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
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 (API) 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 CO₂ 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, florigenesis 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 (Willie 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.
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; Verhae 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 Ca2+ 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 ACRYTHMO (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 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 elf1. 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
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 (Ler) in response to reduced ambient temperatures, Blázquez and colleagues determined the flowering time of mutants in the photoperiod, gibberellin, and autonomous pathway at 23 °C and 16 °C. 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 (Austin et al., 2004). FCA is transcriptionally and post transcriptionally regulated, resulting in an elevated transcription and higher protein levels at 23 °C when compared with 16 °C (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 suppressed 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 Weiigel, 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; Hallday 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 °C and 28 °C (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
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-dependent 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; Martin-Trillo et al., 2006). Furthermore, Martin-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 (Martin-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; Wu 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 (Scortecchi 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 (Scortecchi 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 (Scortecchi 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
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 °C and 27 °C (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 derepression 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
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 SOCI promoters, and binding of FLM to these sites is reduced in the ftc 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 ºC) 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 miRNAs 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, SOCI, AGAMOUS-LIKE 42 (AGL42), a parologue of SOCI (Wang et al., 2009), and miR172, which targets PETALAL2 (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-δ, and FLC directly represses floral activators such as SOCI 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 SOCI 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.
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 over-expression was more pronounced at 16 °C than at 23 °C. 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 *MIR172* 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 *MIR172* 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 °C when compared with 16 °C (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 °C, but had no effect on flowering at 16 °C. Early flowering in these lines has been attributed to increased expression of *TSF* at 23 °C. 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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References
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Prasad PVV, Boote KJ, Allen LH. 2006. Adverse high temperature effects on pollen viability, seed-set, seed yield and harvest index of grain sorghum [Sorghum bicolor (L.) Moench] are more severe at elevated carbon dioxide due to higher tissue temperatures. Agricultural and Forest Meteorology 139, 237–251.


