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RESEARCH ARTICLE

MAF2 Is Regulated by Temperature-Dependent Splicing and Represses Flowering at Low Temperatures in Parallel with \textit{FLM}

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Abstract

Plants enter their reproductive phase when the environmental conditions are favourable for the successful production of progeny. The transition from vegetative to reproductive phase is influenced by several environmental factors including ambient temperature. In the model plant \textit{Arabidopsis thaliana}, \textit{SHORT VEGETATIVE PHASE (SVP)} is critical for this pathway; \textit{sdp} mutants cannot modify their flowering time in response to ambient temperature. \textit{SVP} encodes a MADS-box transcription factor that directly represses genes that promote flowering. \textit{SVP} binds DNA in complexes with other MADS-box transcription factors, including \textit{FLOWERING LOCUS M (FLM)}, which acts with \textit{SVP} to repress the floral transition at low temperatures. Small temperature changes post-transcriptionally regulate \textit{FLM} through temperature-dependent alternative splicing (TD-AS). As ambient temperature increases, the predominant \textit{FLM} splice isoform shifts to encode a protein incapable of exerting a repressive effect on flowering. Here we characterize a closely related MADS-box transcription factor, MADS AFFECTING FLOWERING2 (MAF2), which has independently evolved TD-AS. At low temperatures the most abundant \textit{MAF2} splice variant encodes a protein that interacts with \textit{SVP} to repress flowering. At increased temperature the relative abundance of splice isoforms shifts in favour of an intron-retaining variant that introduces a premature termination codon. We show that this isoform encodes a protein that cannot interact with \textit{SVP} or repress flowering. At lower temperatures \textit{MAF2} and \textit{SVP} repress flowering in parallel with \textit{FLM} and \textit{SVP}, providing an additional input to sense ambient temperature for the control of flowering.

Introduction

The timing of the vegetative to reproductive phase transition in plants is influenced by many environmental stimuli. Perception and integration of a range of environmental signals maximises reproductive success and species fitness. Initiation of the reproductive phase is regulated by environmental variables, such as day-length and temperature, in addition to endogenous
signals such as plant age [1] [2]. In Arabidopsis thaliana these diverse inputs are integrated by regulating the expression of a limited set of genes including FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1). FT and SOC1 are therefore known as floral pathway integrators [3], because several pathways converge on these activators of flowering to translate endogenous and exogenous signals into the decision to flower.

Amongst these stimuli, temperature influences flowering time in two distinct ways. Exposure to prolonged periods of cold promotes flowering through a process known as vernalization [4]. Vernalization enables plants to identify the spring by distinguishing a long period of winter from a transient cold spell in autumn. Two related MADS-box transcription factors, FLOWERING LOCUS C (FLC) and MAF2 have been shown to act in complementary ways in the vernalization pathway to delay flowering until the plant has experienced extended periods of cold. FLC is a repressor of flowering and the amount of FLC expression varies in Arabidopsis ecotypes, determining their vernalization requirements [5]. Expression of FLC is increased in ecotypes with an active FRIDIGIDA (FRI) gene, because FRI activates FLC as part of a transcription complex that binds to the FLC promoter [6] and by binding to mRNA cap binding proteins to link transcriptional regulation with RNA processing [7]. FLC expression is high in FRI + ecotypes, which require vernalization to flower. In contrast, in the Columbia (Col) background, where FRI is mutated, FLC expression is low and vernalization is not necessary [8]. Like FLC, expression of MAF2 is down-regulated after exposure to long periods of cold, although the differing expression kinetics mean that MAF2 ensures that flowering remains repressed despite plants experiencing transient periods of cold sufficient to down-regulate FLC. FLC expression is abolished in Col after ten days of cold treatment, whereas abolition of MAF2 expression requires 85 days of cold exposure [9]. Therefore one role of MAF2 could be to prevent flowering from being induced by a short cold spell, sufficient to down-regulate FLC expression. FLC interacts with SVP and directly represses the floral pathway integrator genes FT and SOC1[10][11]. MAF2 is also capable of interacting with SVP and maf2 mutants have elevated SOC1 and FT expression [9][12].

However, temperature affects flowering time in another way, involving some of the same factors. Changes in the ambient temperature influence flowering time, repressing the floral transition at low temperatures and inducing it at high temperatures [13]. In Arabidopsis even small changes in ambient temperature can modify flowering time [14]. Recently, several genes have been shown to be involved in ambient temperature sensing in flowering; FLOWERING TIME CONTROL LOCUS A (FCA), FVE, SVP, FLM and PHYTOCHROME INTERACTING FACTOR 4 (PIF4) [13][2].

FVE and FCA were among the first genes characterized as belonging to the thermosensory pathway [15]. FVE is part of a chromatin remodelling complex [16]. FCA is involved in cleavage and polyadenylation of mRNAs [17][18][19] and also promotes the processing of specific miRNAs, including miR172, which plays a role in temperature-regulated flowering [20]. miR172 inhibits the expression of several AP2-family transcription factors (e.g. SCHLAFMÜTZE (SMZ) [21], TARGET OF EAT 1 (TOE1) and TOE2 [22]), which would otherwise repress the floral pathway integrator, FT, and inhibit floral transition [23]. Since the abundance of miR172 is greater at 23°C than at 16°C [24], flowering is enhanced at the higher temperature by a reduction in the levels of the AP2 floral repressors leading to an increase in FT. The increase in miR172 abundance at 23°C is dependent on FCA, which is itself regulated by ambient temperature through both gene expression and protein stability [20].

In short days the phytochrome-interacting bHLH transcription factor, PIF4, induces flowering in response to elevated temperature, again by increasing FT expression [25]. Although both PIF4 transcript levels and PIF4 protein stability are somewhat increased at higher temperatures, the enhanced ability of PIF4 to bind to and activate FT at higher temperatures is likely to
be a more significant factor in its contribution to thermosensitivity [25]. The temperature dependency of PIF4 binding to FT is mediated at the chromatin level. Increasing temperature is associated with decreased H2A.Z-nucleosomes at the FT promoter [26], providing greater chromatin accessibility and binding of PIF4 [25].

Finally, multimeric complexes containing the MADS-box transcription factor SVP also play an important role in ambient temperature sensing, by repressing FT and SOC1 expression at low temperatures, thereby delaying flowering [12][27][28]. With respect to flowering time, svp mutants are insensitive to ambient temperature from 16°C to 23°C [29]. SVP-mediated repression of flowering is alleviated at increased temperature, at least in part, because the SVP protein is degraded by the proteasome under these conditions [28]. SVP interacts with several MADS-box transcription factors of the FLC sub-family; FLC, MAF2, MAF4 and FLM [28][12][27], to repress the expression of FT and SOC1 [12]. Strikingly, two of these interaction partners, MAF2 and FLM, have been shown to use temperature-dependent alternative splicing (TD-AS) to transduce the ambient temperature signal into a regulatory effect on flowering time [14][30][28][27].

FLM independently represses flowering in response to both low ambient temperature and short days. The flm mutant is slightly early flowering in long days, but this effect is enhanced in short days, where flm flowers much earlier than wild type controls [31]. Furthermore, analysis of flowering time in flm mutants has shown that FLM represses flowering at low temperatures, but not at higher temperatures around 27°C [27][14]. In Col FLM produces two splice variants, FLM-β and FLM-δ, which generate proteins with differing abilities to repress the floral pathway integrator genes [27][28]. FLM-β can interact with SVP and repress SOC1 whereas FLM-δ interacts with both SVP and FLM-β, but does not repress SOC1, acting instead to decrease the availability of repressive SVP and FLM-β. At 16°C the FLM-β isoform predominates, producing an active repressor of flowering. Increasing the temperature to 27°C causes the FLM-δ isoform to predominate, relieving the repression and allowing flowering.

MAF2, like FLM, is a major determinate of natural variation in Arabidopsis flowering time [32] which is also subjected to TD-AS to respond to ambient temperature and repress flowering at low temperatures [9] [30]. Here we further characterise the role of MAF2 in regulating the floral transition as part of an SVP-containing complex. Although MAF2 and FLM have independently acquired the ability to sense temperature by TD-AS, we show that the effect of the flm maf2 double mutant on thermosensitivity is similar to that of the svp single mutant, demonstrating that MAF2 and FLM both act with SVP to control the ambient temperature pathway.

**Results**

**MAF2 requires SVP to repress flowering**

The FLC and FLM proteins both interact with SVP to repress flowering [11][27]. Since MAF2 is also capable of interacting with SVP [12], we investigated the significance of SVP for MAF2 function by analysing flowering time in single and double mutants. Comparisons of flowering time in WT, maf2, svp, and svp maf2 backgrounds revealed that the early flowering phenotype seen in svp is not significantly enhanced in the svp maf2 double mutant (Fig 1A), suggesting that MAF2 and SVP are in the same pathway and that the MAF2-SVP interaction is required for the repression of flowering by MAF2. Expression analyses show that SVP remains expressed in a maf2 background, possibly even at a higher level than in wild type, and MAF2 also remains expressed in an svp mutant background (S1 Fig). These results show that MAF2 and SVP do not depend on each other for gene expression.

Several alternatively spliced isoforms of MAF2 have either been identified or predicted (Fig 2 and S2 Fig). Over-expression of one of these isoforms, MAF2var1, has previously been
reported to delay flowering in the \textit{Arabidopsis} L1-2 accession [30] (an accession that lacks a functional FLC [33]) in short days. Unexpectedly, overexpression of MAF2\textit{var}1 in \textit{Col} resulted in early flowering, a result that was attributed to non-target effects of MAF2\textit{var}1 expression on MAF paralogs, leading to artifactual early flowering [30]. Co-suppression problems have therefore precluded MAF2 splice variant function from being analysed in the \textit{Col} background. We have repeated these experiments in \textit{Col} (S3 Fig) and found that plants overexpressing MAF2\textit{var}1 do indeed flower later than wild type plants (Fig 1B), in agreement with MAF2 acting as a repressor of flowering. However, over-expressing MAF2\textit{var}1 in an \textit{svp} background does not result in repression of flowering, since 35S::\textit{MAF2}\textit{var}1 \textit{svp} plants flower early and at the same time.

![Fig 1. Comparison of flowering time in WT and mutant backgrounds. A,B,C, the columns indicate the number of rosette leaves (in black) plus the number of cauline leaves (in grey). Plants were grown at 21°C. A, Comparison of flowering time between WT and \textit{maf2}, \textit{maf2} \textit{svp} and \textit{svp} mutants, (no. of plants analyzed 161) B, Flowering time of WT and plants overexpressing MAF2\textit{var}1 in WT and \textit{svp} backgrounds (no. of plants analyzed 100, four independent transformants for each transgenic line). C, Flowering time of \textit{flc} \textit{maf2} double mutants compared to \textit{svp} mutants (no. of plants analyzed 92). D, Quantitative RT-PCR of \textit{FT} in WT and \textit{maf2} backgrounds. Error bars represent the standard error.

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![Fig 2. MAF2 splice variants. MAF2 splice variants observed by RT-PCR are shown with the names used in this report and alternative names reported elsewhere (Rosloski 2013, Ratcliffe 2003 and TAIR website). The names are followed by a schematic representation of the genomic structure of each splice variant. Rectangles represent exons and lines represent introns. The premature termination codon introduced as a result of intron 3 retention in MAF2\textit{var}2 is indicated (TAA).

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Low Temperature Repression of Flowering by MAF2
time as *svp* mutants (Fig 1B). Taken together, these results indicate that MAF2, like its close relatives FLC and FLM, directly interacts with SVP to repress flowering. We also tested the extent to which MAF2 and FLC are responsible for the repression of flowering exerted by SVP at 21°C. *fcl maf2* double mutants flower slightly later than *svp* mutants (Fig 1C), suggesting that SVP may still be capable of repressing flowering at 21°C in the absence of both FLC and MAF2. These results are in accordance with findings that other proteins of the FLC subfamily, such as FLM, can interact with SVP [12].

**The effect of maf2 mutation on FT in Col**

SVP directly represses the floral integrators, *FT* and *SOC1*, through binding to their promoters [11]. In previous experiments, over-expression of MAF2 has also been shown to be capable of repressing both *SOC1* and *FT* [9][12]. In agreement with this, *FT* expression is elevated in *maf2* mutants (Fig 1D), although the increase in expression is small, reflecting the mild phenotype of the *maf2* mutant in the *Col* background (Fig 1A and 1D). Therefore, MAF2, like SVP, FLC and FLM is involved in the repression of *FT* [12].

**MAF2 temperature-dependent alternative splicing**

The steady-state levels of the MAF2 splice variants change in response to ambient temperature [14][30]. We analyzed the relative expression levels of previously published MAF2 splice variants (Fig 2 and S2 Fig) at 16°C, 21°C and 27°C (Fig 3A). When grown at 16°C *MAF2var1*, the isoform that acts as a repressor of flowering in *Col*, predominates (Fig 3A), as was previously demonstrated for plants grown at 4°C [30]. Our analysis at 16°C shows that even a slight decrease in temperature results in this splice isoform predominating. As previously reported in Rosloski et al. 2012, the steady state levels of the splice variants *MAF2var1* and *MAF2var2* are approximately equal at 21°C. However, when plants are grown at 27°C a switch in splice preference is observed, so that *MAF2var2* now predominates at the expense of *MAF2var1*. Therefore, over a small physiological temperature range of just eleven degrees the dominant splice isoform switches from *MAF2var1* (at 16°C) to *MAF2var2* (at 27°C).

Since we showed that MAF2 requires SVP to repress flowering, we used yeast two-hybrid analysis to test the ability of MAF2 splice variants to encode proteins capable of interacting with SVP. MAF2 has been predicted to generate several distinct splice variants ([9], TAIR, S2 Fig). Despite extensive RT-PCR analysis under a range of conditions, we were only able to detect the 3 splice variants shown in Fig 2 in plants, but we artificially generated the undetectable MAF2var6 isoform for completeness. Testing splice variants in yeast two-hybrid assays revealed that MAF2var1, MAF2var5 and MAF2var6 are capable of interacting with full length SVP, but a MAF2var2-SVP interaction was not detected (S2 Fig). MAF2var2 lacks part of the K-domain and all of the C-domain; regions that are involved in the homo- and hetero-dimerisation of MADS-box transcription factors [34][35] and it is likely that exclusion of these domains from the *MAF2var2* isoform renders the resulting protein incapable of forming heterodimers.

**The MAF2var2 and MAFvar5 splice variants**

MAF2var2, the isoform that predominates at high temperatures, was tested for its ability to repress flowering by over-expression. In agreement with Rosloski et al., 2013, 35S::*MAF2var2 Col* plants flower at the same time as wild type controls, indicating that this form, unlike *MAF2var1*, is incapable of repressing flowering (Fig 3B). This finding is consistent with the yeast two-hybrid data that shows that MAF2var2 does not interact with SVP. The intron retention event (Fig 2) that produces *MAF2var2* also introduces a premature termination codon that
could potentially trigger nonsense-mediated mRNA decay (NMD) of this splice variant, leading to degradation of the mRNA and suppression of its steady-state levels. However, expression of MAF2var2 does not significantly increase in the NMD mutants upf1-5, upf3-1 and smg7-1 (S4 Fig), indicating that MAF2var2 is not targeted by NMD. Indeed, none of the MAF2 splice variants appear to be degraded by NMD (S4 Fig). Therefore, although premature stop codons are recognised triggers of NMD in some transcripts and there are examples, such as LHY-UAS4, where temperature regulated splicing produces variants that trigger NMD, MAF2var2 is not targeted by NMD under the conditions tested here. From these analyses we conclude that in wild type plants exposed to elevated temperatures TD-AS favours the production of MAF2var2, a non-functional splice isoform that serves only to decrease the levels of MAF2var1, which would otherwise actively repress flowering.
The third detectable MAF2 splice variant, MAF2var5, which skips the sixth intron and introduces a premature termination codon that removes the C-terminal domain of the protein (Fig 2), was also assessed for its ability to control the floral transition. Unexpectedly, 35S::MAF2var5 plants flower earlier than wild type (Fig 3C), suggesting that MAF2var5 can promote flowering. MAF2var5 is capable of interacting with SVP (S2 Fig) and could therefore promote flowering by competing with FLM for the available SVP, with the resulting complex unable to repress flowering, analogous to the proposed mode of action of the FLM-δ splice variant [27]. However, RT-PCR reveals very low expression of MAF2var5 at all tested temperatures in Col (Fig 3D), calling its contribution to flowering time in this accession into question. Further analysis will be required to determine the significance of MAF2var5, if any, in regulating the floral transition and to assess if this variant plays a significant role in other accessions.

MAF2 represses flowering at low temperatures

MAF2var1 represses flowering and is the predominant isoform at lower temperatures. To study the contribution of MAF2 to the repression of flowering at different temperatures, maf2 mutants were grown at 16°C, 21°C and 27°C and compared to wild type Col plants (Fig 4). maf2 mutants grown at 16°C flower at around the same time as WT plants grown at 21°C and significantly earlier than WT plants grown at 16°C. Similarly, flowering of maf2 mutants at 21°C is comparable to flowering times observed in WT plants at 27°C, indicating that the maf2 mutant is deficient in its response to temperature change across a range of temperatures from 16°C to 27°C. maf2 mutants retain a limited ability to respond to ambient temperature, since they flower later at 16°C than 27°C and this is likely to be due to the presence of FLM and SVP. Even at 27°C MAF2 mildly represses flowering, as evidenced by maf2 plants grown at 27°C flowering slightly earlier than WT plants grown under the same conditions (Fig 4). This residual repression is presumably caused by the limited production of MAF2var1 at 27°C (Fig 3A).

Fig 4. Flowering time of WT and maf2 mutants at different temperatures. (no. of plants analyzed 257) The columns represent the number of rosette leaves (in black) plus the number of cauline leaves (in grey). Error bars represent the standard error.

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MAF2 works in parallel with FLM to repress flowering at low ambient temperatures

FLM and MAF2 encode related MADS-box transcription factors that repress flowering at low temperatures. The early flowering phenotype observed in flm mutants grown at 16°C is comparable with that seen in maf2 mutants, but maf2 flm double mutants show an additive effect, producing a very severe early flowering phenotype at 16°C (Fig 5). This suggests that flm maf2 double mutants are very strongly compromised in their ability to repress flowering in response to low temperatures. Since both FLM and MAF2 repress flowering by interacting with SVP ([27], S2 Fig) and svp mutants are unable to respond to low temperature, we attempted to determine whether all of the low temperature repressive activity of SVP could be attributed to MAF2-SVP and FLM-SVP complexes. flm maf2 double mutants and svp single mutants grown at 16°C show an almost identical severe early flowering phenotype (Fig 5) suggesting that SVP relies mainly on interaction with FLM or MAF2 to repress flowering in response to low ambient temperatures.

Discussion

The role of MAF2 in repressing flowering

MAF2 represses flowering; a function that becomes inactivated either by extended exposure to cold or increased ambient temperature [9][39][this article]. In the vernalization response, when exposure to low temperature decreases the expression of FLC, MAF2 remains expressed for longer and is responsible for the maintenance of a repressed state in brief cold periods [9]. In the ambient temperature response, FLM and MAF2 repress flowering at low temperature, with the active FLM and MAF2 splice variants decreasing in response to increasing temperature. SVP has previously been shown to interact with both FLC and FLM to repress flowering [11][27]. Here we demonstrate that MAF2 also requires SVP to repress flowering. Like FLC and SVP, MAF2 represses both FT and SOC1, possibly by directly binding their promoters as part of a complex with SVP, as is the case for FLC-SVP [11]. In Col, where the FLC transcript abundance is lower than in active FRI ecotypes, FLC causes a mild repressive effect on flowering, similar to the repression exerted by MAF2 and a comparable early flowering phenotype is seen in both flc and maf2 mutants (S5 Fig). The mechanistic basis for this temperature response

Fig 5. Flowering time of WT and maf2, flm, flm maf2 and svp mutants at 16°C. (no. of plants analyzed 147) The columns represent the number of rosette leaves (in black) plus the number of cauline leaves (in grey). Error bars represent the standard error.

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is, however, not the same, since MAF2 is not regulated by FRI [9]. Unlike FLC, expression of MAF2 does not increase in Col FRI* plants (S5 Fig). In ecotypes such as Col, where FRI is inactive, FLC plays a limited role in repression of flowering and it is possible that MAF2 plays a more prominent role.

**TD-AS controls flowering time**

In addition to its role in the vernalization response, MAF2 represses flowering at low ambient temperature. However, the mechanisms through which vernalization and ambient temperature control MAF2 to affect flowering time are completely different. Vernalization affects expression of the MAF2 gene, whereas temperature acts post-transcriptionally on MAF2 splicing. MAF2 is therefore an example of a gene that is controlled by an environmental variable acting through two different mechanisms to influence flowering time. Ambient temperature affects MAF2 splicing through TD-AS, by influencing the relative abundance of two main splice isoforms; a full length MAF2 (MAF2var1) that represses flowering and a truncated MAF2 (MAF2-var2) which is inactive. This differential splicing involves increased retention of intron 3 at elevated temperatures (Fig 2). Increased intron retention at 27°C results in the production of more inactive MAF2var2, at the expense of the active repressor of the floral transition, MAF2-var1 (Fig 3A). Our splice site analysis adds to the published data [39][14] by broadening the temperature range to 27°C and confirming that the progressive increase in MAF2var2 expression continues at temperatures above 21°C. Plants show a progressive temperature-dependent flowering effect, flowering earlier at 27°C than at 21°C and earlier at 21°C than at 16°C. Decreased production of the active MAF2var1 isoform as the temperature increases mediates the concomitant decrease in floral repression. The residual delay in flowering observed in maf2 mutants at 16°C is largely due to the presence of functional FLM in these plants (Figs 4 and 5).

FLM, like MAF2, is also subject to TD-AS, but despite the high sequence similarity between these two genes, TD-AS in MAF2 and FLM shows several differences. TD-AS affects different parts of the encoded MAF2 and FLM proteins, producing an inactive and a dominant-negative variant respectively [27][39]. TD-AS involves exon skipping in FLM and intron retention in MAF2 and the MAF2 isoform that predominates at elevated temperatures contains a premature termination codon [39][27]. Even the rare MAF2 splice variant, MAF2var5, which our experiments suggest has the potential to act as a dominant negative, differs from the FLM-δ dominant negative splice isoform. Whereas MAF2var5 skips the sixth intron and introduces a premature termination codon, FLM-δ skips exon 2 [27]. MAF2 and FLM, which encode two closely related protein partners of SVP, presumably originated through gene duplication and still retain high sequence similarity, making it more likely that they would share the same TD-AS regulatory mechanism. Surprisingly, these differences indicate that, on the contrary, TD-AS mechanisms of thermosensitivity evolved independently in these genes.

Although temperature regulated splicing is a factor in regulating the expression of genes controlling plant adaptation to changing temperatures [40], its mode of action remains unclear. Proteins involved in splice site selection, such as serine/arginine-rich (SR) proteins could be critical for TD-AS [41][42][43][44]. SR proteins have one or two RNA recognition motifs and determine splice site selection in a concentration dependent manner by forming spliceosome complexes [45]. The expression of SR protein encoding genes is modified in response to temperature change [14][41][43] and the SR gene transcripts are themselves subject to TD-AS [42][43][44]. Furthermore SR mutants have various defects that also include flowering time alterations [46][47]. It remains to be seen whether temperature-dependent changes in SR protein levels drive TD-AS of developmental regulatory genes such as MAF2 and FLM.
FLM and MAF2 are not the only examples of temperature dependent splicing affecting flowering time. The circadian clock regulates several genes responsible for influencing flowering time, such as CONSTANS (CO), that acts to regulate the floral integrator FT \[48\] [49] [50]. Alternative splicing of some circadian clock genes such as LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED1 (CCA1), is regulated by temperature \[38\] [51] [52]. These TD-AS events can produce transcripts that are targeted by NMD, as in the case of LHY or generate protein forms that act as a dominant negative, such as CCA1. It will be interesting to discover the full range of TD-AS targets and the nature of the mechanism(s) that link ambient temperature to the choice of splice sites.

FLM and MAF2 repress flowering in parallel at low temperature

The additive effect observed in the flm maf2 double mutant (Fig 5) indicates that FLM and MAF2 work in parallel to repress flowering at 16°C. In addition, the fact that both FLM and MAF2 interact with SVP and the similarity of the early flowering phenotype seen at 16°C in flm maf2 double mutants and svp single mutants, suggests that all the temperature responsiveness of SVP is mediated by its related interaction partners MAF2 and FLM. This agrees with previous findings [12] that show that the extremely weak responsiveness of the svp mutant to temperature change is similar to the responsiveness of flm maf2 flc mutants. It has been suggested that other members of the FLC clade can also weakly influence thermosensitivity [12], although currently MAF2 and FLM are the only two that have been shown to be regulated by small changes in ambient temperature. This, together with our observation that the flm maf2 double mutant flowers similarly to the svp mutant suggests that MAF2 and FLM are the two main FLC clade proteins acting in complex with SVP to determine sensitivity to ambient temperature. Further studies will be required to address the additional contributions of each member of the FLC clade and their mode of action in controlling thermosensitivity.

At 16°C both FLM-SVP and MAF2-SVP repress flowering. As the temperature increases FLM-mediated repression of flowering appears to lift before MAF2-mediated repression, since at 27°C flm mutants flower at approximately the same time as WT plants, whereas maf2 mutants still flower slightly earlier ([14], S6 Fig). The parallel independent regulation of temperature induced flowering shown by FLM and MAF2 is analogous to the independent parallel repression of flowering prior to vernalization caused by FLC and MAF2. In both cases MAF2 adds an additional layer of repression to the control of the floral transition. Since MAF2 is capable of interacting with FLM in addition to its interaction with SVP [12] and MADS-box proteins have been found to be part of very large complexes [53], all three proteins could be part of a larger complex. If this is the case, depleting individual members of the complex could have differential effects, depending on their functional requirement within the complex. For example, the limited effect of individual maf2 or flm mutations, compared to the more significant effect of mutating svp, could indicate a requirement for SVP in the complex, but a degree of redundancy between MAF2 and FLM in complex formation. Future experiments should focus on the composition of the complexes that repress flowering at low temperature and the changes induced when the abundance of components of the complex are changed in response to increases or decreases in temperature.

Materials and Methods

Yeast two-hybrid analyses

Yeast two-hybrid analysis was performed using the yeast strain PJ69-4 and MAF2 splice variants were cloned in pDEST22 and pDEST32 [54]. Bait and prey plasmids were transformed
into two different yeast mating types and mated to obtain the required combinations. Yeast was grown at 21°C on selective media to test for interactions (S2 Fig).

Plant materials

*Arabidopsis Columbia* seeds (Col) were stratified on filter paper at 4°C for three days and then transferred to soil. Plants were grown in growth chambers set at different temperatures (16°C, 21°C, 27°C) in a long day photoperiod (16 hours light, 8 hours dark) and exposed to the same light intensity (230 μmol m⁻² s⁻¹). The statistical significance of differences in flowering time was verified using a two-tailed Student’s t-test.

Genotyping

*maf2* mutants were provided by NASC (SALK_045623), the expression of MAF2 was tested by RT-PCR and found to be undetectable in the *maf2* mutant.

The following primers were used for genotyping: *maf2* SALK_045623 CA245 Atp58, MAF2 WT allele CA245 CA272, s*vp*-32 CA245 AtP58, SVP WT allele CA345 CA346, *flc*-101 CA352 Atp58, FLC WT CA351 CA352, *flm*-3 CA379 AtP58, FLM WT allele CA378 CA379. Primer sequences can be found in S1 Table.

Real time PCR

Quantitative real time PCR was performed with a Bio-Rad CFX96 real-Time System. Retrotranscription was performed with Superscript II Invitrogen retrotranscriptase on total RNA extracted using RNeasy (Qiagen). Relative expression was calculated using the Bio-Rad CFX Manager 3.0 software. Three biological replicates were performed for each experiment. Primers used for Real time quantitative PCR: MAF2 CA279 CA280, SVP CA384 CA385, FT CA388 CA389, FLC CA365 CA366, ACTIN primers ACT2F ACT2R. The primer sequences can be found in S1 Table.

Semi-quantitative PCR to identify splice variant expression

Semi-quantitative PCR was performed by running a sample of the PCR on an agarose gel at different cycle points, to verify that the PCR had not reached the plateau phase. Elongation factor was used for normalization. PCRs of MAF2 were run on a 3% agarose gel to separate the splice variants. The primers used were: ELONGATION FACTOR ELF1A1 ELF1A2, MAF2 var1/var2 CA339 CA340, MAF2 var5 CA339 CA284. The primer sequences can be found in S1 Table. Leaves of young plants (7 leaves) were used for the RNA analyses. Experiments conducted on later leaves gave comparable results.

Overexpression of MAF2 splice variants

Overexpression of MAF2 splice variants was achieved by subcloning the appropriate coding sequence into the alligator vector pFP101 containing a Gateway recombination site downstream of the constitutive 35S promoter [55]. Transgenic seeds were identified by observing the GFP fluorescence in the seeds, only seeds showing a strong signal were sown on soil.

Supporting Information

S1 Fig. Quantitative real time RT-PCR of SVP and MAF2 in WT and mutant backgrounds. Expression is presented relative to WT. (TIF)
S2 Fig. **MAF2 splice variants and yeast two-hybrid results.** A. Published names used for the MAF2 splice variants in different sources: [9] [30]. B. Results of yeast two-hybrid analysis of protein-protein interactions. For completeness we also generated MAF2var6 by assembly PCR, although we were unable to detect this variant in plants. “+” indicates growth of yeast on selective media lacking leucine, tryptophan and histidine, which is indicative of a protein-protein interaction. Representative yeast growth plates are presented on the right, showing both yeast viability (-L-W plates) and interaction tests (-L-W-H plates).

(TIFF)

S3 Fig. **RT-PCRs on WT and lines overexpressing MAF2 splice variants (OV) in WT and svp backgrounds.** Elongation Factor (ELF) is used as a control. All OV samples show a significant increase in expression of the MAF2 splice isoform compared to the WT. Note that despite MAF2var1 svp showing higher levels of MAF2var1 expression than WT, these plants flower early, like svp (Fig 1B).

(TIF)

S4 Fig. **RT-PCR of MAF2 splice variants in WT and NMD mutant backgrounds.** In the NMD mutants upf1-5, upf3-1 and smg7-1, MAF2 splice variants are not expressed at significantly higher levels than in the WT. Elongation Factor (ELF) is used as a control.

(TIF)

S5 Fig. **Comparison between maf2 and flc mutants.** A, Flowering time of WT, maf2 and flc mutants. The plants were grown in LD at 21°C and were not exposed to vernalization (no. of plants analyzed 65). The bars represent the number of rosette leaves (in black) plus the number of cauline leaves (in grey). The error bars represent the standard error. B,C Quantitative real time RT-PCR of, B, MAF2, and, C, FLC in WT and FRI (Arabidopsis Col with an active FRI) background. The graph shows the relative expression compared to WT plants.

(TIF)

S6 Fig. **Flowering time of WT, maf2 and flm mutants at 27°C.** (no. of plants analyzed 93) The columns represent the number of rosette leaves (in black) plus the number of cauline leaves (in grey). The error bars represent the standard deviation.

(TIF)

S1 Table. **Primers used in PCR experiments.**

(XLSX)

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Author Contributions

Conceived and designed the experiments: BD CAA. Performed the experiments: CAA MM. Analyzed the data: CAA MM BD. Wrote the paper: CAA BD.

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