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FLOWERING NEWSLETTER REVIEW

# Control of flowering by ambient temperature

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## Abstract

**The timing of flowering is a crucial decision in the life cycle of plants since favourable conditions are needed to maximize reproductive success and, hence, the survival of the species. It is therefore not surprising that plants constantly monitor endogenous and environmental signals, such as day length (photoperiod) and temperature, to adjust the timing of the floral transition. Temperature in particular has been shown to have a tremendous effect on the timing of flowering: the effect of prolonged periods of cold, called the vernalization response, has been extensively studied and the underlying epigenetic mechanisms are reasonably well understood in *Arabidopsis thaliana*. In contrast, the effect of moderate changes in ambient growth temperature on the progression of flowering, the thermosensory pathway, is only starting to be understood on the molecular level. Several genes and molecular mechanisms underlying the thermosensory pathway have already been identified and characterized in detail. At a time when global temperature is rising due to climate change, this knowledge will be pivotal to ensure crop production in the future.**

**Key words:** Ambient temperature, *Arabidopsis thaliana*, flowering time, MADS, miRNA, thermosensory pathway.

## Introduction

In a constantly changing environment, animals can avoid harsh conditions by migrating to more suitable locations. In contrast, individual plants are sessile and therefore had to develop the means to detect and respond to environmental changes as they occur. As a consequence, plants continuously monitor their surroundings and adjust their growth to daily and seasonal cues, resulting in tremendous developmental plasticity. A trait that in many plants is strongly influenced by the environment is the timing of the transition from vegetative growth to flowering, as proper regulation of flowering time contributes considerably to reproductive success. It is therefore not surprising that plants have evolved an intricate genetic network that monitors endogenous and environmental signals and controls the transition to flowering accordingly. For example, day length and light quality have been shown to regulate flowering time in a large number of species (Poonyarit *et al.*, 1989; Adams *et al.*, 1999; Liu *et al.*, 2001; Weller *et al.*, 2001; Searle and Coupland, 2004; Mattson and Erwin, 2005).

Another important environmental stimulus that contributes to the timing of the floral transition is temperature. In this context it is important to discriminate between the effects of vernalization, the prolonged exposure to cold (overwintering), which many plants have to experience in order to allow the floral transition to occur the following spring (reviewed in Lang, 1965; Amasino, 2004; Choi *et al.*, 2009; Sheldon *et al.*, 2008; Kim *et al.*, 2009; Muller and Goodrich, 2011; Kim and Sung, 2014), and ambient temperature, which can be defined as the physiological, non-stressful temperature range of a given species. The molecular mechanisms underlying the vernalization response in *Arabidopsis thaliana* are quite well understood and have been comprehensively reviewed (Sheldon *et al.*, 2009; Song *et al.*, 2012). Briefly, vernalization in *A. thaliana* requires the progressive trimethylation

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of Lys27 of histone H3 (H3K27me3) at the floral repressor *FLOWERING LOCUS C* (*FLC*) locus, resulting in stable epigenetic repression of *FLC* in response to cold temperature, which is maintained throughout subsequent mitotic divisions. The epigenetic silencing of *FLC* is reset during meiosis by an unknown mechanism, ensuring that each generation again becomes vernalization sensitive (Sheldon *et al.*, 2008; Choi *et al.*, 2009). It should be noted, however, that the role of *FLC* in mediating the vernalization response might not be evolutionarily conserved. For example, it has been shown that in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), vernalization acts on the *APETALA1* (*AP1*) homologue *VERNALIZATION1* (*VRN1*), *VRN2*, and the homologue of *A. thaliana FLOWERING LOCUS T* (*FT*), *VRN3* (reviewed in Trevaskis *et al.*, 2007). These and other findings suggest that the epigenetic machinery that regulates the response to vernalization might be evolutionarily conserved, but might act on different genes in different species.

In contrast to our detailed understanding of vernalization, the molecular mechanisms underlying the regulation of flowering time by ambient temperature were until recently only poorly understood. However, this has been changing thanks to several publications that shed some light on the complex genetic and molecular circuits underlying the control of flowering in response to changes in ambient temperature in *A. thaliana* (reviewed in Samach and Wigge, 2005; Lee *et al.*, 2008; Wigge, 2013).

In plants, ambient temperature fluctuations affect a wide range of physiological and developmental responses besides flowering, such as the rate of net $CO_2$ assimilation and transpiration, the chlorophyll and proline content, the number of chloroplasts, the size of mitochondria, and the seed yield, to name just a few (Smillie *et al.*, 1978; Ozturk and Szaniawski, 1981; Cornic and Ghashghaie, 1991; Todorov *et al.*, 2003; Prasad *et al.*, 2006; Jin *et al.*, 2011). As a consequence, ambient temperature changes can have dramatic effects on plant architecture and biomass, parameters that are of high importance for crop productivity (Patel and Franklin, 2009). Clearly, understanding how plants respond to ambient temperature changes is essential to create crop plants tailored to different climate conditions, especially at a time when global temperature seems on the rise (Thuiller *et al.*, 2005).

## Effect of ambient temperature on flowering time in different species

Flowering time and the responsiveness to thermal induction vary widely between, but also within species and accessions, and probably reflect the adaptation of plants to their native environment. In *A. thaliana*, a moderate temperature increase from 23 °C to 27 °C was shown to be sufficient to induce flowering under an otherwise non-inductive short-day (SD) photoperiod (Balasubramanian *et al.*, 2006). The induction of flowering was accompanied by an increase in the expression of *FT*, which is normally expressed in leaves only under an inductive photoperiod (Song *et al.*, 2013). The FT protein has been shown to act as a long-distance signal (florigen) that conveys the information to induce flowering from leaves to the shoot meristem (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu *et al.*, 2007; Tamaki *et al.*, 2007).

In contrast, flowering has been shown to be delayed at 25 °C when compared with 18 °C in *Boechera stricta*, a perennial relative of *A. thaliana* (Anderson *et al.*, 2011). A similar behaviour has also been described in wild strawberry (*Fragaria vesca* L.), which flowers irrespective of the photoperiod at low temperatures (Heide, 1977). Increased summer temperatures (>20 °C) have also been shown to delay flowering in *Chrysanthemum morifolium*, due to a delay in expression of the *FT* homologue *FT-like 3* (*FTL3*) (Nakano *et al.*, 2013; Oda *et al.*, 2012). In contrast, high temperatures (>25 °C) induced the *FT* homologue (*NtFT*) and promote flowering in the monocotyledonous bulbous geophyte *Narcissus tazetta*, although in this case floral initiation occurs within the bulb located underground. At low temperature (12 °C), however, florogenesis was completely blocked and plants expressed only basal levels of *NtFT* (Noy-Porat *et al.*, 2009, 2013).

In barley, a complex interplay between day length and temperature in the regulation of flowering has been reported. Under long-day (LD) conditions, plants reached more advanced stages of reproductive development at 25 °C compared with 15 °C, whereas the opposite was the case in SDs (Hemming *et al.*, 2012). Furthermore, flowering was significantly delayed in barley grown at 2 °C thermo cycles (18/16 °C day/night) when compared with plants grown at constant temperature (18 °C), suggesting that night temperature is crucial for flowering time regulation in barley (Karsai *et al.*, 2008). Interestingly, unlike in *A. thaliana*, the transcript level of the barley orthologue of *FT*, *VRN3*, is apparently not influenced by temperature, and no clear candidate genes for the integration of thermal signals into the canonical flowering time pathways have been identified so far (Hemming *et al.*, 2012).

In sexually mature poplar (*Populus* spp.) trees, two *FT* homologues have been shown to co-ordinate the transition between vegetative and reproductive development (Hsu *et al.*, 2011). *FLOWERING LOCUS T1* (*FT1*) has been shown to determine the onset of reproductive development in response to cold winter temperatures. In contrast, *FLOWERING LOCUS T2* (*FT2*) promotes vegetative growth and inhibition of bud set in response to warm temperatures and LD conditions (Hsu *et al.*, 2011). Similarly, in subtropical and tropical tree species such as mango, lychee, macadamia, avocado, and orange, flowering is induced by low ambient temperature (Wilkie *et al.*, 2008) and, at least in the case of *Citrus*, this is associated with an increased expression of *CiFT* (Nishikawa *et al.*, 2007).

These examples highlight the fact that the control of the floral transition in response to ambient temperature is regulated differently in different plant species. Interestingly, in many species, *FT*-like genes have been identified as integrators of the response to changes in ambient temperature. However, in most of the cases, the gene regulatory networks that control the expression of these *FT*-like genes have not been characterized in detail.

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## Temperature-sensing mechanisms in plants

Research into how ambient temperature changes modulate flowering, or physiological and developmental processes in general, has been severely hampered by the lack of well-defined temperature sensors in plants. Temperature affects motion of molecules and thus can modulate biochemical reaction rates. However, diverse cellular and molecular mechanisms have also been suggested to participate in temperature sensing in microbial systems, plants, and animals (reviewed in McClung and Davis, 2010; Verhage *et al.*, 2014).

For example, RNA folding has been reported to be influenced by temperature: in the bacterium *Listeria monocytogenes*, high ambient temperature has been shown to promote melting of RNA hairpins in the untranslated regions (UTRs) of a key regulatory gene, thereby regulating the access of ribosomes (Johansson *et al.*, 2002). A similar mechanism has been described in various rhizobial species, in which the 5'-UTR of heat shock operons forms stem–loops that include the ribosome-binding site and the AUG start codon, making this portion of the mRNA accessible only after unfolding at elevated temperature (Nocker *et al.*, 2001). These examples demonstrate that temperature can be perceived by RNA stability/accessibility; however, it is currently unclear if the above mechanism is involved in temperature sensing in plants. Of course elevated temperatures do not only affect mRNA folding but can also disrupt non-covalent interactions at the amino acid level, thereby disrupting protein conformation and function, and ultimately triggering protein degradation (Vogt *et al.*, 1997). Similarly, temperatures can also affect protein–protein and protein–nucleic acid interactions, and indeed temperature-dependent interactions between nucleosomes and DNA have been reported in plants (Kumar and Wigge, 2010; Kumar *et al.*, 2012; Roncarati *et al.*, 2014). Finally, changes in temperature could directly affect the structure of DNA (Wildes *et al.*, 2011).

Changes in membrane fluidity have been suggested to be a primary event in controlling cellular responses to changes in temperature in plants: lipid bilayers become more fluid in response to high temperature, which could alter the activity of membrane-binding proteins such as ion channels (Wallis and Browse, 2002; Falcone *et al.*, 2004; Los and Murata, 2004). For example, transient receptor potential (TRP) ion channels have been shown to be regulated by temperature in *Drosophila melanogaster* and in mice (Moqrich *et al.*, 2005; Rosenzweig *et al.*, 2005; Hamada *et al.*, 2008). Similarly, changes in $Ca^{2+}$ influx have been reported to be one of the earliest events in plants responding to cool temperatures (Knight *et al.*, 1996).

Which of the above mechanisms, or combinations thereof, are ultimately responsible for temperature perception in plants remains to be determined. However, there is evidence for a strong genetic contribution to thermal responsiveness of plants and its cross-talk with other signalling pathways.

## Interaction between light and temperature signalling pathways

The temperature and photoperiod signalling pathways in particular have been reported to be tightly linked in the regulation of flowering time. This is exemplified by the finding that in *A. thaliana*, a reduction in ambient growth temperature from 22 °C to 16 °C was sufficient to suppress the early flowering phenotype of a mutant in the red:far-red (R:FR) photoreceptor phytochrome B (phyB) (Halliday *et al.*, 2003), and also to enhance the late flowering phenotype in plants that are deficient in activity of the blue light cryptochrome 2 (cry2) receptor (Blázquez *et al.*, 2003). Through the perception of the light signals by light receptors within the leaves and subsequently transmission to the apical meristem, plants synchronize their reproductive development according to the seasons (Quail *et al.*, 1995; Kami *et al.*, 2010). *CONSTANS* (*CO*), encoding a zinc finger transcription factor, is the key gene of this pathway (Putterill *et al.*, 1995; Searle and Coupland, 2004). In fact, by a positive regulation of its targets *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), and *LEAFY* (*LFY*), CO promotes flowering in LD conditions (Nilsson *et al.*, 1998; Samach *et al.*, 2000; Hepworth *et al.*, 2002). CO is in turn regulated by light receptors and undergoes degradation due to phyB, whereas cry2 promoted CO stability (Valverde *et al.*, 2004). In addition, it was observed that the delay in flowering caused by low ambient temperature was partially suppressed by a mutation in *EARLY FLOWERING 3* (*ELF3*) and *TERMINAL FLOWER 1* (*TFL1*) (Strasser *et al.*, 2009). Together with ELF4 and LUX ARRHYTHMO (LUX), ELF3 forms the so-called 'evening complex' that functions as a night-time repressor of gene expression in the circadian clock of *A. thaliana* (Nusinow *et al.*, 2011; Herrero *et al.*, 2012). TFL1 is closely related to FT, but carries out the opposite function by repressing flowering and keeping the shoot meristem in an undifferentiated state (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). Interestingly, the *elf3 tfl1* double mutant showed a complete suppression of low temperature-dependent late flowering. In contrast, mutations in *tfl1* abolished the temperature response in *cry2* mutants, but not in the *phyB* mutants, while *elf3* mutations suppressed the temperature response in *phyB* but not in *cry2* (Strasser *et al.*, 2009). Furthermore, *elf3* affected the expression of several photoperiod pathway genes, excluding *SOC1*, which was found to be misregulated in *tfl1*. To explain these findings, Strasser and colleagues proposed the existence of two genetic flowering time pathways that were both influenced by ambient temperature: one associated with the photoperiod pathway that depended on ELF3 activity, and a second one that required TFL1 (Strasser *et al.*, 2009).

## Autonomous and thermosensory pathways share common regulators

The autonomous pathway ensures that *A. thaliana* plants eventually undergo the transition to flowering in the absence of inductive environmental signals through repression of the

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floral repressor *FLC*. Mutations in autonomous pathway genes result in increased *FLC* expression and consequently late flowering. Evidence for a strong connection between the autonomous and the temperature pathway emerged when Blázquez and colleagues showed that two genes of the autonomous pathway, *FCA* and *FVE*, were also involved in the thermosensory pathway. In a search for genes that could account for the delay in flowering of the *A. thaliana* accession Landsberg *erecta* (L*er*) in response to reduced ambient temperatures, Blázquez and colleagues determined the flowering time of mutants in the photoperiod, gibberellin, and autonomous pathway at 23 ºC and 16 ºC. Only *fca-1* and *fve-1* flowered at the same time regardless of temperature (Blázquez *et al.*, 2003). This temperature insensitivity indicated that the function of these two genes is essential for the temperature-dependent regulation of flowering. FCA and FVE both function as regulators of gene expression, yet by different means: FCA is an RNA-binding protein that controls mRNA 3' polyadenylation (Quesada *et al.*, 2003), whereas FVE is a homologue of the retinoblastoma-associated protein and part of a histone deacetylase complex (Ausín *et al.*, 2004). *FCA* is transcriptionally and post-transcriptionally regulated, resulting in an elevated transcription and higher protein levels at 23 ºC when compared with 16 ºC (Fig. 1; Jung *et al.*, 2012). Furthermore, *FCA* is subjected to alternative splicing, and two main transcripts, designated β and γ (Macknight *et al.*, 1997), of which only the latter gives rise to a full-length functional protein, have been identified (Macknight *et al.*, 2002). In contrast, no evidence for temperature-dependent regulation of *FVE* expression or protein has been provided so far (Fig. 1). FVE and FCA participate in the induction of flowering at elevated temperatures through the induction of *FT* independently of the regulation of *FLC* and the upstream activator of *FT*, *CO* (Blázquez *et al.*, 2003).

Genetic analyses demonstrated that FCA and FVE act upstream of the potent floral repressor *SHORT VEGETATIVE PHASE* (*SVP*) (Fig. 1), which encodes a MADS-domain transcription factor, since the late flowering phenotype of the *fca-9* and *fve-3* mutants was supressed by mutations in *SVP* (Lee *et al.*, 2007). Moreover, expression of *SVP* was found to be elevated in *fca-9* and *fve-3* mutants, which would at least partially explain their late flowering phenotype (Lee *et al.*, 2007). Together, these findings indicate that SVP is an important mediator involved in the regulation of flowering time in response to changes of ambient temperature.

## H2A.Z-dependent regulation of flowering

Elevated ambient temperature not only accelerates flowering but also induces a number of other developmental responses such as hypocotyl elongation and leaf hyponasty (Gray *et al.*, 1998; Balasubramanian and Weigel, 2006). These traits are reminiscent of plants grown in shaded conditions and involve phyB signalling, which is also involved in the repression of flowering at high temperatures (Whitelam *et al.*, 1998; Halliday *et al.*, 2003). On the molecular level, the shade avoidance response requires the basic helix–loop–helix (bHLH) transcription factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4). PIF4 therefore constituted a promising point of integration of the shade avoidance response and temperature-dependent regulation of flowering.

Indeed it was recently demonstrated that PIF4, in addition to mediating shade avoidance, is also regulating flowering (Kumar *et al.*, 2012). Interestingly, the ability of PIF4 to regulate flowering seems to depend strongly on photoperiod. Under continuous light, the flowering phenotype of the *pif4* mutant was indistinguishable from that of the wild type at both 22 ºC and 28 ºC (Koini *et al.*, 2009). Similarly, loss of *PIF4* (and related genes) did not appear to delay flowering under LD conditions (Shin *et al.*, 2009). In contrast, when grown under SD conditions, the *pif4* mutant was found to be largely insensitive to elevated temperatures and flowered late even when grown at 27 °C (Kumar *et al.*, 2012).

At the molecular level, the late flowering of the *pif4* mutant can be explained to a large extent by reduced *FT* expression (Kumar *et al.*, 2012). Regulation of *FT* by PIF4 seems to be direct since the two genes are, at least under LDs, co-expressed in leaf phloem companion cells, and chromatin immunoprecipitation (ChIP) assays demonstrated that PIF4 protein was able to bind directly to the *FT* promoter (Kumar *et al.*, 2012). In addition, mutations in *FT* suppressed PIF4 overexpression phenotypes, providing additional support for the idea that temperature-dependent flowering under SDs is mediated at least in part by PIF4 (Fig. 1; Kumar *et al.*, 2012). Recently, it has been demonstrated that PIF4 and a close homologue, PIF5, exert their flower-promoting function predominantly during the night (Thines *et al.*, 2014). Using a 12/12 h day/night cycle, Thines and colleagues found that warm nights induced flowering as efficiently as constant warmth, whereas warm days were less effective (Thines *et al.*, 2014). In addition, PIF4 and PIF5 were found to promote flowering through *FT* during warm nights and through an *FT*-independent mechanism during warm days (Thines *et al.*, 2014).

Interestingly, binding of PIF4 to *FT* appears to be temperature dependent and increased at elevated ambient temperature. Even though *PIF4* expression and protein levels were slightly increased at elevated temperatures, this moderate increase seemed insufficient to account for the strong induction of *FT* (Kumar *et al.*, 2012). Instead, ChIP experiments suggested that the chromatin at the *FT* locus opened up in response to high temperature, allowing PIF4 to bind more efficiently and to activate *FT* expression directly (Fig. 1; Kumar *et al.*, 2012).

A possible explanation for these findings comes from the observation that nucleosomes that contain the histone variant H2A.Z seem to bind DNA more tightly than regular nucleosomes, making the DNA less accessible for transcription factors and slowing down RNA polymerase II (Kumar and Wigge, 2010). H2A.Z has therefore been suggested to act as a thermosensor. Interestingly, incorporation of H2A.Z into nucleosomes was reported to be temperature dependent, and H2A.Z occupancy decreased significantly in wild-type plants in response to higher temperatures at the *FT* promoter (Fig. 1; Kumar and Wigge, 2010; Kumar *et al.*, 2012). Furthermore, gene body H2A.Z has been shown partially to account for

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the increase in abundance of temperature-responsive transcripts in response to high temperature by promoting faster transcription relative to decay (Sidaway-Lee *et al.*, 2014). Since H2A.Z is incorporated into nucleosomes throughout the entire genome, it is not surprising that its incorporation pattern affects not only flowering but also other traits, including immunity, phosphate starvation, etc. (March-Diaz *et al.*, 2008; Smith *et al.*, 2010).

Incorporation of H2A.Z into nucleosomes is a targeted process that requires the *ACTIN-RELATED PROTEIN 6* (*ARP6*) gene, which encodes a subunit of the SWR1 chromatin remodelling complex (Krogan *et al.*, 2003; Mizuguchi *et al.*, 2004). *ARP6* was initially identified as a regulator of flowering in a genetic screen aimed to identify suppressors of the late flowering of *FRIGIDA*- (*FRI*) positive genotypes (Choi *et al.*, 2005; Deal *et al.*, 2005). One of the mutants recovered from this screen, *SUPPRESSOR OF FRIGIDA 3* (*SUF3*), was found to be allelic to *ARP6* (Choi *et al.*, 2005). *ARP6* has also been identified as a component of the temperature pathway in a forward genetic screen that was aimed at identifying mutants with modified thermosensitivity (Kumar and Wigge, 2010). Given that ARP6 is required for H2A.Z nucleosome deposition, it is not surprising that H2A.Z and ARP6 mutants are phenotypically quite similar (Kumar and Wigge, 2010 Coleman-Derr and Zilberman, 2012). In addition, reduced H2A.Z deposition in *arp6-10* resulted in a constitutive high temperature response, and *arp6-10* plants displayed early flowering along with transcriptome changes reminiscent of those of wild-type plants grown at elevated temperatures (Kumar and Wigge, 2010).

However, it is important to note that elevated temperatures induced early flowering even in the *arp6-10* mutant, suggesting that the eviction of H2A.Z-containing nucleosomes contributes to but is not exclusively responsible for the response to high temperature (Kumar and Wigge, 2010). Moreover, depletion of H2A.Z from nucleosomes not only would facilitate the binding of floral activators to DNA, but also would make it easier for floral repressors to exert their function on the very same targets. However, as we will discuss in more detail below, important floral repressors, including SVP, are reduced in their abundance at elevated temperatures, which has been suggested indirectly to increase the probability for activators to bind.

It has been shown that ARP6-dependet deposition of H2A.Z not only affects the expression of *FT* but also regulates the floral repressors *FLC*, *MADS AFFECTING FLOWERING 4* (*MAF4*), and *MAF5* (Choi *et al.*, 2005; Deal *et al.*, 2005, 2007; Martín-Trillo *et al.*, 2006). Furthermore, Martín-Trillo and colleagues showed that the late flowering phenotypes of the autonomous-thermosensory pathway mutants *fve-1* and *fca-1* were suppressed when combined with *esd1-2*, a mutant allele of *arp6* (Martín-Trillo *et al.*, 2006), indicating that *ARP6* functions either downstream of or in parallel with *FCA* and *FVE*.

Taken together, these data support a model in which high temperatures evict H2A.Z from the *FT* locus (and others) allowing its induction by PIF4 and, consequently, the acceleration of flowering (Fig. 1). However, these data also suggest that H2A.Z functions at least partially downstream of the actual temperature-sensing mechanism and that the function of H2A.Z nucleosome occupancy as a thermosensor needs to be revisited.

## MADS-domain transcription factors repress flowering at low temperature

MADS-domain transcription factors form a large gene family in plants. The type II lineage, also known as MICK-type, contains genes involved in various developmental processes such as floral patterning and flowering time regulation. Regulators of flowering time include *SVP*, *FLC*, and the other members of the *FLC* clade, i.e. *FLOWERING LOCUS M* (*FLM*; *MAF1*) and *MAF2–MAF5*, four closely related genes arranged in tandem at the bottom of chromosome 5 (Ratcliffe *et al.*, 2001; Smaczniak *et al.*, 2012). *SVP* and all the genes from the *FLC* clade, with the possible exception of *MAF5*, whose function in flowering time regulation is controversial, have been implicated in the thermosensory pathway (Balasubramanian and Weigel, 2006; Lee *et al.*, 2007; Kim and Sung, 2010; Gu *et al.*, 2013).

Loss of function of either SVP or FLM results in partial temperature-insensitive early flowering (Balasubramanian and Weigel, 2006; Lee *et al.*, 2007, 2013; Posé *et al.*, 2013). However, while SVP has been reported to act mainly in the thermosensory pathway downstream of *FCA* and *FVE* (Fig. 1), *FLM* has been also shown to be regulated by the vernalization and photoperiodic pathways (Scortecci *et al.*, 2003; Sung *et al.*, 2006). In addition, *SVP* and *FLM* have been shown to contribute to the variation of flowering time among natural accessions of *A. thaliana* (Werner *et al.*, 2005; Méndez-Vigo *et al.*, 2013).

## An SVP–FLM repressor complex determines temperature-dependent flowering time

There is evidence that *SVP* and *FLM* interact genetically (Scortecci *et al.*, 2003; Posé *et al.*, 2013). However, the molecular mechanism behind this genetic interaction has been solved only recently (Lee *et al.*, 2013; Posé *et al.*, 2013).

*FLM* has previously been shown to be subject to alternative splicing (Scortecci *et al.*, 2001). Two main splice variants, *FLM-β* and *FLM-δ*, that differ in the incorporation of either the second or third cassette exon and are both translated into proteins, can be detected in the Columbia-0 (Col-0) accession (Jiao and Meyerowitz, 2010; Lee *et al.*, 2013; Posé *et al.*, 2013). Balasubramanian and colleagues observed that the *FLM* splicing pattern changed in response to changes in ambient temperature (Balasubramanian *et al.*, 2006). In particular, they found that a prominent *FLM* splice variant, presumably *FLM-β*, was down-regulated in response to increasing temperature, leading to the hypothesis that this FLM variant repressed flowering particularly at low temperatures (Balasubramanian and Weigel, 2006). Strikingly, it has

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recently been shown that splicing of *FLM-β* and *FLM-δ* is regulated in an opposite fashion by temperature, with *FLM-β* being the prevalent splice variant at low temperature (16 °C), and *FLM-δ* increasing in relative abundance at high temperature (27 °C) (Fig. 1; Lee *et al.*, 2013; Posé *et al.*, 2013). The factors regulating the temperature-dependent splicing of FLM are not known. However, microarray analysis had previously shown that changes in temperature in SDs regulate the transcription of six SR genes, which contribute to splice site selection (Kalyna and Barta, 2004; Balasubramanian *et al.*, 2006). Whether these (or other) SR genes are expressed in a temperature-dependent manner also in LD conditions and whether this is related to the alternative splicing of *FLM* remains to be tested. Similarly, it is unclear whether temperature-dependent regulation of SR genes contributes to the alternative splicing of *FCA* and thus indirectly to the expression of the downstream floral repressor *SVP*.

Interestingly, overexpression of *FLM-β* and *FLM-δ* under the *Cauliflower mosaic virus* (CaMV) 35S promoter resulted in opposite phenotypes. While *FLM-β* overexpression delayed flowering, as expected for a floral repressor, high expression of *FLM-δ* accelerated the transition to flowering (Posé *et al.*, 2013). In addition, *FLM* and *SVP* are co-expressed and the proteins interact physically (Lee *et al.*, 2013; Posé *et al.*, 2013). These findings suggest a model in which the incorporation of a particular FLM isoform determines the activity of the resulting SVP–FLM complex (Fig. 1). Consistent with this hypothesis, SVP and FLM-β share a number of direct transcriptional targets (Tao *et al.*, 2012; Posé *et al.*, 2013). Among them are prominent flowering time and floral homeotic genes, including *SOC1* (Fig. 1), *TEMPRANILLO2* (*TEM2*), and *SEPALLATA3* (*SEP3*) (Posé *et al.*, 2013). *FT*, which genetic and ChIP assays had previously shown to be a target of FLM (Fig. 1; Mathieu *et al.*, 2009; Gu *et al.*, 2013; Lee *et al.*, 2013), was detected only in a single ChIP-seq replicate by Posé and colleagues (2013). In this experiment two binding sites in the *FT* locus could be identified: one located in the first intron, consistent with other reports (Gu *et al.*, 2013; Lee *et al.*, 2013), and a second bound region positioned ~1.5 kb downstream of the *FT* coding sequence (CDS). Interestingly, the latter coincides with a region that is bound by the AP2-like floral repressor SCHLAFMÜTZE (SMZ) (Mathieu *et al.*, 2009), which requires FLM in order to repress flowering (Mathieu *et al.*, 2009). However, whether FLM and SMZ function as part of a complex involving AP2- and MADS-domain proteins to regulate *FT* expression remains to be elucidated.

Electrophoretic mobility shift assays (EMSAs) demonstrated that FLM-β binds DNA *in vitro* only in the presence of SVP. In contrast, FLM-δ was unable to bind DNA *in vitro* and, importantly, prevented SVP from binding DNA in a dose-dependent manner (Posé *et al.*, 2013). Assuming that the FLM-β and FLM-δ protein levels follow those of the corresponding mRNAs, these findings suggest a model in which FLM-δ at elevated temperatures acts as a dominant-negative isoform that renders the SVP–FLM-β complex inactive, thereby indirectly promoting the transition to flowering (Posé *et al.*, 2013). *In vitro* analyses demonstrated that SVP binds both FLM-β and FLM-δ with similar affinity (Hwan Lee *et al.*, 2014), supporting the hypothesis that these two isoforms compete for SVP binding

In contrast to *FLM*, transcription of *SVP* was only moderately affected by temperature (Lee *et al.*, 2007, 2013). However, it has recently been shown that the SVP protein is rapidly degraded via the 26S proteasome in response to elevated temperatures and that these changes affected the abundance of the repressive SVP–FLM-β complex (Fig. 1; Lee *et al.*, 2013). However, it should be noted that the SVP protein degradation assays were performed in the presence of the protein biosynthesis inhibitor cycloheximide. It remains to be tested to what extent *de novo* protein synthesis does compensate for the degradation of existing SVP protein at elevated temperature. Anyway, together these results add a new and important layer to the regulation of flowering by ambient temperature: not only do high temperatures promote the formation of the dominant-negative *FLM-δ* splice variant, but at the same time SVP protein is turned over more rapidly, further reducing the abundance of the repressive SVP–FLM-β complex.

## Role of FLC and related MADS-domain transcription factors in temperature-dependent flowering

As briefly mentioned above, FLC plays an essential role in mediating the vernalization response in winter annual accessions of *A. thaliana*. In contrast, in rapid-cycling accessions with a non-functional *FRI* allele, the role of FLC is limited since its expression is largely repressed by the autonomous pathway (reviewed in Srikanth and Schmid, 2011). Nevertheless, it has recently been shown that *FLC* also contributes to the regulation of flowering in response to ambient temperature. Although to a lesser extent than *svp-32* and *flm-3* mutants, which flower early across a wide range of temperature, the *flc-3* mutant is largely insensitive to changes in ambient temperature between 23 ºC and 27 ºC (Lee *et al.*, 2013). In addition, *FLC* expression was down-regulated in response to increasing temperatures (Fig. 1; Blázquez *et al.*, 2003). Down-regulation of *FLC* was accompanied by a de-repression of known targets of FLC such as *FT*, *SOC1*, and the floral integrator *TWIN SISTER OF FT* (*TSF*), and resulted in the precocious induction of flowering (Lee *et al.*, 2013). Moreover, FLC physically interacts with SVP and binds to *FT* and *SOC1* genomic regions (Li *et al.*, 2008; Deng *et al.*, 2011), supporting a scenario in which higher ambient temperatures reduce not only the abundance of SVP–FLM-β but also that of SVP–FLC. These findings indicate that, similarly to *SVP* and *FLM*, *FLC* also affects flowering in response to ambient temperature (Lee *et al.*, 2013).

*MAF2*, *MAF3*, and *MAF4*, which are related to *FLM* and *FLC*, have been shown to contribute to the repression of flowering under cool ambient temperatures (Ratcliffe, 2003; Gu et al., 2009, 2013). The temperature response is partially disrupted in *maf2*, *maf3*, and *maf4* mutants, and, as a consequence, these genotypes are less sensitive to temperature changes (Gu *et al.*, 2013). Bimolecular fluorescence complementation (BiFC) experiments detected interactions

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of MAF2 and MAF3 with FLM, and of MAF4 with FLM and FLC. Furthermore, MAF3 and FLM bind to the same regions of the *FT* and *SOC1* promoters, and binding of FLM to these sites is reduced in the *flc maf3* double mutant (Gu *et al.*, 2013). Taken together, these data suggest that different complexes formed by FLC-clade MIKC-type MADS-domain transcription factors plus SVP co-ordinately regulate temperature-dependent flowering.

Similarly to *FLM*, *MAF2* is subjected to alternative splicing and the expression levels of two isoforms, named var1 and var2, change with temperature. Splice variant var1 is preferentially formed in the cold (4 °C) whereas var2 is favoured at higher temperatures (21 ºC) (Rosloski *et al.*, 2012). However, unlike *FLM* splice variants, only var1 encodes a potential full-length MIKC-type MADS-domain protein that is sufficient to repress flowering when overexpressed in the *A. thaliana* accession Ll-2, which does not express *FLC* and *MAF2–MAF4* (Lempe *et al.*, 2005; Rosloski *et al.*, 2012). In contrast, var2 encodes a truncated MAF2 protein that does not repress flowering. In addition, plants overexpressing *MAF2* var2 are not early flowering, suggesting that this isoform does not act as a dominant-negative protein, as is the case for FLM-δ (see above).

## miRNAs regulate ambient temperature-responsive flowering

MicroRNAs (miRNAs), a class of small non-coding RNA molecules of 21–22 nucleotides, have emerged as central regulators of plant development (reviewed in Yamaguchi and Abe, 2012). miRNAs arise from single-stranded primary miRNA transcripts (pri-miRNAs), that are subsequently processed into shorter hairpin RNAs (pre-miRNAs) and later into short double-stranded RNAs of ~21–22 nucleotides (reviewed in Zhu, 2008). The mature miRNA is incorporated into the so-called RNA-induced silencing complex (RISC), that mediates direct or indirect transcriptional or post-transcriptional gene silencing (Spanudakis and Jackson, 2014).

Two major miRNAs families, miR156 and miR172, have been identified as important regulators of the juvenile–adult transition and flowering time (reviewed in Huijser and Schmid, 2011). The expression of miR172 and miR156 is regulated oppositely: miR156 is highly expressed in young plants and declines in abundance as plants mature; in contrast, miR172 expression increases with the age of the plant. miR156 targets the mRNAs of 11 out of 17 of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes in *A. thaliana*, the product of one of which, SPL3, has been shown to induce directly the expression of *FT* (Fig. 1; Kim *et al.*, 2012), and the floral meristem identity genes *FRUITFUL* (*FUL*), *LFY*, and *AP1* (Yamaguchi *et al.*, 2009). Similarly, the miR156 target, SPL9, directly up-regulates expression of *FUL*, *SOC1*, *AGAMOUS-LIKE 42* (*AGL42*), a paralogue of *SOC1* (Wang *et al.*, 2009), and miR172, which targets *APETALA2* (*AP2*)-type floral repressor genes such as *AP2*, *TARGET OF EAT1* (*TOE1*), *TOE2*, *TOE3*, *SMZ*, and *SCHNARCHZAPFEN* (*SNZ*) (Fig. 1; Aukerman and Sakai, 2003; Mathieu *et al.*, 2009).

More recently, miR156 and miR172 have been suggested to participate in the temperature-dependent regulation of flowering (Lee *et al.*, 2010; Kim *et al.*, 2012). miR156 levels are increased at 16 ºC when compared with 23 ºC, while miR172 is regulated in an opposite manner (Fig. 1; Lee *et al.*, 2010). It should be noted, however, that plant development progresses at different speeds at different temperatures and that the abundance of mature miR156 and miR172 has been shown to be developmentally regulated. It thus seems possible that the differences in mature miRNA levels observed in 10-day-old seedlings reflect at least in part the developmental stage of the plants rather than temperature-mediated differences in gene expression.

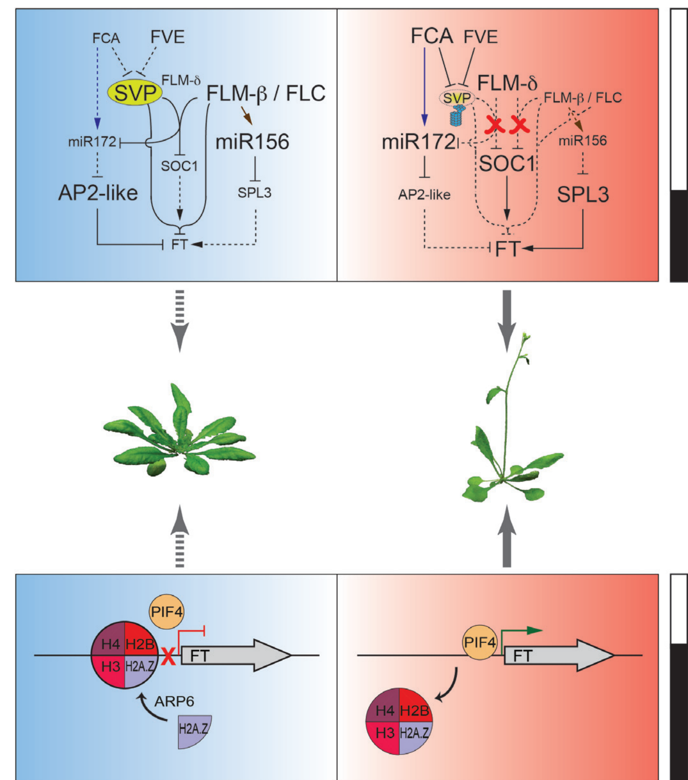


**Fig. 1.** Regulation of flowering time by ambient temperature. Two regulatory subnetworks control the timing of the floral transition in *A. thaliana* in response to ambient temperature. Under LD conditions (top panel), a repressive complex consisting of the MADS-domain transcription factors SVP, FLM-β, and FLC directly represses floral activators such as *SOC1* and *FT*. In addition, the activity of the miR156/SPL- and miR172/AP2-like modules represses *FT* expression at low temperatures. As temperatures increase, SVP protein is actively degraded via the 26S proteasome, reducing the capacity of plants to repress flowering actively. In addition, a dominant-negative version of the FLM protein, FLM-δ, is formed that poisons the repressive MADS-domain transcription factor complex, allowing for activation of *SOC1* and *FT* expression. At the same time, FCA promotes expression of miR172 at higher temperatures, indirectly promoting expression of *FT* through the repression of *AP2*-like floral repressors. Under SD conditions (bottom panel), elevated temperature promotes the eviction of H2A.Z-containing nucleosomes from the DNA, facilitating binding of PIF4 to the promoter and expression of *FT*. Arrows and block lines denote activation and repression, respectively. Dotted arrows and block lines indicate a lower effect of this regulation. The size of fonts indicates abundance. The blue arrow indicates RNA processing. The brown arrow indicates putative regulation. SVP protein degradation at high temperature by the 26S proteasome (blue complex) is represented as a fading green ellipse.

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Both miRNAs eventually modulate, through regulation of their direct targets, the expression of *FT*, which ultimately regulates flowering time in response to temperature (Fig. 1; Jung *et al.*, 2012; Kim *et al.*, 2012). Kim and colleagues demonstrated that the delay in flowering time in response to miR156 overexpression was more pronounced at 16 ºC than at 23 ºC. This was explained by a more efficient down-regulation of SPL3, and subsequently *FT* at low temperatures (Kim *et al.*, 2012). In contrast, overexpression of miR172 caused ambient temperature-insensitive flowering (Lee *et al.*, 2010). In addition, levels of mature miR172 and pri-miR172a were shown to be anticorrelated with SVP activity, suggesting that SVP might directly down-regulate *MIR172* expression (Lee *et al.*, 2010; Cho *et al.*, 2012). In fact, both SVP and FLM bind directly to the *MIR172a* promoter (Tao *et al.*, 2012; Posé *et al.*, 2013), supporting the possibility that these two MADS-box transcription factors may be acting as direct upstream repressors of this miRNA gene. According to this scenario, high levels of the SVP–FLM-β complex at low temperatures should repress *MIR172a* expression and thus contribute to the repression of flowering (Fig. 1).

However, Jung and colleagues found that, while the levels of three out of five *MIR172* primary transcripts were moderately increased at 16 °C when compared with 23 °C, pre-miR172 was low at both temperatures (Jung *et al.*, 2012), suggesting that ambient temperature primarily regulates miRNA processing rather than the transcription of *MIR172* genes. Interestingly, FCA, whose expression and protein levels are up-regulated at 23 °C compared with 16 °C (see above), has been shown to regulate miRNA172 processing by binding to the flanking sequences of the stem–loop within the pri-miR172 transcripts (Jung *et al.*, 2012), suggesting a role for FCA in temperature-dependent regulation of miR172 processing and flowering (Fig. 1; Jung *et al.*, 2012). However, expression of MIR172a was apparently not affected in the *fca-9* mutant (Lee *et al.*, 2010). These data suggest that the FCA-miR172 regulon acts in fine-tuning miR172 accumulation and floral transition, but does not constitute a major hub of temperature-dependent flowering time regulation.

Apart from miR172, miR399, which targets the *PHOSPHATE 2* (*PHO2*) transcript, has also been suggested to contribute to FCA- and temperature-dependent regulation of flowering (Kim *et al.*, 2011; Jung *et al.*, 2012). miR399 levels were higher at 23 ºC when compared with 16 ºC (Lee *et al.*, 2010; Kim *et al.*, 2011). Furthermore, miR399 overexpression and loss of function of its target gene *PHO2* induced early flowering at 23 ºC, but had no effect on flowering at 16 ºC. Early flowering in these lines has been attributed to increased expression of *TSF* at 23 ºC. However, whether this is caused by miR399 directly or is an effect of phosphate accumulation and toxicity remains to be elucidated (Kim *et al.*, 2011). Clearly, more work is needed to unravel the role of miRNAs in the regulation of temperature-dependent flowering.

## Concluding remarks

The current changes in global climate have a pronounced effect on the phenology of plants, including the timing of flowering and species loss (Fitter and Fitter, 2002). Hence, a deeper understanding of the molecular mechanisms underlying the plant responses to changes in ambient temperature is essential, not only from a purely scientific point of view, but also to lessen the impact of global warming on crop productivity.

Over the last few years, a wealth of reports has shed some light onto how ambient temperature controls flowering time. From these studies, it is evident that transcription factors such as SVP, FLM-β, and FLC directly repress flowering at low ambient temperatures. In addition, the level of mature miR156, which is a well-known regulator of phase transitions in plants, is elevated in plants grown at low ambient temperature. The activity of these negative regulators is counteracted by a set of flower-promoting factors, which include PIF4 (especially in SD conditions), FCA, SPL3, and miR172. Ultimately, the balance between repressors and activators determines the outcome and adjusts flowering time in response to changes in ambient temperature.

Despite the progress made, many important questions concerning the regulation of flowering time by ambient temperature remain unresolved. For example, how plants perceive ambient temperature is not fully understood. Another important question that needs to be addressed is how plants respond to temperature under more natural conditions. Most studies so far have been conducted under artificial temperature regimes that did not, for example, include daily temperature fluctuations. Clearly, future studies need to address to what extent the molecular mechanisms discussed above, such as temperature-dependent alternative splicing, protein degradation, or the wrapping of DNA around nucleosomes, contribute to the response to long- and short-term changes in ambient temperature under natural conditions.

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