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A straightforward strategy for reducing variability in flowering time at warm ambient temperatures

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ABSTRACT

Ambient temperature is one of the major environmental factors affecting flowering. As the temperature rises, most plants, including *Arabidopsis*, flower more rapidly. In addition, phenotypic variability in flowering time tends to increase at warm ambient temperatures. The increased variability of flowering time at warm temperatures prevents accurate flowering time measurements, particularly when evaluating the flowering time of *Arabidopsis* plants under short-day conditions in order to restrict the photoperiodic effect. Here, we propose a simple method for reducing the variability of flowering time at warm temperatures. Instead of growing plants at different temperatures from germination, the strategy of first vegetative growth at cool temperatures and then shifting to warm temperatures allows plants to respond more stably and robustly to warm temperatures. Consistent with flowering time measurements, plants grown under the modified growth condition exhibited higher levels of *FLOWERING LOCUS T* (*FT*) gene expression than plants grown exclusively at warm temperatures. This approach enables more precise thermo-response studies of flowering time control in *Arabidopsis*.

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Introduction

As sessile organisms, plants constantly adjust their growth and development in response to environmental changes to maximize fitness and reproductive success. Ambient temperature is one of the most influential environmental factors controlling plant fitness as it affects the rate of every physicochemical reaction. Prolonged exposure to warm temperatures triggers a set of developmental responses, which are collectively referred to as thermomorphogenesis: plants exhibit increased elongation growth of the hypocotyl, petiole, and primary root as well as accelerated flowering at warm temperatures.[1–3]

Flowering, a transition from the vegetative to the reproductive phases, is a critical developmental process determining plant reproductive success. Floral transition is delayed at low ambient temperatures but accelerated at high temperatures, and such temperature effects on flowering are conspicuous under flowering-inhibiting short-day photoperiodic conditions (SDs).[4–7] *FLOWERING LOCUS T* (*FT*) plays a crucial role in flowering time control in *Arabidopsis*. *FT* was originally known to function in the photoperiodic flowering pathway; however, it was recently shown that it also integrates a variety of endogenous and environmental signals. *FT* is also required for the thermal control of *Arabidopsis* flowering.[7–9] At warm temperatures, the transcriptional activation of the *FT* gene promotes flowering.

Plants have evolved complex signaling networks that monitor temperature fluctuations to determine the precise timing of flowering. Multiple transcriptional regulators are engaged in the thermal control of flowering in *Arabidopsis*. Two MADS-box transcription factors FLOWERING LOCUS M (FLM) and SHORT VEGETATIVE PHASE (SVP) play an important role in thermoresponsive flowering by repressing the *FT* gene transcription across a broad ambient temperature range.[10–12] The thermal regulation of the protein activities of FLM and SVP occurs post-transcriptionally and post-translationally, respectively. The *FLM* gene generates various splicing variants including two main isoforms *FLM-β* and *FLM-δ*, which are expressed in a temperature-dependent manner: *FLM-β* accumulates at cool temperatures, whereas *FLM-δ* is induced at warm temperatures.[10,11] FLM-*β* forms a transcriptional repressor complex with SVP, suppressing the *FT* gene transcription and flowering at cool temperatures. At warm temperatures, FLM-*δ* inhibits the formation of the functional SVP-FLM-*β* complex by competitively interacting with SVP to form a nonfunctional SVP-FLM-*δ* complex without DNA binding capacity. In addition, a polyubiquitination-induced protein proteolysis pathway, which is mediated by FLM isoforms, reduces the protein stability of SVP at warm temperatures.[11,12] Warm temperatures also lead to the down-regulation of *FLM* expression through alternative splicing coupled with nonsense-mediated mRNA decay (AS-NMD).[13]

PHYTOCHROME-INTERACTING FACTOR 4 (PIF4), which contains a basic helix-loop-helix DNA binding domain, functions as a transcriptional activator of the *FT* gene by directly binding to the *FT* promoter. Upon exposure to warm temperatures, PIF4 binds more strongly to

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the *FT* promoter to increase *FT* transcription, showing that PIF4 is a positive regulator of thermosensory flowering.[7,14] CONSTANS (CO), which is well known to be an upstream activator of the *FT* gene in the photoperiodic flowering pathway, also positively regulates thermosensory flowering.[7] Both CO and PIF4 physically interact with one another and bind to comparable regions of the *FT* promoter.[7] The delayed flowering and decreased *FT* transcription in the *co pif4* double mutant relative to the respective single mutants at warm temperatures indicate that CO and PIF4 function additively to promoter *FT* transcription, hence favoring flowering at warm temperatures.[7]

When evaluating the flowering time of *Arabidopsis* plants under short-day conditions with a minor photoperiodic effect, it is extremely difficult to measure flowering time precisely at warm temperatures.[7,13,15,16] As the temperature rises, phenotypic variability in flowering time tends to increase.[17] In previous studies, plants were incubated at cool temperatures and then transferred to warm temperatures to examine flowering time and *FT* gene expression, but there is no uniform method for addressing these concerns. Particularly, it is still unknown how long plants should be incubated at cool temperatures in order to optimize the effect of warm temperatures on the induction of flowering.

In this study, we developed a strategy to overcome these obstacles and confirmed that it enabled plants to respond more strongly and consistently to warm temperatures. We discovered that incubating young seedlings at cool temperatures for a sufficient amount of time prior to transferring them to warm temperatures enhances plant temperature responsiveness in flowering time control. This straightforward method will allow for more precise thermo-response studies of flowering time control in *Arabidopsis*.

Materials and methods

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 0.1% Triton X-100 and washed in 70% ethanol. Sterilized seeds were cold-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 short-day conditions (SDs, 8-h light/16-h dark cycles) 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°C or 27°C with a relative humidity of 60%.

The *co-101*, *pif4–101*, and *pif4–101 pif5–3* mutants have previously been described.[18,19] The *co pif4* double mutant was constructed by crossing the single *co-101* mutant with the *pif4–101* mutant. After collecting genomic DNA from F3 seedlings, the *co-101* and *pif4–101* mutations were genotyped using PCR amplification with specific primer sets (LP primer with 5'-TTGCCACAGGAGTATCAGAATG and RP primer with 5'-CCCCTTCTTTCAGATACCAGC for *co-101*; LP primer with 5'-CTCGATTTCCGGTTATGG and RP primer with 5'-RPCAGACGGTTGATCATCTG for *pif4–101*).

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 distilled water 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 4A* (*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.[20] The threshold cycle (C_T) for each reaction was automatically determined by the system's default parameters. The qPCR reactions for the *FT* and *EIF4A* genes were performed using specific primer sets (5'- AGGCCTTCTCAGGTTCAAAACAAGC and 5'-TGCCAAAGGTTGTTCCAGTTGTAGC for *FT*; 5'-TGACCACACAGTCTCTGCAA and 5'-ACCAGGGAGACTTGTTGGAC for *EIF4A*).

Flowering time measurements

For flowering time measurements, seeds were stratified in 0.1% agar solution for 3 days at 4°C in the dark and grown in soil for 10 days at 22°C under SDs after germination. The seedlings were then transferred to either 22°C or 27°C under SDs 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 30 plants were used to examine the flowering time of each genotype.

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.

Results

Plant growth and development are influenced differently by temperatures, depending on the plant's growth stage. Given that seed germination and post-germination seedling establishment are highly temperature dependent,[1–3] the phenotypic variability in flowering time would inevitably rise when plants are grown under different temperature conditions from the germination stage onward. It was hypothesized that the variability in flowering time might be dramatically minimized by conducting temperature shifts at a certain time point after incubating plants under constant temperature conditions at the germination and post-germination growth stages.

Arabidopsis growth stages are characterized by the degree of phenotypic alterations detected with each growth stage.[21] To

optimize the effect of warm temperatures on flowering, we designed an experiment to determine how long plants should be incubated vegetatively at cool temperatures. Seedlings grown at 22°C for a different time duration in soil were transferred to 27°C and incubated until flowering. For comparison, plants were also grown at 22°C and 27°C without temperature shifts. Compared to plants incubated at a constant temperature of 22°C, plants grown at a constant temperature of 27°C exhibited unstable temperature responsiveness with higher variation in flowering time. In contrast, incubating plants at 22°C for several days prior to temperature shifts resulted in more stable flowering at 27°C (Figure 1). Long incubation of plants at 22°C reduced the variability in flowering time up to stage 1.02, when two leaves begin to emerge. However, when plants were incubated at 22°C beyond stage 1.02, the warm temperature responsiveness became unstable again, and the proportion of plants with delayed flowering increased (Figure 1).

Thermal regulation of flowering is dependent on *FT* expression.[3,7,9] We thus examine *FT* expression in individual seedlings of wild-type Col-0 plants grown under different temperature conditions. Surprisingly, plants incubated at 22°C for 10 days until growth stage 1.02 prior to temperature transfer to 27°C exhibited greater sensitivity to warm temperatures in *FT* gene activation than plants grown at a constant

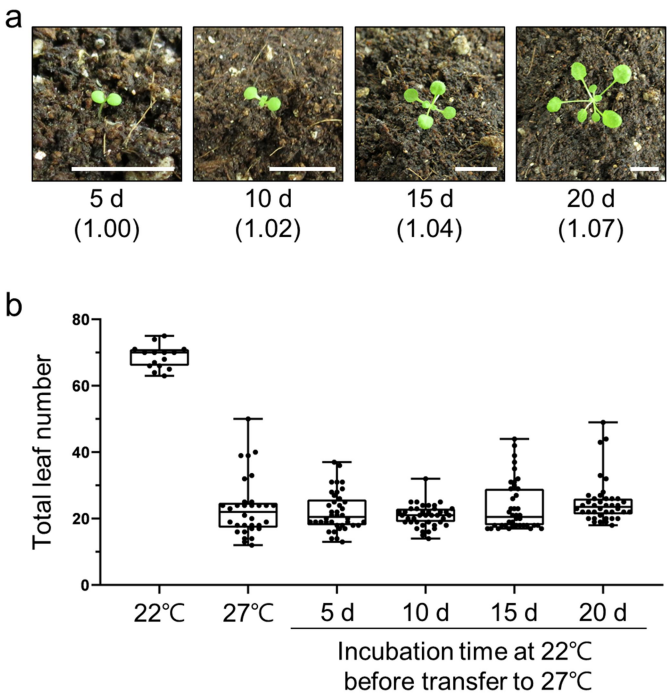

Figure 1. Warm temperatures affect flowering time differently depending on the growth stage. **a** Wild-type Col-0 plants were grown at 22°C for indicated time duration before being transferred to 27°C for flowering time measurements. The numbers in parentheses indicate the growth stage of the plant. Scale Bars, 1 cm. **b** Flowering time measurements under different temperature conditions. Seedlings grown at 22°C for indicated time duration on the soil were transferred to 27°C and grown until flowering. For comparison, plants were also grown at 22°C and 27°C without temperature shift. Flowering time was determined by counting the total number of rosette and cauline leaves when bolting. In box plots, each box is located between the lower and upper quartiles 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.

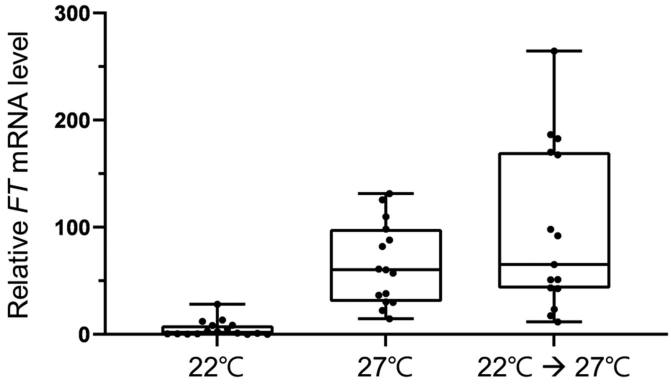

Figure 2. The thermal effect on *FT* gene expression is further increased under the modified conditions. Seedlings were grown at either 22°C or 27°C for 10 days on the soil. 10-day-old seedlings grown at 22°C were shifted to 27°C while control plants were maintained at either 22°C or 27°C. Following seven days of growth, individual seedlings were collected for total RNA extraction. RT-qPCR was used to examine the *FT* mRNA level in each seedling. *FT* mRNA levels were normalized relative to the *EIF4A* gene expression. Each box in box plots is located between the lower and upper quartiles and the whiskers indicate the minimum and maximum values. The central bar in each box represents the median and individual circles represent the relative level of *FT* mRNA on each plant.

27°C (Figure 2). These data suggest that plant competence to warm temperatures is acquired by adequate vegetative growth at cool temperatures rather than longer exposure to warm temperatures during young seedling stages.

Using our modified growing strategy, we verified previous findings about the role of PIF4 and CO transcription factors in the thermal regulation of flowering. The *pif4* mutant was initially reported to be insensitive to warm temperatures as its flowering was not induced by warm temperatures.[7] However, several papers have claimed that PIF4 has a little function in temperature-dependent flowering.[7,13,15] Under the new growth conditions, the increase in *FT* expression at 27°C was attenuated in the *pif4–101* mutant compared to Col-0, but flowering time did not differ significantly between the two genotypes (Figures 3, 4). Unlike the single *pif4–101* mutant, the *pif4–101 pif5–3* (*pif4 pif5*) double mutant exhibited delayed flowering and reduced *FT* expression level at 27°C, suggesting functional redundancy between PIF4 and PIF5 in the thermal control of flowering.

CO transcription factor has been reported to regulate temperature-dependent flowering in conjunction with PIF4.[7] In contrast to the *pif4–101* mutant, the *co-101* mutant exhibited delayed flowering and decreased *FT* expression at 27°C under the modified growth conditions, similar to the *pif4 pif5* double mutant (Figures 3, 4). Although the increase in *FT* expression at 27°C was severely suppressed in the *co-101* mutant, the delay in flowering time at 27°C was moderate in the mutant, suggesting the existence of an unidentified temperature-mediated flowering regulatory mechanism independent of the thermal control of *FT* expression in the leaf.

Discussion

As the temperature rises, the variability of the flowering phenotype increases, which is one of the challenges in the study of temperature-dependent flowering in *Arabidopsis*. This issue is worsened under SDs, where the photoperiodic effect is

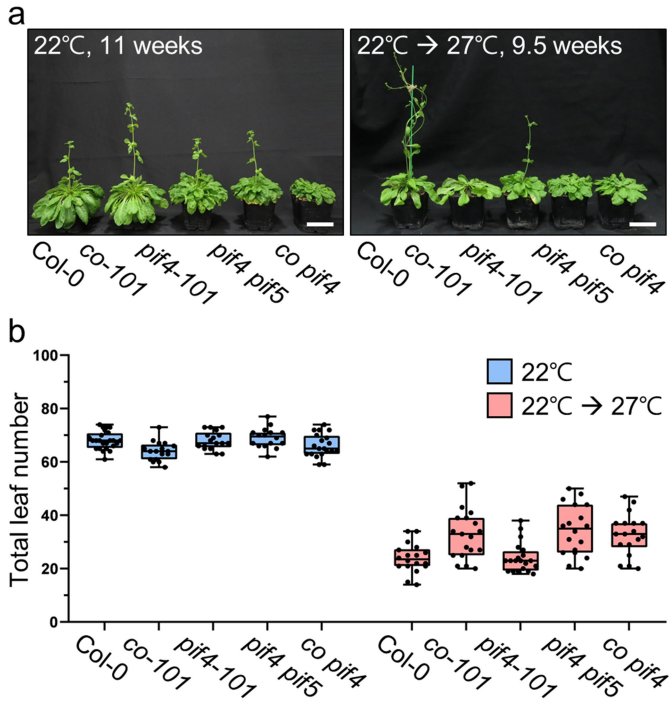

Figure 3. Effect of *co* and *pif4* mutations on flowering at 27°C under the modified conditions. Plants grown in soil for a given period of time under two temperature conditions were photographed (**a**). For each plant genotype, the number of total leaves on at least 16 plants was measured (**b**). 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. Scale bars, 5 cm.

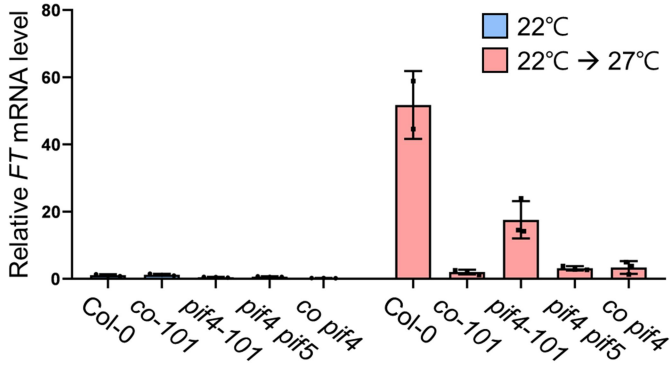

Figure 4. Effect of *co* and *pif4* mutations on the *FT* gene expression at 27°C under the modified condition. 10-day-old seedlings grown at 22°C on soil were shifted to 27°C while the control plants were maintained at 22°C. Following seven days of growth, the seedlings were collected for total RNA extraction. Levels of *FT* mRNA were determined by RT-qPCR.*FT* mRNA levels were normalized relative to the *EIF4A* gene expression. Biological triplicates were averaged for each plant genotype. Bars represent standard errors in the graph.

diminished and the temperature effect is maximized. In this study, we addressed the issue by incubating plants at cool temperatures until they reached the developmental stage 1.02 before transferring them to warm temperatures. Transfer to 27°C after appropriate vegetative growth at 22°C generated a stronger flowering response to 27°C than prolonged exposure to 27°C from germination.

We evaluated the sensitivity of the *pif4* and *co* mutants to warm temperatures by examining the flowering time and *FT* expression under the modified growing conditions. As pointed out in recent studies,[7,13,15] our data also demonstrated that *PIF4* has a minor role in the thermal regulation of *Arabidopsis* flowering under SDs. However, the *pif4 pif5* double mutation reduced the plant sensitivity to warm temperatures, suggesting that the role of PIF4 in the thermal control of flowering should be interpreted in the context of functional redundancy with other PIF proteins.

Unlike the single *pif4* mutant, the single *co* mutant was less sensitive to warm temperatures. In the *co* mutant, it was unusual that at warm temperatures, *FT* expression was significantly suppressed, while flowering time was marginally delayed. How can the *co* mutant retain temperature responsiveness despite its extremely low *FT* expression? *Arabidopsis* might possess a regulatory system that determines the flowering response to temperature changes independently of the *FT* gene. Or, given that a relatively small amount of FT proteins in the *co* mutant can convey temperature signals from the leaf to the apex, temperature sensitivity at the apex would play an important role in determining temperature sensitivity in *Arabidopsis*. It was recently reported that the thermal effect on flowering is mediated by the post-translational control of FT proteins,[9] supporting the latter possibility. By allowing precise thermo-response studies of flowering time control, this straightforward method to reduce flowering time variability will facilitate the identification of new mechanisms governing plant temperature responsiveness.

Disclosure statement

No potential conflict of interest was reported by the authors.

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