT* mRNA levels. RT-qPCR was conducted in biological triplicates, and data for quantification were presented as mean ± SEM. (d) Immunoblot analysis of FD protein abundance in *FD*-com transgenic plants. Whole seedlings grown at 22°C for 10 days under LD conditions were harvested at the ZT16 time point for protein extraction. A part of the membrane stained with Coomassie brilliant blue (C) was added as a loading control. The immunoblot analysis was conducted in biological triplicates, and data for quantification were presented as mean ± SEM.

2.4 | Total RNA extraction, cDNA synthesis, and gene expression analysis

Total RNA was extracted using TRI Reagent (ThermoFisher Scientific), and cDNA was synthesized from 2 μg of total RNA using RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific) according to the manufacturer's recommendations. cDNAs were diluted to 60 μL with H_2O, and 1 μL of diluted cDNA was used for PCR amplification. Quantitative PCR reactions (qPCR) were performed in 96-well blocks using TOPreal SYBR Green qPCR PreMIX with low ROX (Enzynomic) in a final volume of 20 μL. Gene expression levels were normalized relative to the *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*EIF4A*) gene (*At3g13920*). All qPCR reactions were conducted in biological triplicates. The comparative $\Delta\Delta C_T$ approach was used to evaluate the relative amounts of each amplified product in the samples (Livak & Schmittgen, 2001). The threshold cycle (C_T) for each reaction was automatically determined by the system's default parameters. The qPCR reactions for the *FT*, *FD*, and *EIF4A* genes were performed using specific primer sets (5′- AGGCCTTCTCAGGTTCAAAACAAGC and 5′-TGCCAAAGGTTGTTCCAGTTGTAGC for *FT*; 5′-GCAAGACTCAAGAGACAACAAG and 5′- CAAAATGGAGCTGTGGAAGAC for *FD*; 5′-TGACCACACAGTCTCTGCAA and 5′-ACCAGGGAGACTTGTTGGAC for *EIF4A*).

2.5 | Immunoblot analysis

Approximately 40 mg of the seedlings grown on MS-agar plates were used for the immunoblot analysis. Seedlings were collected and processed by grinding them in liquid nitrogen. To extract the proteins, the ground tissue was boiled in 2 × SDS loading buffer, separated through SDS-PAGE, and then transferred onto PVDF membranes (Bio-Rad). Anti-FLAG (F1804, Sigma-Aldrich) and anti-MYC (05-724, Sigma-Aldrich) antibodies were used to detect FT and FD proteins, respectively, along with anti-mouse antibody conjugated to horse radish peroxidase (HRP) (sc-516102, Santa Cruz Biotechnology). Super-Signal ECL western blotting substrate (34580, Thermo Scientific) was used for membrane development, and protein signals were detected using the ImageQuant LAS 500. Bio-Safe Coomassie G-250 stain (1610786, Bio-Rad) was used to obtain loading controls by staining the membranes. All immunoblot analyses were performed in biological triplicates and analyzed statistically.

2.6 | Quantification and statistical analysis

Data for quantification analysis were presented as mean ± standard error of mean (SEM). Statistical analyses were performed using Prism (GraphPad) software. To determine statistical significance, one-way ANOVA and Tukey's multiple comparisons tests were conducted. Differences were considered significant at $P < .001$ levels (Figures 1b and 3b).

3 | RESULTS

3.1 | Generation and characterization of *FT* transgenic plants

Multiple transgenic lines expressing the *FT* gene have been reported so far, but none of them have been designed so that the *FT* gene is expressed under the control of the *FT* native promoter (Abe et al., 2005; Chen et al., 2021; Chen & Penfield, 2018; Mathieu et al., 2007; Wigge et al., 2005). Given that the long promoter and 3′ downstream regions of the *FT* gene are required for the precise regulation of *FT* gene transcription (Adrian et al., 2010; Helliwell et al., 2006; Takada & Goto, 2003; Zicola et al., 2019), we cloned the *FT* genomic fragment for the generation of *FT* transgenic plants, which contains an 8-kb promoter upstream of the transcription start site and a 3-kb downstream region of the 3′-UTR. To detect functional FT proteins in the transgenic plants, the *FT* transgenic plants were created to express the N-terminal FLAG-tagged FT fusion protein in *ft-10* mutant background (Figure 1a).

We successfully generated two *FT* transgenic lines that restored the delayed flowering of the *ft-10* mutant to that of the wild-type Col-0 plant (Figure 1b). These transgenic plants will be referred to as *FT*-complementation (*FT-com*) plants in this study. To examine the expression of the *FT* gene in the *FT-com* plants, we conducted RT-qPCR and immunoblot assays. Of the two *FT-com* plants, line #3 exhibited higher levels of *FT* mRNA and FT protein (Figure 1c,d), supporting a stronger flowering phenotype in line #3 compared with line #2 (Figure 1b). Our data show that a construct containing over 13 kb of *FT* genomic fragment was successfully transformed into the *ft-10* mutant and that the *FT* gene expressed in the resulting *FT-com* plants is functional.

3.2 | Thermal and photoperiodic regulation on *FT* mRNA content in *FT* transgenic plants

We initially examine the response of *FT-com* plants to temperature changes by measuring their flowering time at 22 and 27°C under SD conditions. Please note that temperature effects on flowering time are more prominent under SD conditions than flowering-inductive LD conditions (Balasubramanian et al., 2006; Fernández et al., 2016). Our observations showed that there were no significant differences in flowering time at 22°C, but at 27°C, *FT-com* plants exhibited earlier flowering similar to the Col-0 plant compared with the *ft-10* mutant (Figure 2a). To verify if the *FT* gene plays a crucial role in the thermal acceleration of flowering in *FT-com* plants, the diurnal expression patterns of *FT* mRNA were examined in Col-0 and *FT-com* line #2 plants at both temperatures. At 22°C, Col-0 showed the highest level of *FT* mRNA expression at the end of the day, and this trend was more pronounced at 27°C (Figure 2b). Similar patterns were observed in the *FT-com* plant at both temperatures, indicating that the temperature response of the *FT-com* plant to regulated flowering time is normal.

In order to investigate the influence of photoperiod on *FT* expression in *FT-com* plants, we measured *FT* mRNA levels under SD and LD conditions at 22°C. Consistent with the previous flowering time data (Figures 1c and 2a), we found that *FT* expression was nearly absent in the *ft-10* mutant, whereas Col-0 and *FT-com* plants exhibited significantly high *FT* expression (Figure 2c). Furthermore, we analyzed the diurnal expression patterns of *FT* gene under both SD and LD conditions. Col-0 exhibited higher *FT* expression under LD conditions compared with SD conditions, with the highest *FT* mRNA level being detected at the end of the day under LD conditions (Figure 2d). The *FT-com* plant also exhibited similar diurnal patterns of *FT* gene expression under both SD and LD conditions, indicating that the *FT* gene is normally expressed in response to photoperiod as well as temperature in *FT-com* plants.

3.3 | Generation and characterization of *FD* transgenic plants

FD, a bZIP transcription factor, interacts with FT to promote the floral transition in response to environmental cues conveyed by FT from the leaf to SAM (Abe et al., 2005; Collani et al., 2019; Mathieu et al., 2007; Wigge et al., 2005). To examine how FD is environmentally regulated at the protein level, we created transgenic plants expressing the N-terminal MYC-tagged FD fusion protein under the native *FD* gene promoter in the *fd-2* mutant background. A 7.8 kb of the *FD* genomic fragment, which includes the promoter and the 3′ downstream region of the *FD* gene, was cloned and transformed into the *fd-2* mutant in a manner similar to how we created the *FT-com* plants (Figure 3a). We first measured flowering time under LD conditions at 22°C. The *fd-2* mutant showed significantly delayed flowering under our plant growth conditions, whereas *FD* transgenic plants exhibited a similar flowering to Col-0 (Figure 3b), showing that the late flowering phenotype of the *fd* mutant was rescued in the transgenic plants. These transgenic plants are hereafter referred to as *FD*-complementation (*FD-com*) plants.

To confirm the successful complementation in transgenic plants, we conducted RT-qPCR experiments to measure FD gene mRNA levels. The *fd-2* mutant exhibited minimal *FD* expression, whereas the *FD-com* plants showed *FD* mRNA levels similar to Col-0 (Figure 3c). Additionally, we performed immunoblot assays using anti-MYC antibody to verify if the FD protein was expressed as a MYC-tagged fusion form in the transgenic plants. MYC-FD fusion proteins were detected in both *FD-com* plants, but not in Col-0 and *fd-2* (Figure 3d). These data demonstrate that the MYC-FD fusion protein expressed in the *FD-com* plants is functional in rescuing the delayed flowering phenotype of the *fd* mutant.

3.4 | Thermal regulation of FT and FD protein accumulation

Compared with the extensive research on the photoperiodic regulation of the FT–FD module, the thermal regulation of this module remains less explored. Previous studies mainly focused on the

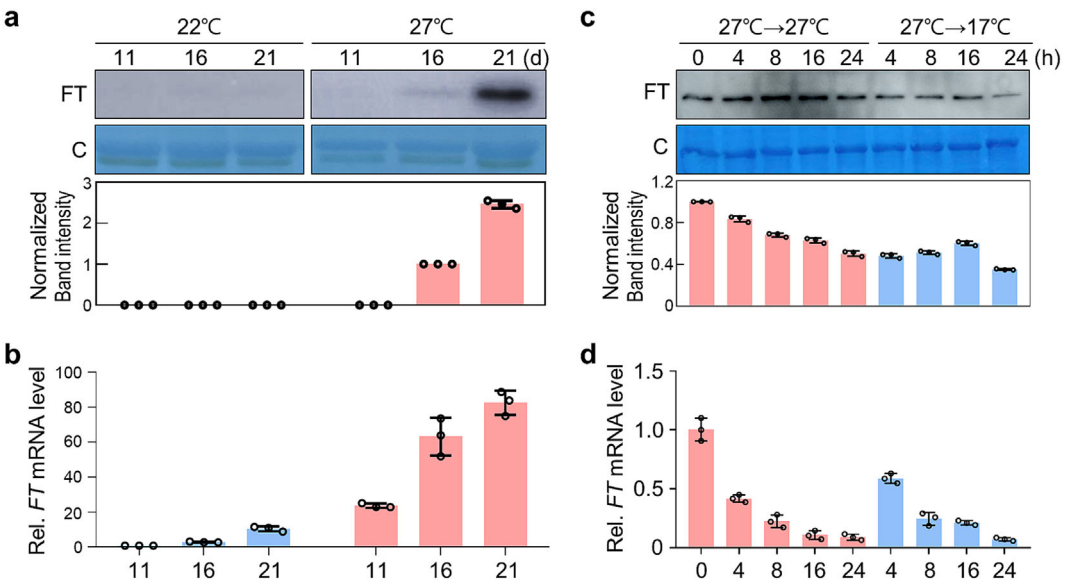

FIGURE 4 Thermal regulation of FT protein abundance. All immunoblot analysis and RT-qPCR were conducted in biological triplicates, and data for quantification were presented as mean ± SEM. As a loading control in (a) and (c), parts of the membranes stained with Coomassie brilliant blue (C) were added. In (a) and (c), the *FT-com* line #3 plants were used to examine FT protein abundance at different temperatures. (a) and (b) Temperature effects on temporal changes of *FT* expression levels under SD conditions. Whole seedlings of *FT-com* transgenic plants grown under SD conditions for indicated time durations at either 22 or 27°C were harvested at the ZT16 time point for immunoblot analysis (a). The levels of *FT* mRNA in Col-0 seedlings grown under the same conditions were also examined by RT-qPCR (b). (c) and (d) Effect of transient temperature changes on FT protein accumulation. Ten-day-old seedlings grown at 27°C under LD conditions were incubated further either at 27 or 17°C under continuous dark conditions for the indicated time. Whole seedlings were harvested for immunoblot analysis (c). RT-qPCR analysis of *FT* mRNA levels was also performed on Col-0 seedlings grown under the same conditions (d).

regulation of *FT* transcription by temperature (Brightbill & Sung, 2022; Jin & Ahn, 2021), and little is known about how the FD gene responds to temperature, particularly at the protein level. Using two types of transgenic plants, we examined how the FT–FD module activity is changed under different temperature conditions.

We first examined the temporal patterns of FT protein accumulation at different temperatures. FT proteins were not detected under SD conditions at both 22 and 27°C on day 11. At 22°C, FT protein was undetectable even in the late growth stages, whereas at 27°C, the level of FT protein increased dramatically as the days

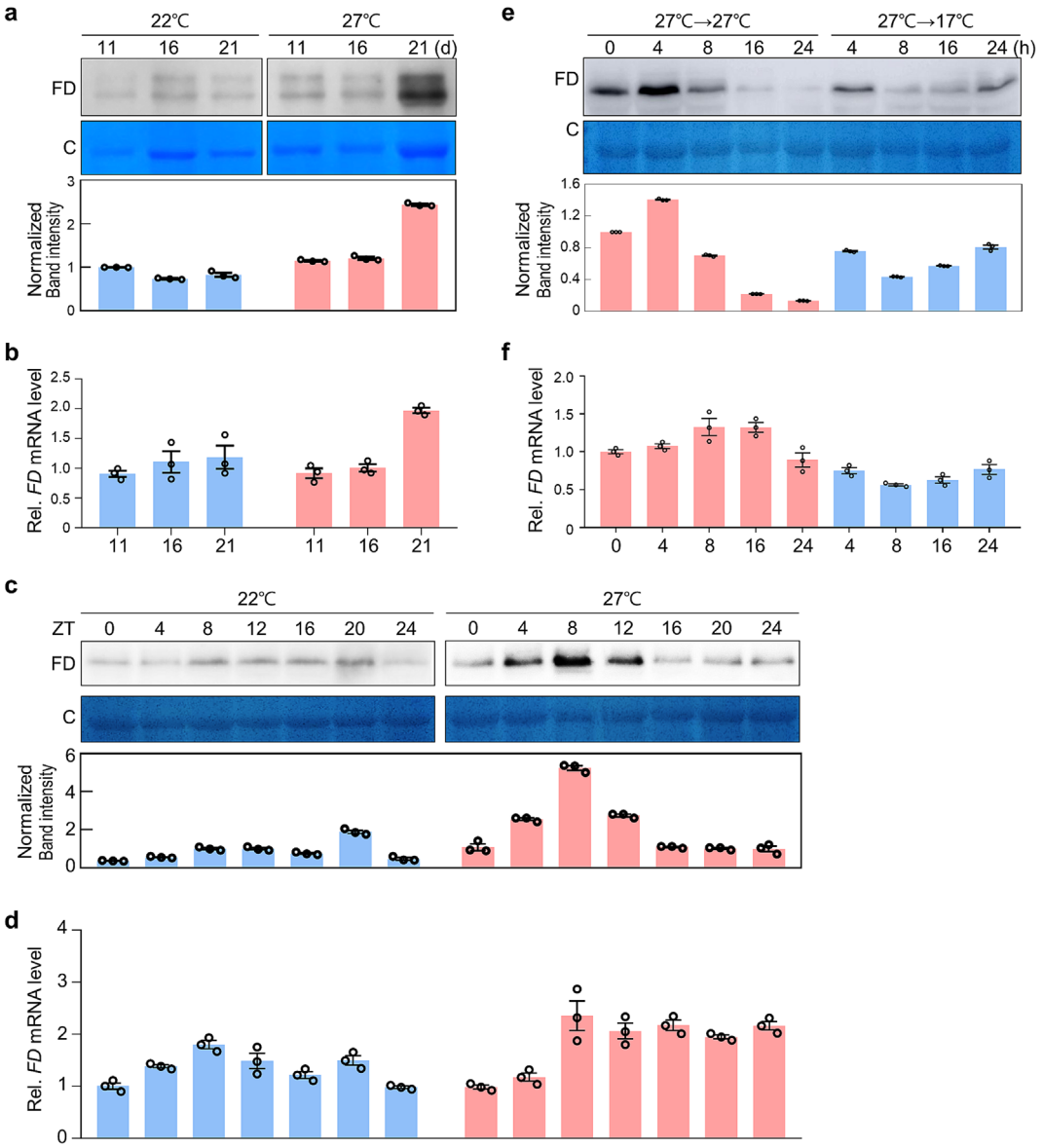

FIGURE 5 Thermal regulation of FD protein abundance. All immunoblot analysis and RT-qPCR were conducted in biological triplicates and data for quantification were presented as mean ± SEM. As a loading control in (a), (c), and (e), parts of the membranes stained with Coomassie brilliant blue (C) were added. The *FD-com* line #8 plants were used to examine FD protein abundance at different temperatures. (a) and (b) Temperature effects on temporal changes of *FD* expression levels under SD conditions. Whole seedlings of *FD-com* transgenic plants grown under SD conditions for indicated time durations at either 22 or 27°C were harvested at the ZT8 time point for immunoblot analysis (a). The levels of *FD* mRNA in Col-0 seedlings grown under the same conditions were also examined by RT-qPCR (b). (c) and (d) Diurnal accumulation pattern of FD proteins at different temperatures. Seven-day-old *FD-com* plants grown at 22°C under SD conditions were transferred to SD 22 or 27°C at dawn (ZT 0) and were incubated further for 7 days. Whole seedlings were harvested at the indicated Zeitgeber time (ZT) points for immunoblot analysis (c). RT-qPCR analysis of *FD* mRNA expression was conducted using Col-0 seedlings grown under the same conditions (d). (e) and (f) Effect of transient temperature changes on FD protein accumulation. Ten-day-old *FD-com* plants grown at 27°C under LDs were transferred to LD 27 or 17°C at dusk (ZT 16) and incubated under continuous dark conditions for the indicated time. Whole seedlings were harvested for immunoblot analysis (e). RT-qPCR analysis of *FD* mRNA levels was also performed on Col-0 seedlings grown under the same conditions (f).

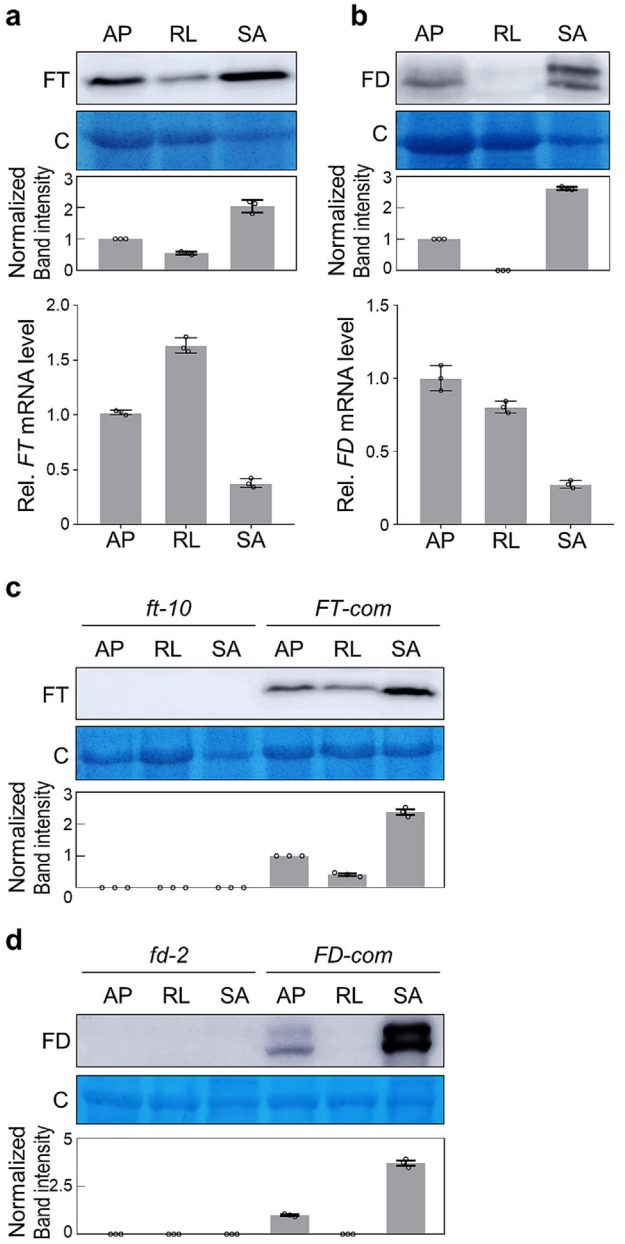

FIGURE 6 Tissue-specific expression of *FT* and *FD* genes. The homozygous F3 progeny derived from the cross of *FT*-com #3 and *FD*-com #8 transgenic plants were used. Same plant samples were used to examine the tissue-specific gene expression of *FT* (a) and *FD* (b). *FT*-com and *FD*-com transgenic plants, together with *ft-10* and *fd-2* mutants, respectively, were also used for the tissue-specific gene expression analyses of *FT* (c) and *FD* (d). Aerial parts (AP) of transgenic plants grown under LD conditions at 22°C for 21 days were divided into rosette leaves (RL) and shoot apices (SA) and harvested at ZT16 time point. In (a) and (b), immunoblot analyses were conducted in biological triplicates and Coomassie brilliant blue (C)-stained membrane parts were added as a loading control (upper panels). RT-qPCR analysis of *FT* and *FD* mRNA expression was also performed on Col-0 plants harvested under the same conditions (lower panels). RT-qPCR was conducted in biological triplicates. *FT*-com and *FD*-com transgenic plants, together with *ft-10* and *fd-2* mutants, respectively, were also used for immunoblot analyses (c, d). Data for quantification of immunoblot and RT-qPCR analyses were presented as mean ± SEM.

passed (Figure 4a). Changes in *FT* mRNA levels were very similar to those in FT protein levels (Figure 4b), suggesting that the effect of high temperatures on FT protein accumulation in a growth stage-dependent manner is mediated primarily through transcriptional regulation.

To investigate the temperature-dependent accumulation of FT protein in more detail, the *FT*-com plants grown for 10 days at 27°C under LDs were transferred to either 27 or 17°C at the end of the day. Our transient temperature shift assays revealed that unlike *FT* mRNA levels, which declined dramatically during the night period (Figure 4d), FT protein levels did not decrease much during the night period (Figure 4c). However, although *FT* mRNA levels did not differ between the two temperatures, FT protein levels decreased relatively more at 17°C than at 27°C, indicating that FT protein can accumulate at higher temperatures independent of transcriptional regulation.

In the same way, we examined how FD protein levels varied with temperatures. In assays measuring the growth stage-dependent expression of the *FD* gene, the difference in *FD* mRNA between temperatures was minimal, whereas FD protein levels were higher at 27°C than at 17°C at all three growth stages examined (Figure 5a,b). Diurnal patterns of *FD* gene expression at different temperatures also revealed that FD proteins accumulate more at high temperatures, particularly at dusk (Figure 5c,d). To confirm the high temperature-induced accumulation of FD protein, we performed temperature shift assays using the *FD*-com plants, as previously described (Figure 4c,d). Consistent with our previous findings, FD protein levels declined steeply over the night, although the reduction was more pronounced at 17°C than at 27°C, particularly during the early phases of the night period (Figure 5e). The difference in *FD* mRNA levels is less pronounced (Figure 5f), suggesting that FD protein accumulates more at higher temperatures regardless of transcriptional control.

3.5 | Tissue-specific expression of *FT* and *FD* genes

Under flowering-inductive conditions, the *FT* gene is transcribed in phloem companion cells of leaves, and its protein moves to the SAM, where it forms a transcriptional activator complex with the SAM-specific transcription factor FD (Corbesier et al., 2007; Jackson & Hong, 2012; Jaeger & Wigge, 2007; Mathieu et al., 2007). We verified the tissue-specific expression patterns of FT and FD proteins using homozygous F3 plants generated by crossing *FT*-com and *FD*-com transgenic plants.

As expected, *FT* mRNA level was higher in the leaf than in the shoot apex, whereas FT protein level was prominently detected in the shoot apex, supporting the leaf-to-SAM transport of FT protein (Figure 6a). Likewise, FD protein was strongly detected in the shoot apex but not in the leaf (Figure 6b). Intriguingly, *FD* mRNA was still detectable in the leaf at the vegetative growth stage examined (Figure 6b), suggesting a regulatory mechanism that prevents FD

protein expression in the leaf. Consistent with the findings, individual *FT*-com and *FD*-com transgenic plants displayed comparable tissue-specific patterns of FT and FD proteins, respectively, to F3 progeny generated by the cross between *FT-com* and *FD-com* transgenic plants (Figure 6c,d).

It is interesting to note that the FD protein bands are often observed in two layers: the lower band is strongly detected throughout the whole seedling, whereas the upper band is barely detected except in the shoot apex (Figure 6b,d). Given that phosphorylation of FD is essential for FD to promote flowering through interacting with FT (Abe et al., 2005; Kawamoto et al., 2015), tissue-specific phosphorylation of FD is plausible.

4 | DISCUSSION

We created *FT-com* and *FD-com* transgenic plants by introducing genomic fragments containing the promoter and downstream region of 3′-UTR of each gene and confirmed that the delayed flowering of each mutant was restored to the level of the wild-type plant in the transgenic plants. Although previous studies have reported the generation of *FD* transgenic plants using the native *FD* gene promoter (Abe et al., 2019; Collani et al., 2019; Jung et al., 2016; Minow et al., 2022), there has been no report of *FT* transgenic plants in which a large region of *FT* genomic fragment is inserted for precise regulation of *FT* expression. In this study, we successfully generated *FT-com* plants that showed a similar *FT* gene expression and resultant flowering time to those of the Col-0 plant. Moreover, temperature- and photoperiod-dependent changes in *FT* mRNA levels were comparable with those of Col-0, indicating that the transformation of a 13 kb of *FT* genomic region into the *ft* mutant reproduced the actual *FT* expression pattern in *FT-com* plants.

Using two *FT-com* and *FD-com* plants, we examined the effect of temperature changes on the protein abundance of FT and FD. Surprisingly, we discovered that both FT and FD proteins accumulate more at higher temperatures. FT and FD tend to be more transcribed at higher temperatures, but we found that their expression can also be induced by high temperatures at the protein level, independent of transcription. Arabidopsis flowering is promoted by high temperatures even under non-inductive SD conditions, where the *FT* mRNA levels are much lower than those under inductive LD conditions (Balasubramanian et al., 2006; Fernández et al., 2016; Kumimoto et al., 2008; Song et al., 2013). This suggests that even very low levels of FT protein signals can be sufficient to promote flowering at high temperatures (Fernández et al., 2016). The existence of factors that enhance temperature sensitivity at the SAM, the final destination of FT, would be essential for this effect. Given that FD interacts with FT in the SAM, the higher accumulation of FD proteins at increasing temperatures may play a critical role in determining the plant temperature sensitivity at the SAM.

How does temperature affect the accumulation of FT and FD proteins? Previous studies have shown that FT protein is degraded in a protease-dependent manner, and phosphorylation of FD is important for FD to stimulate flowering (Abe et al., 2005; Kawamoto et al., 2015; Ryu et al., 2014; Taoka et al., 2011). Further investigation is necessary to elucidate the thermal regulation of these regulatory mechanisms and their contribution to the temperature-dependent regulation of FT–FD protein accumulation. Our *FT-com* and *FD-com* plants will serve as valuable tools for exploring the role of the FT–FD module in regulating flowering time using various approaches.

AUTHOR CONTRIBUTIONS

Kyung-Ho Park, Sol-Bi Kim, and Jae-Hoon Jung conceived and designed the experiments. Kyung-Ho Park and Sol-Bi Kim generated transgenic plans and performed experiments using them. Kyung-Ho Park, Sol-Bi Kim, and Jae-Hoon Jung analyzed the data and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors did not report any conflict of interest.

PEER REVIEW

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SUPPORTING INFORMATION

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