Ambient temperature-directed flowering time regulation

The role of alternative splicing

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Chapter 1

Introduction

A role for alternative splicing in ambient temperature-directed floral timing?

Flip-flops, shorts and a t-shirt: the perfect outfit for a hot summer day. But when evening falls and it becomes chilly, a warm sweater is not an unnecessary luxury. We as human beings are able to sense relatively small differences in ambient temperature, and recognize them as pleasant or unpleasant. To circumvent long exposure to unpleasant temperatures, we take actions like dressing appropriately or seeking shelter. The reason for this lays in the fact that even small differences in temperature can have a profound effect on bodily functions. Obviously, this does not only apply to humans, but to all living organisms. In animals ranging from flies to humans, temperature fluctuations are perceived by ion channels in the skin that get activated by temperature changes, leading to transmission of an action potential to the brain (Barbagallo and Garrity, 2015; Gracheva and Bagriantsev, 2015). In plants, the object of this thesis, homologs of the proteins that constitute these kind of thermoreceptors seem to be non-existing. Nevertheless, the ability to sense and react to ambient temperature changes is just as important. Indeed, plants show a high phenotypic plasticity in relation to ambient temperature. For example, *Arabidopsis thaliana* is known to have elongated petioles and hypocotyls, and show early flowering at higher temperature (Figure 1). This latter feature, the moment of flowering, is an important event in the life cycle of plants, since reproductive success depends on it. For example, when a plant flowers too early, and a period of frost arrives when the plant is in full bloom, flowers may die and the plant will not be able to propagate. Similarly, a plant that depends on insects for pollination needs to flower at the moment these pollinators are around, otherwise no seeds will be produced and again, the plant will not reproduce.
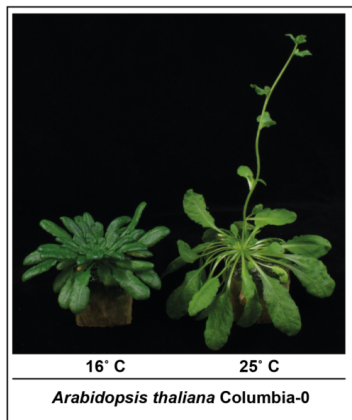
Figure 1. Arabidopsis thaliana Columbia-0 plants grown under continuous 16° C and 25°C conditions. Compared to 16° C, plants grown under 25° show an altered morphology, including early flowering.

Moreover, from a human perspective, optimal plant propagation is essential in many aspects of our lives, like production of food, shelter and biofuel. Flowering time has been shown to highly affect the yield of all kinds of crops. Earlier flowering means a shorter vegetative phase and for crops that are produced for their vegetative organs, such as cabbage (*Brassica*

oleracea), sugar beet (*Beta vulgaris*) and fodder grasses like *Lolium perenne*, this leads to reduced yields (Jung and Müller, 2009).

The climate of our planet is an ever-changing system, and over the past century, there has been a global trend of climate warming, that will probably proceed in the coming decades (Stocker et al., 2014). Since plants react on small changes in ambient temperature, it is likely that this global temperature rise will affect the flowering time of many of the plant species that colonize the Earth. In most ecosystems, flowers play an integral role, serving as a food source for insects and birds, which in turn take care of pollination. The organisms of an ecosystem are all interconnected, and disturbing the equilibrium, for example by altered flowering time of plants, can have highly unfavourable effects.

For most plants, it is easy to establish whether they are flowering or not, and throughout history, people have been documenting sightings of the first moment of flowering for a wide range of species. Several studies have used these data together with temperature data to investigate how rising temperature correlates with flowering time, and showed that a higher temperature coincides with earlier flowering for many plant species. This includes for example a study on 43 North American species using data over three time spans of which the first series was based on observations of the famous author and naturalist Henry David Thoreau, collected between 1852 to 1858 in Concord, Massachusetts, USA (Miller-Rushing and Primack, 2008). The authors showed that temperature increased 2.4° C during the total length of their study (1852-2006), and determined that the studied plants had flowered progressively earlier over the past 150 years, with an average total change of flowering time of seven days. The earlier flowering was strongly correlated with warmer mean temperatures in the months January, April and May for almost all species. Moreover, they showed that there is a big variation in the increase in flowering time, with extremes of 21 and 32 days earlier flowering for highbush blueberry (Vaccinium corymbosum) and yellow wood sorrel (Oxalis europaea), respectively.

Another study on desert shrubs in the Sonoran desert, the hottest desert in North America that has even become hotter over the last century (Weiss and Overpeck, 2005), used modelling and herbarium species to show that flowering time has probably advanced 20-41 days from 1894 to 2004 (Bowers, 2007).

Closer to home, a study on more than 400 plant species in the UK that were assessed over a 250 years timespan, showed a significant correlation between temperature and flowering time (Amano et al., 2010). For every 1° C increase in temperature, plants flower five days earlier on average.

The rising temperatures will also affect crop species. A study on cereal crops in Northern and Central Europe has investigated this, and developed models to predict how the climate forecasts for 2040 will affect flowering time in the future. Using flowering data obtained from field trials between 1985 to 2009, and climate data obtained from weather stations close to the field experiments, they predicted that flowering time of winter wheat, spring wheat, spring oats and maize will advance from several days to more than two weeks,

depending on the crop, the region and the climate model that was used (Olesen et al., 2012). A comparable study in wheat cultivars in Australia showed similar results, and concluded that the current germplasm lacks variation to maintain the current length of the pre-flowering season in the future (Zheng et al., 2016). The authors concluded that breeders will have to develop new genetic sources for later flowering.

For this, understanding how plants perceive ambient temperature and adjust flowering time is an essential prerequisite. However, research on the molecular mechanisms by which plants sense temperature and adapt flowering time started relatively recent, and much is still unknown (see Chapter 2 for a review on the current state of knowledge). This thesis was designed to investigate the molecular pathways that enable the plant to sense temperature and adapt flowering time accordingly. Based on an observation that many MADS-box transcription factor genes that are involved in flowering time show alternative splicing, we decided to investigate the possibility that alternative splicing is a key mechanism in temperature perception and flowering time adaptation in plants. Therefore, the foundations of this research are transcriptome-wide alternative splicing analyses of plants that face changes in ambient temperature. The results are presented in the next chapters. Below, I will explain the basic principles of alternative splicing, and describe what is known about causes and consequences of differential alternative splicing.

The basics principles of alternative splicing

Alternative splicing is the molecular mechanism by which more than one messenger RNA (mRNA) can be produced from a single gene. The majority of eukaryotic genes are organized in domains called introns and exons. In general, the introns are spliced out during transcription and the exons are joined together to form the coding sequence from which the protein will be translated. However, besides this canonical way of splicing, differential selection of splice sites can lead to intron retention (IR), exon skipping (ES), and alternative splice site selection at the 5' and/or 3' end (as reviewed by (Reddy et al., 2013), Figure 2). In this way, a single gene can produce several to many different mRNA isoforms.

In the model plant *Arabidopsis thaliana*, approximately 60% of the intron-containing genes show alternative splicing (Marquez et al., 2012). In most cases, alternatively spliced genes produce a mix of different splice forms. However, the proportion of each splice form in this mix is not constant, and varies depending on developmental stage, tissue type, or environmental fluctuations (Filichkin et al., 2010). This ratio change of splice forms is what we call "differential (alternative) splicing".

Box 1: Alternative is mainstream

Alternative splicing. It sounds like something that is the exemption from the rule. But the term is deceptive in several ways. The majority of eukaryotic genes consist of exon and introns, and after the gene is transcribed into pre-messenger RNA (pre-mRNA), the introns are spliced out at the exon-intron borders, the exons are joined together, and mature mRNA is formed. This is what text books call "conventional splicing". However, splicing can occur differently from this rule, since exons can be skipped, introns can be included, or splicing takes place at a different position than at the canonical exon-intron border. It seems logical that this would be called "alternative splicing", but this is not the case. The term "alternative splicing" is used when one gene produces more than one mRNA isoform. This is achieved by non-conventional splicing, for example a gene that besides producing a conventional form, also produces an isoform that retains the second intron. However, when a gene produces only one mRNA isoform that is not produced according to the rules of conventional splicing, this gene is not regarded to be alternatively spliced, since alternative splicing requires more than one isoform per gene. Having the definition of alternative splicing clarified, the question remains how 'alternative' alternative splicing is. In 2003, the estimates for alternative splicing in plants was 1.2% (Zhu et al., 2003) of the genome. The explosive growth of next generation sequence experiments throughout the last years (Kodama et al., 2012) gives a totally different view though: the latest study assessing the whole Arabidopsis transcriptome shows that 61% of the intron containing genes undergo alternative splicing (Marquez et al., 2012). Thus, the majority of intron containing genes are alternatively spliced, and alternative splicing turns out to be the rule rather than the exception.
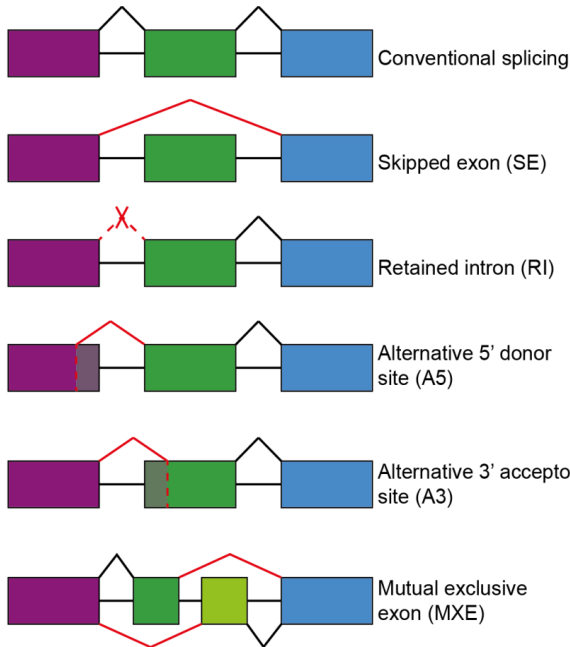
Figure 2. Splicing events. Overview of splicing events that can occur. Alternative events are depicted in red, conventional events are depicted in black. Boxes represent exons, lines represent introns.

The spliceosome

At the root of either conventional or alternative splicing lays the spliceosome, a large cellular machinery that catalyses the splicing reaction. The spliceosome is a ribonucleoprotein (RNP) with a highly dynamic structure that assembles *de novo* at each splice site in a stepwise manner. It consists of five different small nuclear (sn)RNP subunits and numerous protein cofactors of which the composition changes with every step. The snRNPs are composed of an snRNA, several RNA-binding Sm proteins and other complex-specific proteins (as extensively reviewed by (Chiou and Lynch, 2014; Matera and Wang, 2014; Papasaikas and Valcárcel, 2016; Wahl et al., 2009)). Higher eukaryotes, including plants, contain two types of spliceosomes: the major U2-type spliceosome and the minor U12-type spliceosome. The latter is involved in the splicing of a rare class of introns with non-canonical splice sites, called the AT-AC or U12-dependent introns. In Arabidopsis, less than 1% of the introns belong to this class (Zhu and Brendel, 2003). Hence, I will focus on the major U2-type to explain spliceosomal functioning. Moreover, most information regarding the assembly of the spliceosome comes from work in budding yeast and human cells. The key assembly steps are highly conserved between these species (Wahl et al., 2009), and can be regarded to be applicable in plants as well. Moreover, most animal spliceosomal proteins are conserved in plants, suggesting a similar composition of the spliceosome (Wang and Brendel, 2004).

For accurate splicing, the spliceosome requires 4 intron-defining sequences: the two splice sites, a branch point upstream of the 3' splice site (ss) and a polypyrimidine tract at the 3' ss. The first step in the formation of the spliceosome is the recognition of the 5' ss by the U1 snRNP, at which the U1 snRNA interacts with the 5' ss through base paring. This ATP-dependent interaction is weak, but can be stabilized by serine/argenine-rich (SR) proteins and the cap-binding complex (Cho et al., 2011; Pabis et al., 2013; Staknis and Reed, 1994). Consequently, there is cooperative binding of the U2 auxiliary factor subunits U2A65 and U2A35 to the polypyrimidine tract and the 3' ss, and the SF1 protein that binds at the BPS and U2A65 (Berglund et al., 1998; Ruskin et al., 1988). At this stage, the spliceosome has formed the **Early or E complex**.

To form the **pre-spliceosome or A complex**, the U2 snRNP binds to the BPS of the pre-mRNA through ATP-dependent base pairing, displacing SF1. Again, this is a weak interaction, and stabilization is accomplished by binding of the multi-subunit complexes SF3a and SF3b (Gozani et al., 1996).

For the next step, at which the **precatalytic spliceosome or B complex** is formed, a pre-assembled tri-snRNP complex composed of U4/U6 and U5 is recruited (Maroney et al., 2000). Subsequently**, complex B***, that is catalytically active, is formed through several major conformational and compositional changes facilitated by several RNA helicases. During these rearrangements, U1 and U4 are released (Fabrizio et al., 2009).

Now that the spliceosome is catalytically activated, the first splicing step can be performed, at which the 5' ss is cleaved, and the **catalytic splicesome or complex C** is formed. Rearrangements, assisted by a DExD/H ATPase, take place to facilitate the second catalytic step at which the 3' ss is spliced (Konarska et al., 2006). Splicing is now finalized, and the **post-catalytic spliceosome** disassembles in an ATP-dependent manner, assisted by several RNA helicases (Ilagan et al., 2013; Schwer and Gross, 1998).

Despite the fact that splicing is a highly controlled process with extensive quality control, it also has a large degree of plasticity, shown by the fact that differential selection of splice sites is occurring constantly, and can be differential in time, tissue and upon environmental influences. Besides the key assembly elements mentioned above, the spliceosome recruits a whole array of factors that aid in splice site recognition, stabilization and destabilization, quality control and more, influencing the splicing decision. As yet, 395 splicing related proteins are identified, that are all assumed to play a role in the splicing process (Wang and Brendel, 2004). Moreover, besides the splice sites, branch point and polypyrimidine tract that define the intron, the spliceosome makes use of other cis-regulatory elements that can act as splicing enhancers or silencers (as reviewed by (Syed et al., 2012)). These elements can be both intronic and exonic, and can be directly bound by splicing related proteins like SR proteins and heterogeneous nuclear RNP (hnRNP) proteins, which control splice site selection by recruiting other splicing related proteins, forming multicomponent interactions

that influence where the spliceosome assembles (as extensively reviewed by (Matlin et al., 2005)).

The plant splicing related proteins remain relatively poorly understood. Most progress has been made on the group of SR proteins, although the lack of plant-derived *in vitro* splicing assays impeded biochemical characterization (Reddy and Shad Ali, 2011). SR proteins show a high level of conservation between metazoa and plants. However, flowering plants contain in general twice as many SR genes as other organisms (Richardson et al., 2011). For example, Arabidopsis contains 18 SR genes, rice contains 22 and soybean encodes even 25 SR proteins. In contrast, humans only have 12 SR genes. Moreover, plants evolved plant-specific SR proteins that are structurally different from all other species, showing a unique domain organization. The high number of SR proteins together with the fact that plants evolved a novel group of SR proteins are indications of the importance of these proteins in these organisms.

Other factors that influence the splicing decision

The splicing process in not only regulated by spliceosomal proteins, but involves many more layers of regulation. Factors like chromatin structure, nucleosome positioning, DNA methylation, histone marks, RNA polymerase II (polII) elongation speed, the secondary structure of the pre-mRNA and long non-coding (lnc)RNAs, all play a role in the splicing outcome (as reviewed by (Naftelberg et al., 2015), (Buratti and Baralle, 2004; McManus and Graveley, 2011; Yoon et al., 2013).

RNA polymerase II

An important factor in splicing regulation is the fact that splicing is not an independent process, but is actually coupled to transcription, and occurs when the transcript is still in contact with RNA polII. Therefore, factors that are involved in transcription, may also be involved in splicing ((Naftelberg et al., 2015). RNA polII plays an important role in the coupling between transcription and splicing. The C-terminal domain of the large subunit is responsible for guiding splicing factors to the site of transcription through direct interaction (Misteli and Spector, 1999). This role in splicing factor recruitment is often specified as "spatial coupling", to distinguish it from another important characteristic called "kinetic coupling". Kinetic coupling is based on the fact that the splicing outcome is dependent on the elongation rate of RNA polII. The idea behind this is that when two splice sites compete, the event more upstream will have an advantage over the more downstream event because the upstream position is earlier reached by RNA polII. This advantage for the upstream event is bigger when polII speed is high, and smaller when polII speed is low (as reviewed by (Saldi et al., 2016)). PolII elongation rate is highly dynamic, and varies between genes and even between different regions within a gene (Jonkers and Lis, 2015). This is also where the chromatin comes into play. The nucleosomes in the chromatin can act as "speed bumps", pausing elongation. Hence, a higher nucleosome density of a certain gene can affect the

splicing outcome. The assembly or disassembly of these nucleosomal road blocks is in turn influenced by chromatin remodellers and histone chaperones. Also histone marks are thought to influence polII elongation rate by controlling nucleosome occupancy, although they also affect splicing by recruitment of splicing factors (Kornblihtt et al., 2013).

DNA methylation is also influencing splicing through kinetic coupling. The DNA binding protein CCCTC-binding factor (CTCF) is able to affect splicing by pausing polII. However, in regions with DNA 5-methyl-CpG methylation, the methyl-CpG-binding protein MeCP2 is recruited, which inhibits binding of CTCF (Marina et al., 2016; Maunakea et al., 2013; Shukla et al., 2011).

RNA secondary structure

RNA molecules are known to form complex secondary and tertiary structures *in vivo*. The secondary structure of the mRNA has shown to influence splicing, for example by preventing recognition of splice sites and branch points by the spliceosome (as reviewed by (Buratti and Baralle, 2004)). Most research has focussed on the effect of local secondary structure, since initial RNA folding occurs co-transcriptionally (McManus and Graveley, 2011). Conserved secondary structures have been found to be enriched at alternative splice sites (Shepard and Hertel, 2008), indicating that local structures are indeed important in splicing. However, there are also examples of long-range mRNA interactions that influence the splicing decision. In Drosophila, the *Down syndrome cell adhesion molecule* (*Dscam*) gene contains a "docking" sequence in the first intron, and the subsequent introns contain "selector" sequences that can base pair with the docking sequence at varying strengths. The strength of this interaction affects the frequency of exon 6 variant inclusion (May et al., 2011).

mRNA secondary structure also appears to be related to RNA polII speed, in a reciprocal fashion: RNA structure influences polII elongation rate (at least *in vitro*) (Zamft et al., 2012), and polII speed affects how nascent transcripts fold (at least *in vitro* and in prokaryotes, as reviewed by (Pan and Sosnick, 2006)).

Long non-coding RNAs

A newly emerging research field is that of the long non-coding (lnc)RNAs, non-protein coding sequences of more than 200 nucleotides in length. For a long time, these molecules were deemed as transcriptional "noise", but recently they are shown to have regulatory functions, both in pre- and post-transcriptional processes. There are a couple of examples in which lncRNAs play a role in splicing regulation, by binding splicing factors and modulating their concentration (Yoon et al., 2013). However, up till now, there are only a few examples, and it is yet to be determined whether the role of lncRNAs in splicing is a more general feature.

The consequences of alternative splicing

As mentioned earlier, in Arabidopsis approximately 60% of the intron containing genes are alternatively spliced. The number of different mRNA transcripts is more than twice the

amount of genes (Marquez et al., 2012). Thus, due to alternative splicing, the transcriptome is more than twice the size of the genome. But what are the consequences of the expansion of the transcriptome? Does it implicate an equal expansion of the proteome?

Nonsense-mediated decay

One of the possible outcomes of splicing is the introduction of a premature stop codon (PTC) due to a frame shift. In eukaryotes, part of PTC-containing transcripts are eliminated by nonsense-mediated decay (NMD), a surveillance pathway to prevent translation of these mRNAs into aberrant proteins. By equilibrating functional transcripts and transcripts that are targeted for NMD, alternative splicing becomes a way to regulate gene expression at a post-transcriptional level.

There are several studies showing NMD of alternatively spliced transcripts. For example, the serine/argenine-rich splicing factors (SR genes) produce many alternative transcripts that are targeted for NMD (Palusa and Reddy, 2010). Also, some transcripts of the core clock components *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *EARLY FLOWERING 3* (*ELF3*) are degraded through the NMD pathway (Kwon et al., 2014). A study using a double mutant for the core NMD machinery genes *UPF1* and *UPF3* shows that 17% of intron-containing protein coding genes generate NMD-targeted splicing variants (Drechsel et al., 2013). NMD is thus an important outcome of alternative splicing, but leaves the majority of alternative transcripts unaffected.

Non-functional proteins

Transcripts that are not targeted for NMD will be translated. However, not all of the resulting proteins will be functional, for example because functional domains are disrupted or completely skipped, or because targeting signals are absent and the protein is not transported to the appropriate destination in the cell. The eventual effect is comparable with NMD - affecting the total amount of functional protein - but then at a post-translational level. The major source of non-functional proteins is probably the production of unstably folded proteins. Alternative splicing can introduce differences in amino-acid sequence that affect the structural integrity of the protein, resulting in a non-functional protein isoform. There has been a lot of debate about the extent of stably versus unstably folded proteins, due to a lack of proteomic evidence for the majority of isoforms (Hao et al., 2015; Melamud and Moult, 2009; Tress et al., 2008; Tress et al., 2007). A recent study tried to predict the amount of stably folded isoforms from the human transcriptome using a modelling approach (semi-supervised learning) and predicted that ~$1/3^{rd}$ of isoforms with an exon-skipping event produces stable proteins (Hao et al., 2015). Thus, at least for human splice isoforms resulting from exon skipping, a considerable amount of proteins will likely be unstable and therefore non-functional.

Functional proteins

Splicing isoforms that are not degraded by NMD and are not translated in unstably folded proteins, will form functional proteins that that may be structurally and functionally different from the canonical protein. For example the isoforms can have different DNA, RNA and/or protein-binding specificities, modified enzymatic properties or altered localization (Kelemen et al., 2013). Alternatively, splice forms can adopt dominant-negative activity. For example, the JASMONATE-ZIM-DOMAIN PROTEIN 10 (JAZ10) in Arabidopsis, which regulates jasmonate (JA) signalling. In the absence of JA, the canonical protein inhibits the JA response. Upon elevated levels of the hormone, the CORONATINE INSENSITIVE 1 (COI1) protein causes degradation of JAZ10 by interaction with the Jas domain. This releases the repression of the JA response, and consequently activates expression of genes involved in plant growth, development and defense (Staswick, 2008). One of the alternative splice forms of JAZ10 lacks the Jas domain and is therefore highly resistant to JA-induced degradation. Because the other domains are still functional, it functions as a repressor of the JA response that cannot be released by elevated JA levels, making the plant hypersensitive to JA (Chung and Howe, 2009; Moreno et al., 2013).

Concluding words

Alternative splicing is a highly complex and dynamic process that contains many layers of regulation. Although new studies on the splicing mechanism are appearing continuously, many aspects of the splicing process and regulation remain enigmatic. Nevertheless, there is a growing body of evidence that alternative splicing plays an important role in the regulation of many different processes, which can be influenced by environmental fluctuations. Hence, in pursuit of finding out how plants can alter floral timing by ambient temperature variation, we decided to investigate the role of alternative in this process in more detail.

Aim and outline of this thesis

The aim of this thesis was to elucidate the molecular pathways underlying the response in flowering time upon ambient temperature fluctuations, with a focus on the putative role of alternative splicing in this process.
In **Chapter 2**, we reviewed the current state of knowledge on molecular pathways regulating ambient temperature-directed flowering time. In **Chapter 3**, we used transcriptome-wide tools to investigate how changes in ambient temperature affect alternative splicing in three different genotypes (two accessions of *Arabidopsis thaliana* and one variety of *Brassica oleracea* (cauliflower). We showed that amongst the differentially spliced genes, splicing related genes are overrepresented, indicating that the genes encoding the spliceosome are prone to differential splicing when temperature changes. Investigation of a mutant for one

of the splicing related genes showed an altered flowering time response under different ambient temperatures, compared to wild type plants. We proposed a two-step model in which splicing related genes are targeted for differential splicing, which results in changes in the composition of the spliceosome, causing differential splicing of downstream genes that affect the development and architecture of the plant.

In **Chapter 4**, we showed that *FLOWERING LOCUS M* (*FLM*) is a key regulator in ambient temperature floral timing, which is regulated by temperature-dependent alternative splicing. In chapter 3 we already found *FLM* to be differently spliced under different temperatures, and in this chapter we showed that this is indeed responsible for the flowering time response in *Arabidopsis thaliana* Col-0. In this accession, the two major splice forms are *FLMβ* and *FLMδ*. At lower ambient temperature, relatively more *FLMβ* is produced, and at a higher ambient temperature, more FLMδ is produced. We showed that FLMβ forms a repressive complex together with SHORT VEGETATIVE PHASE (SVP) that directly binds and represses floral integrator genes, and hence represses flowering. FLMδ, that is increasingly produced when the temperature raises, functions as a dominant negative form that is not able to bind DNA, but titrates out SVP from the repressive complex, clearing the road for floral induction. In **Chapter 5** we commented on a study in another Arabidopsis accession with different FLM splicing, and clarified several misconceptions about investigating the flowering time response to different ambient temperature. With **Chapter 6** we aimed to get insight into the mechanism behind differential splicing of FLM by investigating the role of chromatin position and transcription-initiation speed. We showed that different chromatin positions indeed cause different *FLMβ*/*FLMδ* splicing ratios.

In **Chapter 7**, we studied the functional conservation of *FLC*-clade genes in *A. thaliana*, which consist of *FLC*, *FLM* and *MAF2-5*, at the intra- and interspecific level. Despite the high functional conservation at the intraspecific level, *FLM* and *MAF* orthologues are not widely present, as we could only detect them in the Brassicaceae family. However, in several species belonging to other plant families, varying numbers of *FLC*-like genes can be found. This raises the question what the function of these genes is. By investigating this in tomato, we showed for the first time that thermosensitive floral timing by alternative splicing of *FLC*-like genes might be a conserved mechanism in flowering plants.

To conclude, in **Chapter 8** I discussed the findings of this thesis and put them into context, and made suggestions for further research.

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Chapter 2

Research on floral timing by ambient temperature comes into blossom

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Abstract

The floral transition is an essential process in the life cycle of flower-bearing plants, because their reproductive success depends on it. To determine the right moment of flowering, plants respond to many environmental signals, including day length, light quality, and temperature. Small changes in ambient temperature also affect the flowering process, although our knowledge of the genetic and molecular mechanisms underlying this flowering pathway is limited. However, recent advances in *Arabidopsis* (*Arabidopsis thaliana*) have uncovered multiple molecular mechanisms controlling ambient temperature regulation of flowering, which modulate both repressing and activating factors of flowering time. At a time when temperatures are rising worldwide, understanding how plants integrate ambient temperature signals can be crucial for crop production.

A short history of flowering time research

Plants are sessile organisms that cannot migrate to more optimal locations when the environmental conditions are not favourable. Therefore, they need strategies to adapt and cope with these conditions, enabling them to increase their chance of survival and, ultimately, reproduction. It has long been noted that plants integrate environmental cues in their developmental programs to achieve this adaptation. The moment of flowering, which is heavily influenced by environmental conditions, is a key step in the life cycle of flowering plants and successful production of progeny depends on this process. When flowering starts under unfavourable conditions (e.g., just before a period of frost), seed production cannot be guaranteed.

The first descriptions of molecular mechanisms that integrate environmental cues to control flowering time date back to the early 1990s (Martinez-Zapater and Somerville, 1990). Over the succeeding years, it has become clear that different environmental signals that influence flowering time, such as day length (photoperiod), light quality, and vernalization (see Glossary), are perceived through different molecular pathways in the plant. These pathways converge at the so-called 'floral integrators', a small set of genes where all flowering time pathways come together. *FLOWERING LOCUS T* (*FT*)*, SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *LEAFY* (*LFY*) are regarded to be floral integrator genes (Simpson and Dean, 2002). Besides knowledge on the floral integrators, a plethora of genes involved in different pathways has been revealed following the first report on molecular mechanisms controlling flowering time, including *FLOWERING LOCUS C* (*FLC*) in the vernalization pathway (Michaels and Amasino, 1999), and *CONSTANS* (*CO*) (Simon and Coupland, 1996), phytochromes, and cryptochromes (Lin, 2000) in both the photoperiod and light-quality pathways. By contrast, the first reports on the influence of ambient temperature on flowering time appeared much later (Blázquez et al., 2003) and (Halliday et al., 2003), despite the fact that small fluctuations in temperature have dramatic effects on flowering time. However, growing signs of a rapidly

changing climate with temperatures rising worldwide puts a spotlight on the importance to better understand the processes whereby fluctuations in ambient temperature influence flowering time.

The first analyses of flowering-time responses of *Arabidopsis* mutants and ecotypes to different temperatures highlighted several candidate genes as potential key regulators of the ambient temperature pathway. Most *Arabidopsis* ecotypes flower earlier at elevated temperatures, whereas some mutants and ecotypes are less affected by a temperature switch. Among these are mutants for the MADS-box genes *SHORT VEGETATIVE PHASE* (*SVP*) and an ecotype with a deletion of *FLOWERING LOCUS M* (*FLM*), which have lost their ability to respond to fluctuation in ambient temperatures (Balasubramanian et al., 2006) and (Lee, 2007). Until recently, the exact underlying molecular mechanism was unknown. However, recent studies (Kumar et al., 2012b), (Kumar and Wigge, 2010), (Lee et al., 2013a), (Posé et al., 2013), (Kim et al., 2012) and (Lee et al., 2010) show the presence of multiple mechanisms, such as alternative splicing and stability of encoding proteins, involved in ambient temperature-regulated floral timing for genes previously placed in different flowering pathways (Scortecci et al., 2003), (Li et al., 2008), (Aukerman and Sakai, 2003) and (Yang et al., 2011). Here, we review newly identified genetic and molecular mechanisms involved in ambient temperature-regulated flowering time and explore the question of how these different mechanisms and pathways relate to each other.

Glossary

Alternative acceptor site: in alternative splicing, usage of an alternative 3' splice junction.

Alternative splicing: the process at which particular exons or introns are excluded or included from the precursor of mRNA to form multiple mature mRNAs.

ChIP: Chromatin Immunoprecipitation, a technique used to study binding of a protein of interest to DNA *in vivo*.

Co-immunoprecipitation: technique used to study the proteins bound to a protein of interest by precipitating the intact protein complex along with the known protein.

Cryptochromes: photoreceptors sensitive for blue light.

EMSA: Electrophoretic mobility shift assay, technique used to study binding of a protein of interest to a DNA probe of interest *in vitro*.

Epistatic: when expression of one gene depends on the presence of another –nonallelic– gene

Floral integrator: gene that integrates signals from different flowering time pathways that can initiate flowering when sufficiently activated.

Hypocotyl: the stem of a seedling below the cotyledons.

Juvenile phase: early phase of vegetative development at which the plant is not yet competent to flower.

Mutually exclusive exon: in alternative splicing, one of two exons is retained in a splicing event, but not both.

Orthologous genes: genes from different species that share a common ancestral gene.

Paralogous genes: genes derived from a duplication within a genome.

Petiole: the stalk of a leaf that attaches the leaf blade to the stem.

Phytochromes: photoreceptors sensitive for red and far-red light.

Proteasome: protein complex that degrades unneeded or damaged proteins.

RNA Polymerase II: an enzyme responsible for the synthesis of mRNA by transcription of DNA.

Vernalization: a prolonged period of cold, winter-like temperatures by which the plant acquires the competence to flower.

Open sesame: does warmth evict H2A.Z to open the gates for transcriptional activation?

Is there a role for chromatin remodeling in the regulation of floral timing by ambient temperature? The DNA of eukaryotes is organized into chromatin, which comprises repeating units called nucleosomes. Each nucleosome presents a core comprising eight proteins (histones) with DNA wrapped around it. Similar to most eukaryotes, plant nucleosomes contain two copies of each of the canonical histones H2A, H2B, H3, and H4. The basic structural function of nucleosomes is that they tightly pack the DNA to fit into the nucleus. However, they also have an important function in the control of gene expression. This can be achieved through post-translational modifications of histone tails, such as methylation, acetylation, SUMOylation, and phosphorylation, and replacement of the canonical histones with several histone variants (Nelissen et al., 2007). One of the important histone variants is H2A.Z, which can be incorporated into chromatin by histone replacement, whereby the canonical histone H2A is replaced by H2A.Z.

Plants with a mutation in *ACTIN-RELATED PROTEIN 6* (*ARP6*), which is part of the SWR1 complex that is responsible for H2A.Z deposition (Deal et al., 2007), show serrated leaves and a bushy phenotype, and occasionally have flowers with more than four petals (Choi et al., 2005). Moreover, these mutant plants display early flowering and petiole and hypocotyl elongation, resembling phenotypes of warm-grown wild type plants (Kumar and Wigge, 2010). The transcriptome of the *arp6* mutant grown at 12°C was compared with wild type plants transferred to a higher temperature and revealed a substantial overlap between mis-expressed genes in the mutant and the differentially expressed genes from wild type plants shifted to higher temperatures. This indicated that the *arp6* mutant, which is impaired in H2A.Z incorporation, was also affected in terms of its temperature response. To investigate the putative role of H2A.Z in temperature responses, chromatin immunoprecipitation (ChIP) analysis was used to study H2A.Z occupancy at the transcriptional start site (TSS) of several loci. When plants were shifted to a higher temperature, all studied loci showed a significant decrease in H2A.Z occupancy, independent of their transcriptional response. The nucleosomes positioned directly upstream (−1) and downstream (+1) of the TSS in the locus encoding the temperature-responsive protein HEAT SHOCK PROTEIN 70 (HSP70) were used to investigate the nucleosomal dynamics in more detail. At the +1 nucleosome, which contains H2A.Z at relatively low temperatures, shifting to a higher temperature decreased H2A.Z occupancy and to a lesser extent occupancy of the control histone H3. At the −1 nucleosome, which is devoid of H2A.Z, the same temperature shift did not reduce histone H3 occupancy, indicating that nucleosome occupancy in general is not affected by a temperature shift. This led the authors to suggest that the presence of H2A.Z in the nucleosome contributes to the dynamic responses to temperature. However, whether the authors used the appropriate position to study H2A.Z dynamics is under debate, because more recent research has shown that it is H2A.Z enrichment in the gene body rather than at the TSS that correlates with gene responsiveness (Coleman-Derr and Zilberman, 2012). Nevertheless, *arp6* mutants showed a constitutive open conformation of the +1 nucleosome

of *HSP70*, and phenocopy warm-grown plants, which is in line with the hypothesis that H2A.Z is involved in temperature responses. Regarding the biological function of the suggested H2A.Z mechanism, it is important to realize that *arp6* mutants are still temperature responsive, including earlier flowering upon higher temperature. This suggests that, in a wild type situation, H2A.Z depletion functions as an enabler, rather than an activator of the higher temperature response. In such a scenario, transcription factors can be differentially regulated upon shift to a higher temperature and can only access their targets when H2A.Z depletion has occurred. With respect to flowering control, H2A.Z functions as an additional control post and a gatekeeper that circumvents transcription of flowering genes at lower temperatures, despite the presence of inductive signals (Figure 1A).
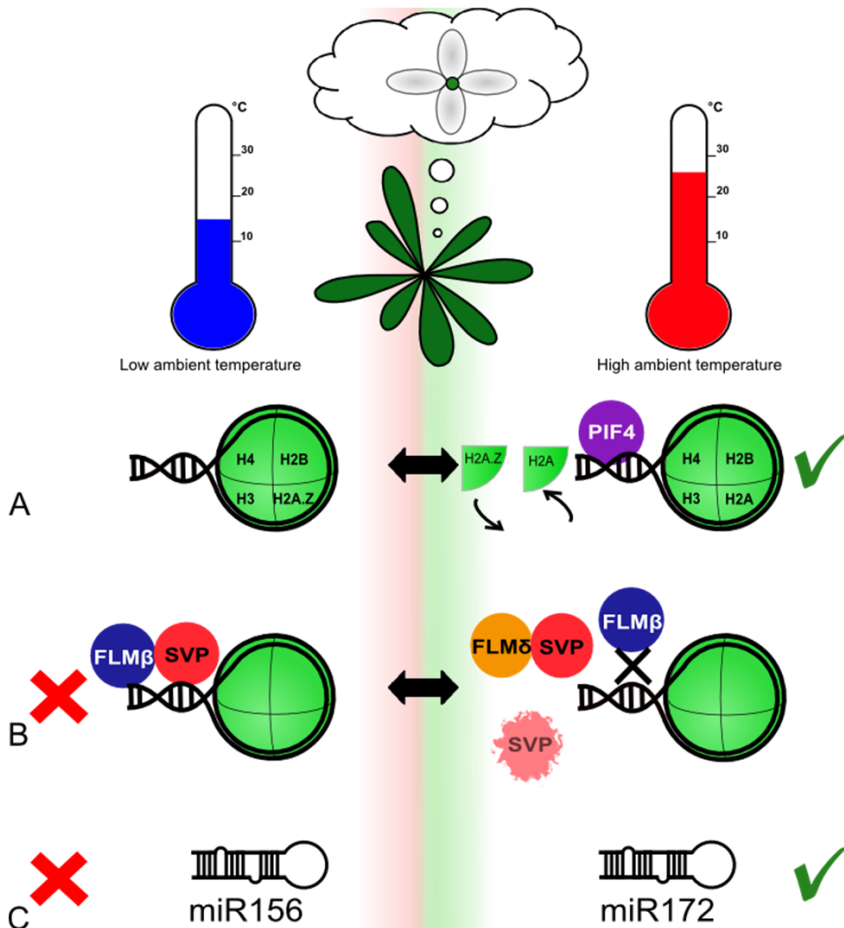
Figure 1. Possible molecular mechanisms underlying ambient temperature-regulated floral timing. When a plant has to decide whether it should start flowering (thinking cloud), **(A)** H2A.Z is evicted from the nucleosome at high ambient temperatures (red thermometer) and the activating factor PHYTOCHROME INTERACTING FACTOR 4 (PIF4) can bind floral activating genes (Kumar et al., 2012a) and (Kumar and Wigge, 2010). **(B)** The repressive *FLOWERING LOCUS M–SHORT VEGETATIVE PHASE* (*FLMβ*–SVP) complex binds DNA at low ambient temperatures (blue thermometer), and gets

outcompeted by formation of FLMδ-SVP complexes and by degradation of the SVP protein at higher ambient temperatures, resulting in activation of flowering (Lee et al., 2013b) and (Posé et al., 2013). **(C)** miR172 has a putative repressing action at low ambient temperatures (Lee et al., 2010), whereas miR156 has a putative activating function at high ambient temperatures (Kim et al., 2012). The figure shows suppressing mechanisms on the left and activating mechanisms on the right.

A role for chromatin remodelling and H2A.Z is not an unknown phenomenon in flowering time regulation, because it is well established that the floral repressor *FLC* is epigenetically silenced upon prolonged cold, winter-like temperatures (vernalization) (He, 2012) and (Crevillén and Dean, 2011). When expressed, FLC interacts with SVP, and these two proteins may function together in repressing *FT* and *SOC1* (Li et al., 2008) (Figure 2). Under nonvernalizing conditions, the FRIGIDA (FRI) protein recruits chromatin modifiers at the TSS of the *FLC* locus, among which is the RNA polymerase II (Pol II)-associated factor 1 complex (PAF1c). This leads to modification of nucleosomes and dynamic exchange of H2A by H2A.Z, which facilitates transcription of *FLC* by Pol II. At least part of this system seems to be conserved for paralogs of *FLC*, named *FLOWERING LOCUS M* (*FLM*) and *MADS AFFECTING FLOWERING* (*MAF*)*2-5*, because mutants deficient in components of PAF1c showed a modest (in case of *MAF2*) to marked silencing of *FLM* and *MAF3-5*, just like *FLC* (Oh et al., 2004). Moreover, H2A.Z enrichment has been reported at the *MAF4* and *MAF5* loci (Deal et al., 2007). As discussed below (see 'Alternative splicing and protein stability of repressive MADS-domain transcription factors'), FLM, and to a lesser extend MAF2–MAF4, have an important function in ambient temperature-regulated floral timing. It remains unclear whether putative transcriptional regulation of *FLM* and the *MAF*s at the chromatin level in an *FLC*-like manner is of biological relevance, because the *MAF*s, and especially *FLM*, seem to be regulated mainly by alternative splicing rather than by alternative expression. However, recently correlations have been found between chromatin status and alternative splicing in mammalians, leaving all possibilities open (Luco et al., 2011).
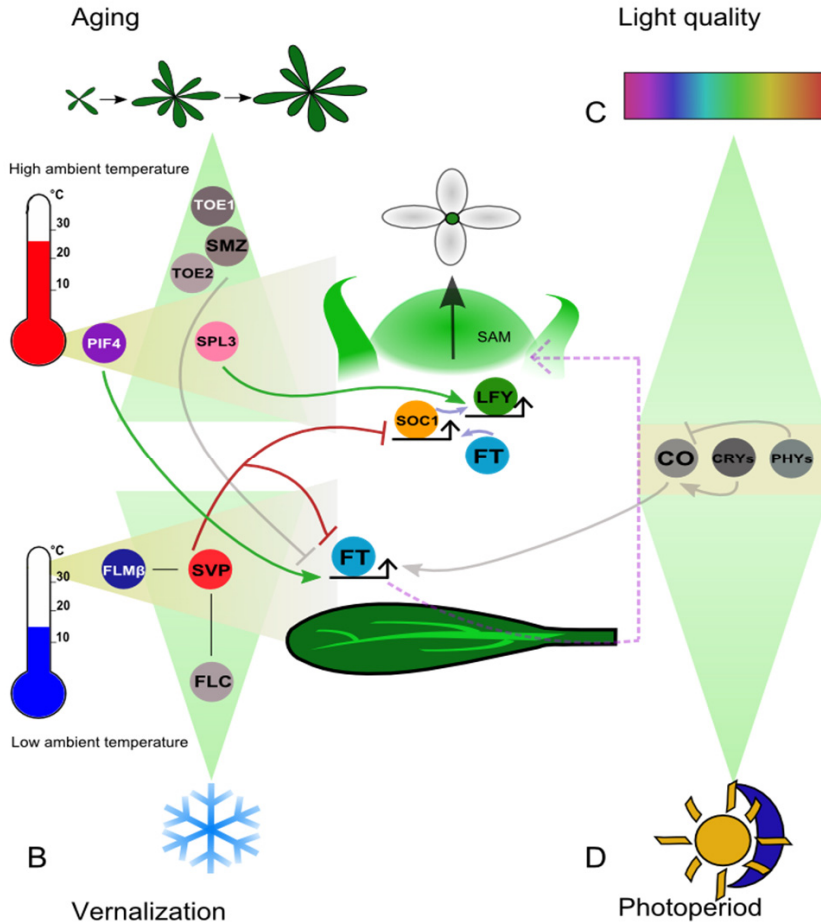
Figure 2. Integration of the ambient temperature flowering time pathway in other known flowering time pathways. Besides the factors from the ambient temperature pathway, a few major factors from **(A)** the aging pathway (Wu and Poethig, 2006) and (Mathieu et al., 2009), **(B)** the vernalization pathway (He, 2012) and (Crevillén and Dean, 2011), **(C)** the light-quality pathway (Cerdan and Chory, 2003), and **(D)** the photoperiod pathway (Lin, 2000) are shown. The aging pathway (A) and the vernalization pathway **(B)** show overlap with the ambient temperature pathway. For each factor, the interactions with the loci of floral integrators (*SOC1* and *FT*) are shown by arrows (activation) or stops (repression). The floral integrator *FT* is expressed in the phloem (Yamaguchi et al., 2005) and (Corbesier and Coupland, 2006), after which the protein travels (dashed arrow) to the shoot apical meristem (SAM), where it activates *SOC1* (Corbesier and Coupland, 2006), which in turn activates the floral meristem identity gene *LFY* (Corbesier and Coupland, 2006). Direct activation of *SOC1* and *LFY* by the shown flowering time pathway regulators also takes place in the SAM. Abbreviations: CO, CONSTANS; CRYs, cryptochromes; FT, FLOWERING LOCUS T; FLC, FLOWERING LOCUS C; FLM, FLOWERING LOCUS M; LFY, LEAFY; PHYs, phytochromes; PIF4, PHYTOCHROME INTERACTING FACTOR 4; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CO 1; SMZ, SCHLAFMÜTZE; SPL3, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3; TOE1, TARGET OF EAT1.

PIF4: an activating factor under non-inductive photoperiod

A protein involved in light signaling has also been shown to function in ambient temperature-regulated floral timing. PHYTOCHROME INTERACTING FACTOR 4 (PIF) is a member of the PIF subfamily of phytochrome-interacting basic helix loop helix (bHLH) transcription factors that function in a variety of light-mediated biological processes, extensively reviewed in (Leivar and Quail, 2011). PIF4 was first identified as a protein interacting with the light-activated form of phytochrome B (phyB) in the light-quality pathway, controlling elongation growth under shade conditions, where the red:far-red light ratio is low. As mentioned above, light quality also influences flowering time, but in contrast to phyB (Cerdan and Chory, 2003) and (Endo et al., 2013), PIF4 has not been reported to function in light quality-regulated floral timing (Huq and Quail, 2002). This is in accordance with studies showing genetic separation of signaling pathways regulating either elongation or floral timing by light quality (Cerdan and Chory, 2003) and (Botto and Smith, 2002). Regulation of PIF4 by light quality occurs at the protein level, where PIF4 is stable under low red:far-red light, or dark conditions. Upon perception of high red:far-red light, the protein is rapidly degraded by the proteasome after interaction with light-activated phyB.

A striking phenotypic similarity between plants grown in shade conditions and at higher ambient temperatures has been noted (Koini et al., 2009), including extended stems and leaf hyponasty. *pif4* mutants were grown at different temperatures to test their involvement in ambient temperature signaling and, indeed, the mutant showed a severe reduction in petiole elongation and leaf hyponasty response to higher temperature. This indicates that response to shade and high ambient temperature share a common mechanism that includes PIF4. However, the *pif4* mutant still showed accelerated flowering upon growth at higher temperatures, leading the authors to conclude that PIF4 is not involved in ambient temperature-regulated flowering time (Koini et al., 2009). Nevertheless, a more recent study (Kumar et al., 2012b) showed that specifically and only under short-day (SD) conditions, the *pif4* mutant does show a strongly decreased flowering time response upon growth at a higher temperature. A plausible explanation for the lack of a flowering time phenotype reported by the authors of (Koini et al., 2009) could be explained by the fact that, in their study, mutant and wild type plants were grown under continuous light (LL), under which PIF4 is probably unstable. In the *pif4* mutant grown under SD, a strong decrease in expression of the floral integrator *FT* was noted, indicating that PIF4 might target *FT* (Figure 2). Direct interaction between PIF4 and the *FT* locus could be shown by ChIP experiments, and this interaction appears to be temperature sensitive, because a higher enrichment of PIF4 at the *FT* locus was demonstrated in plants grown at higher temperature (Kumar et al., 2012b). This higher enrichment might be a consequence of more PIF4 protein at higher temperatures and, therefore, transcript and protein quantities were measured. Despite the fact that some differences could be shown at both the transcript and protein levels, the authors reasoned that this was not enough to explain the observed decrease in flowering time. Inspired by previous work on the role of H2A.Z in the ambient-temperature response,

the authors hypothesized that PIF4-dependent regulation of early flowering under higher temperature is controlled at the level of chromatin accessibility of the *FT* promoter (Kumar and Wigge, 2010) (Figure 1A). Indeed, in the *arp6* mutant, which is defective in H2A.Z deposition, PIF4 binding is strongly increased compared with wild type plants. Altogether, PIF4 seems to be a factor that positively influences flowering time upon higher ambient temperature, but solely under a non-inductive SD photoperiod.

Alternative splicing and protein stability of repressive MADS-domain transcription factors

Two MADS-domain proteins that act in a complex to control flowering time have been shown to be individually regulated by ambient temperature. MADS-domain transcription factors are known to have important roles in the development of plants throughout their whole life cycle, including flowering time (Smaczniak et al., 2012). For example, the floral integrator SOC1 and the floral repressor FLC belong to this group. The MADS-domain proteins FLM and SVP have been implied to be involved in ambient temperature-regulated flowering time (Balasubramanian et al., 2006), (Lee, 2007), (Scortecci et al., 2001), (Werner, 2005), (Hartmann et al., 2000) and (Ratcliffe et al., 2001), but until recently, the exact mechanism by which they do so had not been elucidated. Recent discoveries revealed a combinatorial role for FLM and SVP, with ambient temperature modulating both factors in different ways (Lee et al., 2013b) and (Posé et al., 2013).

FLM is a member of the FLC clade (Becker and Theißen, 2003) that, besides FLC and FLM, contains the four close homologs MAF2-5 (Scortecci et al., 2001) and (Ratcliffe et al., 2001). In 2006, it was reported that *FLM* is differentially alternatively spliced under different ambient temperatures (Balasubramanian et al., 2006), but the exact identity of these splice forms and whether they are biological relevant was not clear. A recent study demonstrated that FLM is a key regulator of ambient temperature-regulated floral timing, and that the proteins encoded by its two main splice forms function antagonistically through interaction with SVP (Posé et al., 2013). It was shown that lower ambient temperatures favor the production of the *FLMβ* splice form, whereas more of the *FLMδ* splice form is produced at higher ambient temperatures. To investigate the function of these two splice variants, *FLMβ* and *FLMδ* open reading frames were expressed under the constitutive *CaMV35S* promoter in Col-0 wild type plants, which demonstrated that expression of *FLMβ* delayed flowering time, whereas *FLMδ* accelerated flowering. This indicates that these two different splice forms of *FLM* work in an antagonistic manner, with FLMβ being a repressor and FLMδ being an activator of flowering. By using an electrophorese mobility shift assay (EMSA), the authors showed that this works through an intricate mechanism, in which both FLMβ and FLMδ interact with SVP. Upon interaction, the FLMβ–SVP complex binds to DNA as a repressor of flowering (Figure 1B), putatively by direct binding to, for example, the *SOC1* locus (Figure 2), given that ChIP-seq experiments uncovered this floral integrator as a direct target. The interaction between SVP and FLMδ results in a functionally ineffective complex

that functions as a dominant negative factor by competing for SVP interaction with FLMβ, which leads to the formation of less repressive FLMβ–SVP complexes (Figure 1B).

The other FLC clade members, MAF2–MAF5, have all been studied for their putative role in flowering time control (Ratcliffe et al., 2003), (Rosloski et al., 2013), (Gu, 2013) and (Kim and Sung, 2010), and they have all been shown to be able to repress flowering when overexpressed. *maf2*, *maf3*, and *maf4* single mutants showed a reduced flowering-time response upon growth at higher ambient temperature, but not as strong as the *flm* mutant (Gu et al., 2013), and double mutants of *flm* and one of the other *mafs* showed different degrees of additive effects on flowering time compared with *flm*. Furthermore, all the FLC clade members are alternatively spliced. Although these data imply that MAF2–MAF5 might function mostly similar and additive to FLM, this seems to be not the case completely. For MAF2, a function in a slightly lower-than-ambient temperature (between 4°C and 21°C) has been proposed (Rosloski et al., 2013). *MAF2* is spliced in a different way than *FLM*, because *FLMβ* and *FLMδ* are different by a mutually exclusive exon, whereas *MAF2* splice forms display an alternative acceptor site and premature transcript termination (Severing, 2012). *MAF2* has at least three splice forms, of which two, var1 and var2, have been functionally characterized in more detail. *MAF2* var1 and var2 are differentially abundant at 21°C and 4°C, where var1 goes up when transferred to 4°C and var2 goes down. In the *A. thaliana* accession LI-2, which is null for wild type expression of *FLC* and *MAF2–MAF4*, overexpression of var1 delayed flowering under SD conditions, whereas var2 did not change flowering time. *MAF2* var1 encodes the full-length protein, whereas var2 encodes a truncated protein lacking the K-box, which is supposed to mediate protein–protein interactions. Given that var2 still contains the MADS-box DNA-binding domain, it could be hypothesized that the protein can function as a dominant-negative to the var1 protein, but lack of a flowering-time phenotype in plants overexpressing the var2 splice form contradicts this idea. Altogether, MAF2–MAF4 probably act partly additively and partly redundantly to FLM in thermosensory floral timing, and appear to have evolved different temperature sensitivities.

In the above-mentioned research on flowering time control by the repressing FLMβ–SVP complex, regulation is achieved through ambient temperature-controlled alternative splicing of *FLM*. Also recently, another study reported another mechanism that regulates the FLMβ–SVP complex through protein stability of SVP (Lee et al., 2013b). This research showed that, in plants expressing *SVP-HA* grown at different temperatures, the SVP protein became gradually less abundant in a temperature range of 16°C, 23°C, and 27°C, indicating that SVP is less stable at higher temperatures. Through co-immunoprecipitation, it was shown that this decrease in SVP protein led to a lower abundance of the repressing FLMβ–SVP complex, which consequently resulted in decreased binding to the putative target loci *FT*, *SOC1* (Figure 2), and *TWIN SISTER OF FT* (*TSF)*. When the researchers added a proteasome inhibitor, degradation of SVP was inhibited, indicating that SVP might be degraded by the proteasome at higher temperatures (Figure 1B); however, which factor is responsible for targeting SVP to the proteasome remains unknown.

In a recent paper (Lee et al., 2014), the binding affinity of FLMβ and FLMδ for SVP was determined by *in vitro* pull-down experiments and appeared to be similar for both FLM variants, supporting the hypothesis that the two protein isoforms of FLM compete with each other, as suggested by (Posé et al., 2013). In the same study (Lee et al., 2014), it was confirmed that the ratio between *FLMβ* and *FLMδ* transcript levels changed upon temperature changes, although the absolute levels at 27°C were the same. Also in plants grown constantly at different temperatures (16°C, 23°C, and 27°C), the FLMβ transcript levels gradually dropped at higher temperatures, supporting the idea that FLM splicing is of major importance.

The FLMβ–SVP complex seems to target floral integrators directly, because both studies (Lee et al., 2013b) and (Posé et al., 2013) mentioned above found *SOC1* as a direct target. *FT* and *TSF* were also found as targets by Lee and colleagues (Lee et al., 2013b), although they were not found reproducibly as significantly enriched in the genome-wide ChIP-seq experiments of Posé and colleagues (Posé et al., 2013).

The regulation of FLM isoforms competing for SVP, together with the regulation of SVP protein abundance, can be strong mechanisms for repressing flowering under lower ambient temperatures that are then released when the temperature rises. To what extent these mechanisms contribute to thermosensory flowering-time regulation remains to be determined.

Repressive and activating functions for miRNAs?

miRNAs that have previously been shown to regulate flowering time in the aging pathway (Aukerman and Sakai, 2003), (Wu and Poethig, 2006) and (Wu et al., 2009) have also been proposed to regulate floral timing by ambient temperature input. miRNAs are small, noncoding RNAs that were only discovered relatively recent in plants (Reinhart et al., 2002). They are encoded by miRNA genes and, after transcription, are processed into short double-stranded (ds) RNAs of approximately 22 nucleotides (Rogers and Chen, 2013). Subsequently, one of the strands is incorporated in the RNA-induced silencing complex (RISC), which selectively targets mRNAs and directs them for degradation or translational inhibition.

During *Arabidopsis* development, miR156 and miR172 are of particular importance. Besides timing of the juvenile phase, these two miRNAs have a role in the timing of the phase change from vegetative to reproductive (Aukerman and Sakai, 2003), (Wu and Poethig, 2006) and (Wu et al., 2009). The expression of miR156 and miR172 is inversely correlated: miR156 is highly expressed in young plants and gradually drops as the plant matures, whereas miR172 shows the inverse pattern. This is achieved by the indirect negative regulation of miR172 by miR156, which is mediated by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9) (Wu et al., 2009). miR172 regulates flowering time by translational inhibition of several *APETALA 2* (*AP2*)-like transcription factors, including the flowering time repressors *TARGET OF EAT1* (*TOE1*), *TOE2* (Aukerman and Sakai, 2003), and SCHLAFMÜTZE

(SMZ) (Mathieu et al., 2009) (Figure 2). Besides regulation of flowering time through miR172, miR156 has been shown to regulate flowering time in a miR172-independent way by targeting another *SPL* gene family member, *SPL3* (Wu and Poethig, 2006), which in turn functions as a flowering time activator, (Figure 2) probably by direct activation of *LFY, FRUITFULL (FUL)*, and *AP1* (Yamaguchi et al., 2009). Activation of *FT* has also been reported (Kim et al., 2012); however, given that both non-responsiveness and responsiveness to SPL3 have been reported for this gene (Kim et al., 2012) and (Yamaguchi et al., 2009), its potential role as target of SPL3 remains ambiguous.

A putative role for miRNAs in the thermosensory pathway has been investigated by comparing expression levels of miRNA genes between 10-day-old plants constantly grown at 16°C and 23°C under long-day (LD) conditions (Lee et al., 2010). Higher miR172 expression was observed at 23°C than at 16°C, and the opposite was true for miR156 genes. This suggests that the miR156–miR172 system is also involved in the thermosensory flowering-time pathway (Figure 1C), although a direct involvement has not been shown. In the same report (Lee et al., 2010), it was speculated that miR172 might function downstream of SVP, because the expression of one of the miR172 genes appeared to be higher in an *svp* mutant background than in wild type, both at 16°C and 23°C. However, as in wild type plants, expression of miR172 was still temperature responsive in *svp* mutant plants, which does not directly support a role for miR172 downstream of SVP. More recently, the role of miR156 was examined (Kim et al., 2012), and it was shown that downregulation of miR156 and overexpression of an miRNA-insensitive *SPL3* showed a decrease in leaf number ratio at the onset of flowering (indicating the responsiveness of the plant to temperature shifts) in plants grown at 16°C compared with plants grown at 23°C, which can be interpreted as decreased temperature responsiveness (Figure 2). Unfortunately, complete knockouts for miR156 genes could not be generated and, hence, definitive proof of temperature-insensitive flowering and a role for miR156 in the thermosensory pathway could not be shown. Obviously, more research is needed to show indisputably a role for these miRNAs in the ambient temperature-regulated flowering time pathway.

How do the newly discovered factors relate?

The recent reports on several factors that control thermosensory floral timing through various molecular mechanisms, raises the question of how they relate to each other. Light has already been shed upon the link between H2A.Z (Kumar and Wigge, 2010) and PIF4 (Kumar et al., 2012a), because it was shown that these factors are probably epistatic (Kumar et al., 2012b). However, we can only speculate on the relations of the other factors. An essential observation is that the factors described act either in a flowering-repressing manner that can be released by the underlying molecular mechanism at higher temperatures to allow a faster transition to flowering, or in an activating manner that can be abolished by the underlying molecular mechanism through which flowering time is delayed. H2A.Z, PIF4, and miR172 belong to the first group of factors, which function at higher

ambient temperatures to trigger flowering, whereas *FLM* alternative splicing, SVP instability, and miR156 are negative factors that suppress flowering at lower temperatures. For the positive factor PIF4, and the negative factors FLM and SVP, no evidence exists that they are epistatic, neither molecularly nor genetically. The fact that *PIF4* mutants, and even higher-order *pif* mutants (*pif1pif3pif4pif5*) flower normally under LD conditions (Shin et al., 2009), whereas *flm* and *svp* mutants show a flowering phenotype under an inductive LD photoperiod (Lee et al., 2013b) and (Posé et al., 2013), supports this idea. Mutants for *FLM* or *PIF4* still seem to show a slight temperature responsiveness (Kumar et al., 2012b), (Lee et al., 2013b) and (Posé et al., 2013), indicating that there might be more than one pathway controlling thermosensory floral timing. Thus, PIF4 and FLM/SVP appear to act in distinct pathways that are differentially regulated by ambient temperatures and both converge at the floral integrators where they directly activate (PIF4) or repress (FLMβ–SVP) expression of the floral integrators. However, a double mutant that would give definite proof has not yet been reported. The two mechanisms proposed for FLM–SVP control of the ambient temperature pathway (i.e., *FLM* splicing and SVP stability) seem to be independent, although both boil down to the amount of FLMβ–SVP suppressor complex present. In principal, positive pathways at higher temperatures and negative pathways at lower temperatures can both reinforce each other as well as conferring more control and can provide the possibility for precise timing. However, there is some contradiction in the positively acting H2A.Z depletion at higher temperatures (Kumar and Wigge, 2010), because H2A.Z depletion at the *FT* locus could also allow repressing factors to bind, which does not agree with the fact that plants flower earlier when deficient in H2A.Z deposition. One could argue that, at higher temperatures, the level of repressing factors is low and the presence of more abundant positive factors could shift the balance towards flowering induction. The question still remains, however, why mutants deficient in H2A.Z deposition also flower early at lower temperatures. Thus, more research is necessary to clarify the relation between H2A.Z and repressing factors of flowering time.

Outlook and concluding remarks

Flowering time is an important trait in crop production, which is controlled by several external and internal signals. Recent advances in knowledge of the molecular and genetic mechanisms underlying the effect of ambient temperatures on *Arabidopsis* flowering time could be of use in breeding programs of crops. *Arabidopsis* plants flower earlier when grown under higher ambient temperatures, but other species respond in an opposite manner or stay largely independent of temperature fluctuations. Knowledge of the evolutionary conservation of the genes and mechanisms involved is necessary to enable the prediction and control of flowering time in other species. Recent analysis of the genome of Chinese cabbage (*Brassica rapa*) revealed the presence of three orthologs of *PIF4* (Song et al., 2014), and research of bHLH proteins in rice (*Oryza sativa*) showed that two close orthologs of *PIF4* and *PIF5* exist in this monocotyledonous plant (Nakamura et al., 2007), indicating that *PIF4* might be conserved. However, whether there is also functional conservation cannot be

inferred from these genomic data and, hence, more research is needed to answer this question. Histone variant H2A.Z is conserved among eukaryotes, and has been proposed to mediate warm temperature signals in budding yeast (*Saccharomyces cerevisiae*) as in *Arabidopsis* (Kumar and Wigge, 2010). Therefore, concerning the conservation of the H2A.Z–PIF4 mechanism, H2A.Z is likely not to be the variable factor. The repressive factor SVP also appears to be conserved, repressing floral meristem identity and delaying flowering in both monocots and dicots (Hemming and Trevaskis, 2011). However, its interaction partner FLM seems to be less conserved, because there are no published reports on orthologs of the *FLM* gene in species outside the *Arabidopsis* genus. However, orthologs of the closely related *FLC* gene are present in three major eudicot lineages (Reeves et al., 2007), and recent research even shows the presence of *FLC* in monocots (Ruelens et al., 2013). In several species, more than one *FLC*-like gene can be distinguished, and it cannot be ruled out that, in these species, a similar sub-functionalization as for *FLM* in *Arabidopsis* has occurred for another *FLC*-like gene.

The recently uncovered molecular mechanisms in *Arabidopsis* controlling thermosensory flowering time can be placed in at least two parallel pathways: a positively acting pathway where H2A.Z and PIF4 activate flowering under higher ambient temperatures, and a negatively acting pathway where SVP and FLM suppress flowering at lower ambient temperatures. Despite the substantial progress that has been made in understanding the molecular mechanisms, it still remains unclear how temperatures are sensed by the plant. It is imaginable that there is a direct physical effect of higher temperatures on the folding, molecular interactions, and stability of the chromatin, mRNA, and proteins involved (Murata and Los, 1997), (Los and Murata, 2004), (Knight et al., 1996), (Spadafora et al., 1979), (Nocker et al., 2001) and (Vogt et al., 1997). These possibilities are reviewed in Box 1. Further research is needed to shed light on this intriguing question and to uncover fully the thermometer of flowering time control in plants.

Box 1. Possible ways in which plants sense temperature

Although recent studies identified key regulatory genes involved in flowering time regulation upon ambient temperature changes, an important question that remains is how plants perceive temperature. In animals, special temperature receptors have been identified (Patapoutian et al., 2003), (Patapoutian et al., 2003)(Patapoutian et al., 2003) but there is no evidence for similar structures in plants. Biological systems are governed by the laws of physics, and temperature changes affect molecular properties and cellular processes. In Table I, we review possible physical and biochemical characteristics that might be involved in temperature sensing by plants, some examples of which are discussed in the main text.

Table I. Physical and biochemical characteristics that might be involved in temperature sensing by plants				
Level	Effect on:	Description	Possible consequences	Refs
General	Kinetic energy	Energy of the motion of molecules increases as temperature rises, reaction speed increases	Altered transcription and translation rates	
Cellular	Membrane fluidity	Lipid bilayers of membranes in the cell become more fluid at higher temperatures	Altered activity of membrane-bound proteins and altered membrane transport	(Murata and Los, 1997)
	Ion channel gating	Gates of ion channels might be opened by temperature	Altered ion concentration in the cell	(Knight et al., 1996)
Molecular	Chromatin structure	Higher temperatures can unravel the chromatin, which might allow binding of elements such as transcription factors and splicing factors	Altered transcription and altered alternative splicing	(Spadafora et al., 1979)
	DNA structure	Higher temperatures can change DNA bending, unwind, and separate the double helix structure by breaking the hydrogen bonds	Altered transcription and altered transcription factor binding	
	mRNA structure	Temperature can affect the stability and 3D structure of mRNA with altered exposure of splice sites as a consequence	Altered translation and altered alternative splicing	(Nocker et al., 2001)
	Protein stability	Temperature can disrupt noncovalent interactions in a protein structure and disrupt its native conformation	Disrupted protein function and degradation of proteins	(Vogt et al., 1997)
	Protein–protein and protein–DNA interactions	Temperature can affect interactions between proteins and between protein and DNA	Lower affinity between proteins in a complex (e.g. transcription factor dimers) and weaker DNA binding by transcription factors	

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Chapter 3

Splicing-related genes are alternatively spliced upon changes in ambient temperatures in plants

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Abstract

Plants adjust their development and architecture to small variations in ambient temperature. In a time in which temperatures are rising world-wide, the mechanism by which plants are able to sense temperature fluctuations and adapt to it, is becoming of special interest. By performing RNA-sequencing on two Arabidopsis accession and one Brassica species exposed to temperature alterations, we showed that alternative splicing is an important mechanism in ambient temperature sensing and adaptation. We found that amongst the differentially alternatively spliced genes, splicing related genes are enriched, suggesting that the splicing machinery itself is targeted for alternative splicing when temperature changes. Moreover, we showed that many different components of the splicing machinery are targeted for ambient temperature regulated alternative splicing. Mutant analysis of a splicing related gene that was differentially spliced in two of the genotypes showed an altered flowering time response to different temperatures. We propose a two-step mechanism where temperature directly influences alternative splicing of the splicing machinery genes, followed by a second step where the altered splicing machinery affects splicing of downstream genes involved in the adaptation to altered temperatures.

Introduction

As a consequence of a sessile lifestyle, plants are continuously facing fluctuating environmental conditions. In order to both benefit maximally and to protect them from the environment, plants evolved ways to sense and respond to many environmental cues. Ambient temperature is one of these signals that plants sense and adapt to in order to enhance their chance of survival and reproduction. Small changes in ambient temperature can have major effects on plant architecture and development (Koini et al., 2009). One of these adaptations is the moment of flowering, which is an important event in the life cycle of a plant, since reproductive success depends on it. For the widely-used model plant *Arabidopsis thaliana* Col-0, it is known that it flowers earlier when the ambient temperature is higher (Balasubramanian et al., 2006).

The mechanism of how a plant senses temperature and how this affects its phenotype are hardly understood. It has been suggested that alternative splicing (AS) - the phenomenon that one gene produces more than one form of messenger RNA (mRNA) - plays an important role in temperature sensing, since environmental changes trigger differential AS (Ding et al., 2014; Filichkin et al., 2010; Leviatan et al., 2013; Ling et al., 2015). Moreover, various studies showed the impact of environmentally induced AS. For example, genes encoding the components of the circadian clock are prone to AS upon temperature fluctuations. One of them, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), is besides temperature responsive, also alternatively spliced upon high light intensity (Filichkin et al., 2010) and is proposed to regulate the period of the clock through this mechanism (Seo et al., 2012). Another example is *FLOWERING LOCUS M (FLM)*, which produces different ratios of splicing

variants upon shifting ambient temperature, and in this way regulates floral timing (Balasubramanian et al., 2006; Posé et al., 2013; Sureshkumar et al., 2016).

An intron-containing gene can potentially produce several to numerous different splice forms, depending on the intron-exon structure. This is achieved by combining conventional splicing, in which all introns are spliced out at the exon-intron border, with alternative selection of splice sites, leading to retention of introns (RI), skipping or mutual exclusion of exons (SE or MXE), or alternative splice site selection at the 5' and/or 3' end (A5 or A3), rendering different transcripts (as reviewed by (Reddy et al., 2013)) (Figure 1A). The driving force behind gene splicing is the spliceosome, a large cellular machinery that catalyses the splicing reaction. The spliceosome is a ribonucleoprotein complex with a highly dynamic structure that assembles de novo at each splice site in a stepwise manner. Many different proteins and riboproteins orchestrate splice-site selection, exon and intron definition, two catalytic splicing steps, and extensive quality control (as reviewed by (Matera and Wang, 2014; Wahl et al., 2009)). The spliceosome recognizes splicing signals located at exon-intron boundaries, but also numerous *cis*-regulatory sequences that act as splicing enhancers or silencers (as reviewed by (Syed et al., 2012)).

As mentioned above, numerous studies report differential AS upon environmental changes, at gene-specific and transcriptome-wide levels. Although small-scale studies already showed that slight changes in temperature can have a significant effect on AS (Streitner et al., 2013), transcriptome-wide studies in relation to AS have mainly focussed on extreme stresses, such as high light intensity, salt, dehydration, cold and heat (Ding et al., 2014; Filichkin et al., 2010; Leviatan et al., 2013), and much less on ambient conditions like ambient temperature. Moreover, these studies only used a single accession of the model species *Arabidopsis thaliana*, and therefore do not provide information about the conservation of the response or the underlying mechanism. In this study we analysed ambient temperature-directed AS in two accessions of *A. thaliana* and in one genotype of *Brassica oleracea* ssp. *botrytis*. We show that splicing related genes are overrepresented amongst the genes that exhibit a different splicing pattern upon ambient temperature changes. Because many different classes of splicing related genes were affected, it suggests that the whole spliceosome is sensitive to ambient temperature fluctuations. Furthermore, when comparing the three different genotypes we used in this study, we demonstrated that although we could not find proof for conservation of the temperature-dependent splicing response at the single gene level, splicing related genes are prominent amongst the conserved cases. Analysis of a mutant of a splicing-related gene that was alternatively spliced in one of the *A. thaliana* accession and in cauliflower, showed an altered flowering time response under different ambient temperatures, indicating that AS of splicing-related genes functions as a key molecular mechanism in the plant's temperature response.

Results

The effect of ambient temperature on alternative splicing

We performed ambient temperature shifts on *Arabidopsis thaliana* Col-0 towards both higher and lower temperatures (23°C to 27°C and 23°C to 16°C, respectively) and harvested temperature treated plants together with the non-treated control plants 24 hours after the shift. We used paired-end Illumina HiSeq RNA sequencing and reads were mapped onto the Arabidopsis genome (The Arabidopsis Information Resource 10 (TAIR10)). Detection of AS was performed for each splicing event, rather than whole isoform level, in order to facilitate a better quantitation analysis. The TAIR10 transcriptome was used for annotation-guided splice site detection, complemented with *de novo* assembly since the TAIR10 transcriptome is not exhaustive. To validate the generated data and bioinformatics analysis, qRT-PCR was performed on a selection of splicing events. By normalizing each tested event to an internal control, a region in the mRNA that is present in all transcripts of the respective gene, we were able to show that both the prediction of splicing events as well as the quantification of these events under different ambient temperatures was highly accurate (Figure S1).

Comparing the overall distribution of splicing events with the distribution of events that were differential upon a shift to high or low ambient temperature, we noticed some changes (Figure 1B). The high temperature shift induced significantly less A3 events whereas low temperature induced significantly less A5 events. Moreover, both temperature shifts induce more RI events.
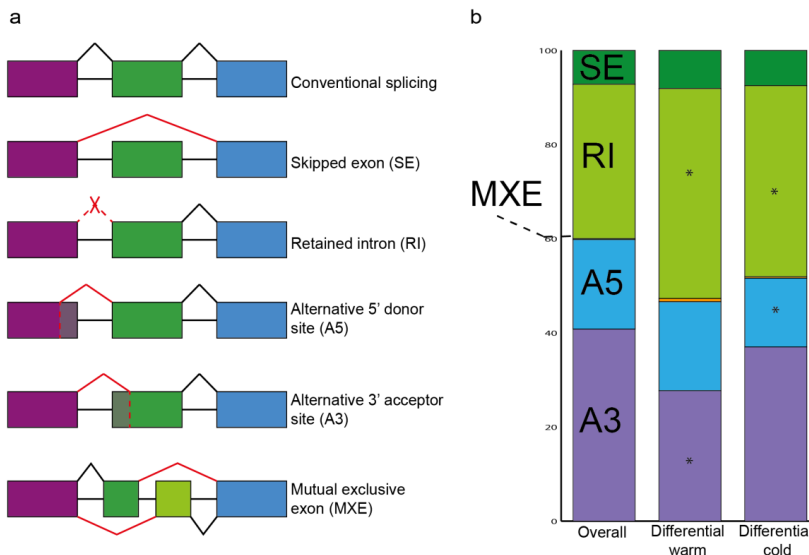

Figure 1: Splicing events. (a) Overview of splicing events that can occur. Alternative events are depicted in red, conventional events are depicted in black. Boxes represent exons, lines represent introns. **(b)** Distribution of the differential splicing events upon shifts to higher or lower ambient temperature, compared to the events in the total dataset. The asterisk * indicates a significant difference of the abundance of the event compared to the overall abundance (Using Pearson's Chi-square test, for data used for significance test, see Table S2).

In addition to identifying splicing events, we determined the relative abundance of splicing events for each alternatively spliced gene, and compared this abundance between the control and temperature-treated plants. We observed differential splicing of around 140 and 290 genes (Table S3, S4 and S5) upon the shift to higher or lower ambient temperature, respectively. When comparing our results to previous small-scale studies, we could reproduce several described cases, supporting the quality of our dataset. For example, we observed differential alternative splicing of *PSEUDO-REPONSE REGULATOR 7 (PRR7)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, two genes that have been shown to mediate the response of the circadian clock to cold treatment (4° C) through AS (Filichkin et al., 2010; James et al., 2012). Also *FLOWERING LOCUS M (FLM)*, a flowering time gene for which ambient temperature dependent AS was discovered (Balasubramanian et al., 2006; Posé et al., 2013), displayed a similar response in our transcriptome-wide data set. Like for many genes, we could identify several different AS events in *FLM* (Figure 2A). The two most abundant splice forms of *FLM* that regulate flowering time, *FLMβ* and *FLMδ*, distinguish from each other by a mutually exclusive exon (MXE) event. We found this event to be differential in both the high and low ambient temperature and in a reciprocal fashion. This confirms the data from the original paper describing temperature-dependent regulation of flowering by antagonistic FLM variants (Posé et al., 2013), Also two genes closely related to *FLM*, *MADS AFFECTING FLOWERING 2* (*MAF2*) and *MAF3*, were found to be differentially spliced upon ambient temperature changes in our dataset. *MAF2* has been shown to regulate flowering time by ambient temperature-directed AS in a slightly different manner than *FLM* (Airoldi et al., 2015; Rosloski et al., 2013). Here we show that also *MAF3* is differentially spliced upon ambient temperature changes (Figure 2B). *MAF3* has been shown to act as a regulator of flowering time as well (Gu et al., 2013), and our findings suggest that this occurs through temperature-directed alternative splicing comparable to *FLM* and *MAF2*. *MAF3* undergoes several splicing events, but only one event, the skipping of exon 2, shows temperature sensitivity in our dataset. Figure 2B displays *MAF3* sequencing reads at 23° C and 16° C, showing the difference in exon 2 abundance between the two temperatures. When we performed RT-PCR on *MAF3* on the same RNA samples, we could detect six different splice forms of which two isoforms undergo the differential skipped exon (SE) event (Figure 2C/S2)). Interestingly, from this semi-quantitative gel it becomes clear that the two isoforms undergoing the SE event (isoform 2 and 6) do not respond to temperature in a similar fashion: the intensity of the fragment representing isoform 2 shows hardly any difference upon the temperature shift, whereas the fragment representing isoform 6 is clearly reduced. This suggests that temperature influences the abundance of splicing events in the context of the whole transcript isoform, and that this isoform and/or other events of this isoform influence the final splicing outcome, at least for *MAF3*.
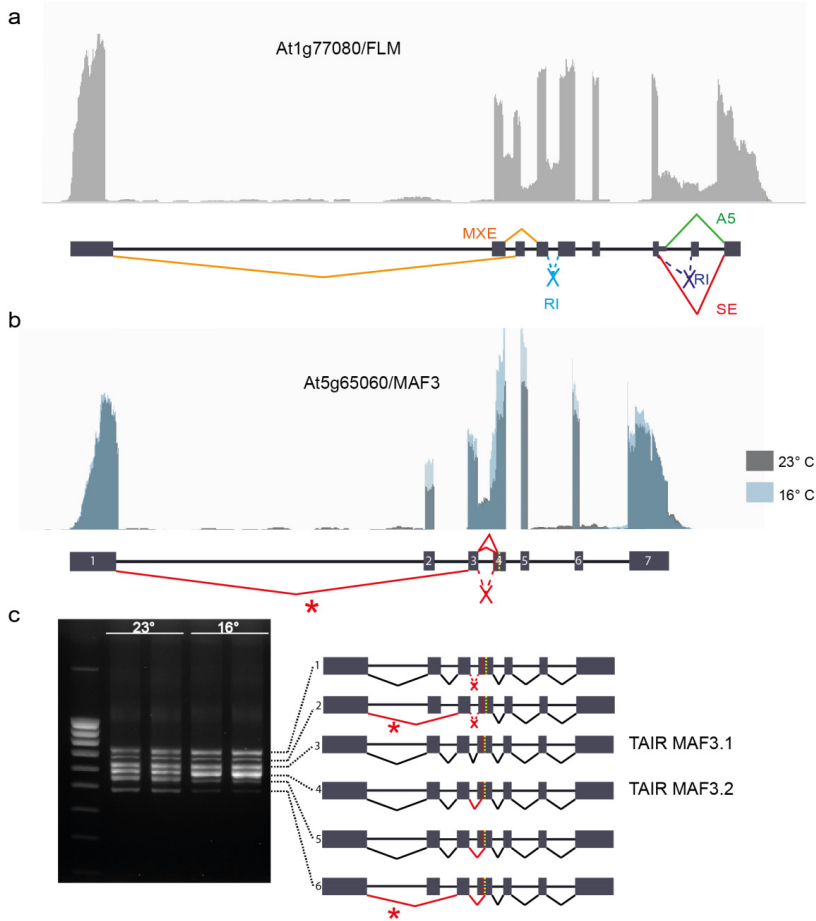
Figure 2: Alternative splicing in *FLM* and *MAF3*. (a) Alternative splicing events in *FLM*. Top: raw reads in Integrative Genomics Viewer (IGV). Bottom: intron/exon structure of *FLM* with all detected splicing events. **(b)** Alternative splicing response of *MAF3* upon low ambient temperature in the IGV browser. Up: raw RNAseq reads of *MAF3* visualised in the IGV browser and the intron/exon structure of *MAF3* with all alternative splicing events. In grey the reads of the control temperature of 23°C, in blue the reads of the 16° temperature treatment. The skipping of exon 2 is differential between the two temperatures, indicated by the asterisk (*). **(c)** RT-PCR on the same samples with *MAF3* primers at the start and stop codons and loaded on a 2% agarose gel. On the left the control temperature of 23° C, on the right the 16° C temperature treatment, each temperature with two biological replicates. The four different splicing events result in six different splice forms, as depicted next to the gel and was confirmed by Sanger sequencing (Figure S2). Note that isoforms 3 and 4 are the only splice forms annotated in TAIR10. (N=2 (16 plants per sample).

Splicing-related genes are overrepresented

To get insight into the biological processes targeted by ambient temperature directed alternative splicing, we performed a gene ontology (GO) analysis on all genes that show differential splicing after lower or higher ambient temperature treatment. We tested for overrepresentation of biological functions amongst all intron-containing genes that were expressed in our dataset (≥ 1 read) and corrected for multiple testing. The results showed clear enrichment of GO terms describing genes involved in the circadian clock (corrected P<0.005) (Figure 3), which confirms earlier reports on AS of clock genes by temperature fluctuations (James et al., 2012; Seo et al., 2012). Related to this, we found enrichment of the GO term "Photoperiodism, flowering", the term that describes flowering time genes that react to changes in photoperiodism. Because alteration of flowering time is one of the responses of the plant to changes in ambient temperature, it is an interesting finding that this group of flowering time genes are highly affected in splicing pattern upon ambient temperature shifts. For the flowering time gene *FLM* it was already shown that changes in splicing pattern enables the plant to modify flowering time in response to ambient temperature shifts (Posé et al., 2013), but our results suggest that AS occurs for numerous flowering time genes and hence is a more general mechanism of the plant to regulate flowering time under different ambient temperatures.

Surprisingly, the GO term analysis also revealed a strong enrichment for several categories representing RNA processing and splicing-related genes (corrected P<0.005) (Figure 3). This implies that shifts in ambient temperature change the splicing pattern of splicing-related genes, the genes that encode the spliceosome and its accessory proteins. Although this might at first appear as an unexpected outcome, splicing of splicing-related genes can potentially influence the composition of the spliceosome, and subsequently alter the splicing of many downstream targets, like genes that function in the temperature response of the plant. To get a better insight into the differentially spliced splicing-related genes in our dataset, we made use of the Arabidopsis Splicing Related Gene Database, which contains a comprehensive list of almost 400 splicing-related protein-coding genes (Wang and Brendel, 2004). Comparison with our dataset showed that about 8.5% of the differentially spliced genes could be classified as splicing related genes (Table S3). Chi-square testing showed that this is a highly significant enrichment (P<0.01, taking into account all intron containing genes that showed expression in our dataset). Analysis of the individual cool and warm datasets revealed that splicing-related genes represent 8.3% and 10% of the total differentially spliced genes respectively, showing that both lower and higher ambient temperature shifts induce differential splicing of splicing-related genes to the same extent, suggesting an important role for these genes in temperature sensing.
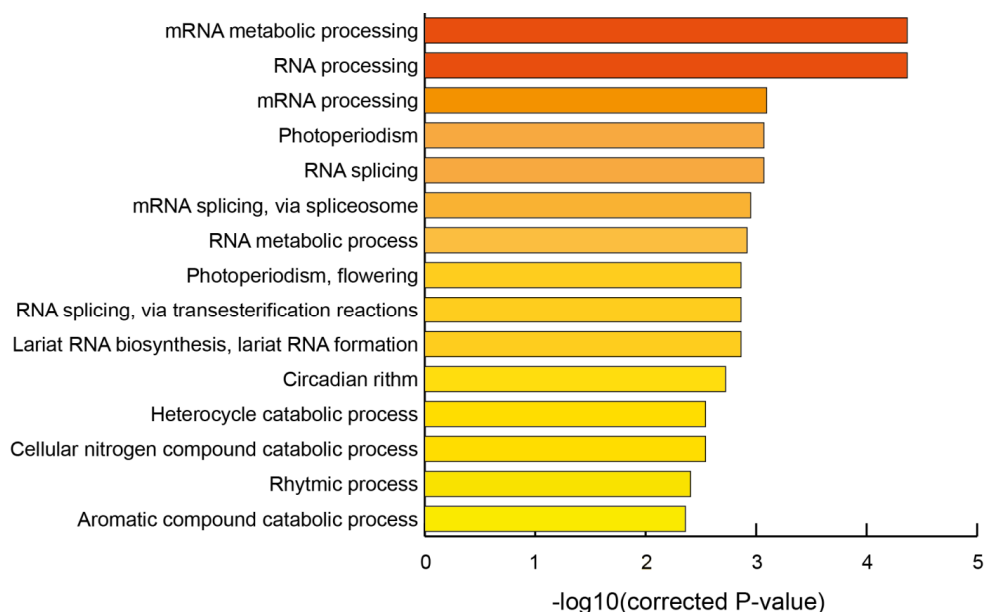
Figure 3: GO-term enrichment of differentially spliced Arabidopsis Col-0 genes upon ambient temperature treatment. GO terms with a corrected P-value < 0.005. –log10(corrected P-value) is shown for visualisation purposes.

The spliceosome is target of ambient temperature-induced alternative splicing

The spliceosome is a large cellular machinery, comprising many different factors. Therefore, we categorized the differentially spliced genes according to the classification of the Splicing Related Gene Database (Wang and Brendel, 2004). As it turns out, many classes of splicing related genes were represented in our dataset (Figure 4). For example, three out of the seven annotated 17S U2 associated protein coding genes, six out of 18 serine/arginine-rich proteins (SR proteins) and two out of 11 DEAD/H box helicases are differentially spliced in our dataset. Thus, many genes involved in the splicing process seem to be targeted for ambient temperature induced splicing.
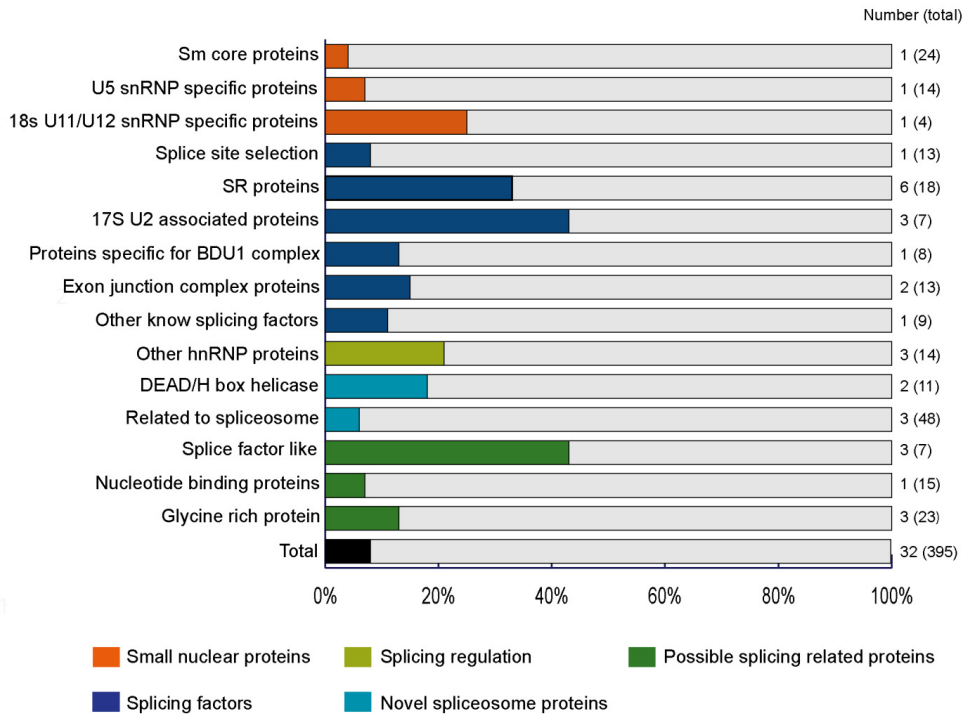
Figure 4: Classes of splicing related Arabidopsis Col-0 genes that show differential splicing of at least one of the class members. Bars represent percentage of genes that is differentially spliced in each class. Total numbers of differentially spliced genes and all genes in each class (between brackets) are given at the end of each bar.

Alternative splicing of splicing-related genes in other genetic backgrounds

To get a better idea about the importance and the level of conservation of our finding that many splicing-related genes are differentially spliced upon ambient temperature treatment, we performed temperature shift experiments followed by RNAseq on another Arabidopsis accession (*A. thaliana* Gy-0) and on *Brassica oleracea* var. *botrytis* (cauliflower). A. thaliana Col-0 is known the be sensitive for ambient temperature shifts and responds by changing its architecture and developmental program, amongst which adapting floral timing. For *B. oleracea*, an early study showed that it also reacts to higher ambient temperature by adjusting flowering time (Booij, 1987), but opposite to Col-0, i.e. early flowering at lower temperature. However, for the Gy-0 accession of *A. thaliana*, nothing was known about the sensitivity for ambient temperature fluctuations. Therefore, before performing RNAseq on temperature-treated plants, we first performed an analysis on the effect of different temperatures in this accession, and it turned out that Gy-0 responded to a higher ambient temperature by later flowering (Figure S3), similar to cauliflower.

Since both cool and warm treatments showed similar effects on AS of splicing-related genes in *A. thaliana* Col-0, we decided to focus only on high temperature treatment in these two other genetic backgrounds. In the Gy-0 accession, we detected differential splicing of~100 genes upon a shift to higher ambient temperature, which is about the same number as in the Col-0 accession (Table S3 and S6). Strikingly, comparing Gy-0 with Col-0 revealed that only 10.9% of the genes that are differentially spliced in the Col-0 accession are also differentially alternatively spliced in Gy-0. Apparently, the ambient temperature directed AS response is not very conserved, not even between these two accessions. Nevertheless, two out of the 15 conserved genes, SR34 and MEE5, are listed in the splicing-related gene database, and a third gene, AtRH3, is a DEAD-box RNA helicase that has recently been shown to play an important role in intron splicing, but has not been listed in the database as of now (Gu et al., 2014).

The cauliflower temperature treatment (21°C → 27°C) rendered a comparable number of differentially spliced genes (156 genes, Table S3 and S7). However, as could be expected taking the Gy-0 results into account, the number of genes being differentially spliced upon the temperature shift that overlapped with the *A. thaliana* Col-0 accession or Gy-0 accession was even lower, being 2.2% and 1% respectively. Again, from the three genes overlapping between cauliflower and the Col-0 accession, one gene was a splicing-related gene (SCL30a). The same is true for the only gene overlapping between the Gy-0 accession and cauliflower (RS40).

Moreover, when we assessed all genes that were alternatively spliced in the Gy-0 accession, 11.5 % could be classified as splicing-related genes. In cauliflower, this was~ 9.5% (taking into account all differentially spliced genes for which we could identify an Arabidopsis homologue). This is very comparable with the 10% in the Col-0 accession treated with high ambient temperature. Thus, despite the low overlap of the temperature-directed splicing response at the individual gene level, the effect on splicing of splicing factors appears to be a conserved mechanism. This indicates that AS of splicing-related genes might be an important process in the temperature response of the plant.

A role for the differentially spliced splicing-related gene *ATU2AF65A* in thermosensitive floral timing

An interesting case of a splicing gene that is alternatively spliced in our dataset is *ATU2AF65A*. We found this gene to be alternatively spliced in *A. thaliana* Col-0 upon the shift to lower ambient temperature, whereas in cauliflower we found it to be alternatively spliced upon the higher ambient temperature shift. In *A. thaliana* Col-0, the cooler ambient temperature treatment suppresses the production of a transcript that retains the second intron of the coding sequence, as well as a transcript that has an alternative 3' splice site of the second exon. In contrast, the warmer ambient temperature treatment in cauliflower

shows the exact opposite. These outcomes strongly suggest a biological function for the ambient temperature dependent splicing of *ATU2AF65A*. Therefore, an Arabidopsis T-DNA insertion line (SALK_144790) was ordered to test for temperature responsiveness. *atu2af65a* homozygous plants were grown together with the wild type that segregated from the heterozygous T-DNA insertion line at two different ambient temperatures. Initially, we grew plants under short day at 16° C and 25°C. In the vegetative state, *atu2af65a* plants did not show any morphological difference compared to wild type plants, and the mutant plants showed a similar response to the high ambient temperature regarding petiole and hypocotyl elongation and leaf hyponasty (Figure 5B). However, the *atu2af65a* mutant turned out to be late flowering. Since this complicated the counting of the number of leaves due to decomposition of the leaves, we repeated the experiment under long day, at 22°C and 27°C. AT 27°C, *atu2af65a* plants flowered around the same moment as wild type plants, whereas at 22°C, the mutant flowered significantly later than the wild type (Figure 5A), showing that mutant reacts differently on ambient temperature.
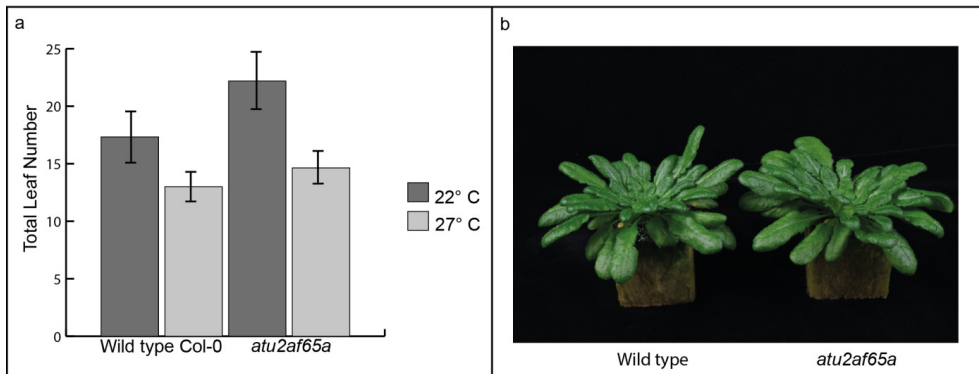

Figure 5: *atu2af65a* mutant has a flowering time phenotype and adapts differently to ambient temperature differences. (a) *atu2af65a* showed a significantly delayed flowering time compared to wild type Col-0 plants, only under lower ambient temperature. N=21 plants (wild type 22° C, 27° C and *atu2af65a* 22° C) and 22 plant (*atu2af65a* 27° C). Error bars represent standard deviations. **(b)** wild type and *atu2af65a* plants grown at 16° C. Besides the flowering time phenotype shown in panel A, *atu2af65a* looks like wild type.

Discussion

The splicing machinery as a target for regulation

Our results show that plants that undergo variation in ambient temperature, display changes in AS. GO-enrichment analysis shows that the main targets of this response are the splicing related genes themselves. In Arabidopsis, almost 400 different splicing related genes have been identified (Wang and Brendel, 2004), and although the overall structure and dynamics of the spliceosome are relatively well understood, the exact function of all these different

factors remains enigmatic. Nevertheless, most of the identified factors can be attributed to different components of the spliceosome. We detected differential splicing in many of these classes. Our study unveiled that, upon small temperature changes, spliceosomal genes are overrepresented amongst the differentially spliced genes, and moreover, that this included many classes of splicing related genes. The relevance of the regulation of the splicing machinery by AS is supported by a recent study on AS in different genotypes of grapevine (Vitulo et al., 2014). The authors showed that there is considerable variability in splicing between different genotypes. When they performed a principle component analysis (PCA) on the ratio's between the first two highly expressed isoforms in different tissues of the genotypes, the different tissues cluster together with the first two components, but taking into account the third component, the samples further separate on genotype. They speculated that the differences amongst genotypes might be partly caused by differences in the general splicing program, which supports the idea that evolutionary adaptation might be achieved by modifications of the splicing machinery.

The splicing response in different genotypes

The above mentioned study might also partly explain why we find a very low overlap between the genes that are differentially spliced upon ambient temperature treatment between the different genotypes we have analysed. The Gy-0 accession of Arabidopsis and the cauliflower cultivar showed a high degree of differential splicing in the splicing related genes, although there is hardly overlap between the individual genes. However, we showed that the Gy-0 accession did not respond the same way to different temperatures as the Arabidopsis Col-0 accession for all developmental aspects, since Gy-0 flowered later when grown under higher ambient temperature, whereas Col-0 is known to flower earlier when subjected to higher ambient temperatures. Also cauliflower is known to flower later when ambient temperature are higher (Booij, 1987). Moreover, the two accessions of Arabidopsis originate from regions with different climate conditions. Col-0 originates from Columbia, Missouri, in the mid-west of the USA, which is a region with sharp seasonal temperature differences. On the other hand, Gy-0 originates from a region in northern central France, in which the climate can be classified as more mild, with a narrower annual range of temperatures. Hence, the difference in the differential splicing response between the three genotypes might reflect the different environmental regimes in which they evolve, suggesting that the splicing machinery co-adapts to facilitate optimal survival. Mechanistically, this could in part be due to SNPs in splice sites or splicing regulatory regions, as shown in a small-scale study on splicing in the *A. thaliana* C24 and Col-2 ecotypes (Streitner et al., 2012).

ATU2AF65A is involved in ambient temperature directed floral timing

The mutant for _ATU2AF65A_, a gene that showed differential splicing in both _A. thaliana_ Col-0 and cauliflower in our temperature experiments, displayed an altered flowering time response when subjected to different temperatures, compared to wild type. Since this gene did not show any significant differences in expression in our datasets, these outcomes strongly suggest a biological function for the ambient temperature directed splicing of _ATU2AF65A_, although we cannot exclude other mechanisms of regulation as yet. ATU2AF65A is a U2 small nuclear ribonucleoprotein (snRNP) auxiliary factor, and part of the spliceosome. In mammals, its homologue U2AF[65] binds to polypyrimidine (Py) tracts to promote the assembly of the spliceosome (Hoffman and Grabowski, 1992; Zamore and Green, 1989). Recently it was shown that this protein can promote the exclusion of alternative exons, and regulation of U2AF[65] leads to differential alternative gene splicing in human cells (Cho et al., 2015). On _A. thaliana_ ATU2AF65A, limited knowledge is available. Structurally, it seems to closely resemble its mammalian counterpart, comprising three RNA recognition motifs. Moreover, like in mammals, the third RRM is able to interact with (an A. thaliana homolog of) splicing factor 1 (SF1) (Cho et al., 2015). Thus, a similar function in AS of _ATU2AF65A_ in Arabidopsis is not unlikely. However, further investigation, like complementation of the mutant with genomic _ATU2AF65A_ and the different splice forms, is necessary to get more insight in the function of this gene in the ambient temperature response of the plant.

Temperature sensing through alternative splicing: a two-step model

Because variation in ambient temperatures eventually causes differences in development and architecture of the plant, the question arises how AS of splicing related genes can achieve this. A plausible scenario is that the differential splicing of splicing related genes results in an altered spliceosomal composition. Consequently, this could establish an altered specificity of the spliceosome for splice sites of target genes that control developmental pathways of the plant. Due to this changed specificity, the developmental genes become differentially spliced, which results in a temperature-adapted phenotype (figure 6). Besides our results on _ATU2AF65A_, our proposition that alternative splicing of splicing related genes leads to differential splicing of downstream genes that control the temperature response of the plant is supported by emerging findings on splicing factors that are essential for certain developmental processes. For example, several studies overexpressing or knocking out SR proteins in Arabidopsis showed a range of developmental and growth phenotypes (as summarized by (Barta et al., 2008)). Despite the pleiotropic effect of most of these mutations, it seems that distinct functions can be attributed to different splicing factors. For example, SR45 was shown to negatively regulate glucose and abscisic acid signalling during early seed development (Carvalho et al., 2010), and ectopic expression of RSZ33 resulted in cell expansion and a changed polarization of cell elongation and division (Kalyna et al., 2003).

Overexpression of a partial cDNA of *SRL1*, a putative splicing factor, conferred increased tolerance to high salt (Forment et al., 2002). For SR45, it was even shown that two different splicing isoforms have distinct roles during plant development (Zhang and Mount, 2009). Moreover, in human cells, different SR proteins are shown to have distinct functions in AS *in vitro*, and display diverse sequence-specific RNA binding abilities, as summarized in (Long and Caceres, 2009). Thus, there is a growing body of evidence that the different splicing factors possess functional specificity, also in plants. An illustrative example is cyclin-dependent kinase G (CDKG1), a novel spliceosomal protein that is differentially spliced in our Col-0 low temperature treatment. Cyclin-dependent protein kinases were originally characterized as cell cycle regulators, but recent data shows that CDKG1 is involved in splicing (Huang et al., 2013). Moreover, the authors showed that it regulates pollen wall formation through alternative splicing of *CALLOSE SYNTHASE5* (*CalS5*). In addition, another publication showed that CDKG1 is essential for synapsis and male meiosis at high ambient temperature. How ambient temperature is able to direct CDKG1 functioning has not been uncovered yet, but the fact that we find *CDKG1* differentially spliced upon ambient temperature variations, it is tempting to speculate that AS is the mode of action by which CDKG1 confers the ability for synapsis and male meiosis at higher ambient temperatures. Nevertheless, deeper investigation like mutant analysis and splice-form specific complementation is necessary to prove this hypothesis.

In conclusion, we have shown that ambient temperature fluctuations have a pivotal effect on gene splicing and based on our detailed analyses, we propose that ambient temperature perception through alternative splicing works at least partially as a two-step model: first the splicing genes, such as *ATU2AF65A*, are changed in their splicing pattern, followed by AS of downstream genes (figure 6).
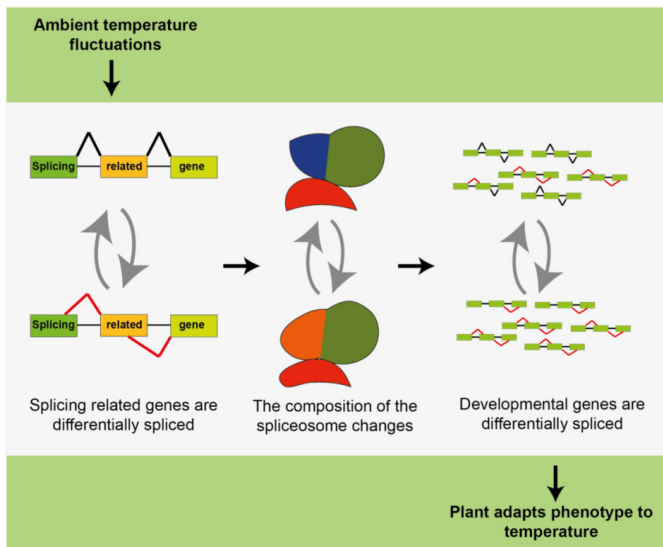

Figure 6: two-step model showing how ambient temperature may regulate alternative splicing. Upon fluctuations in ambient temperature, splicing related genes are changed in their splicing pattern. This alters the composition of the spliceosome, which in turn affects splicing of many downstream genes.

Materials and methods

Plant material and growth conditions

For RNAseq experiments, *Arabidopsis thaliana* Col-0 and Gy-0 plants were sown on rockwool blocks and stratified for 2-3 days at 4°C and subsequently transferred to 23°C SD conditions in controlled climate cabinets for 3 (in case of the Col-0 and Gy-0 high temperature experiment) or 6 (in case of the Col-0 low temperature experiment) weeks. Plants in vegetative state were transferred to 27°C (Col-0 and Gy-0) or 16°C (Col-0) for 24 hours, after which all above-ground tissue of both control (23°C) and temperature-treated seedlings were harvested and flash-frozen in liquid nitrogen. Each sample contained 18 plants and two biological replicates were collected for each sample.
Brassica oleracea var. *botrytis* "Lindurian" F1-hybrids were sown on sowing soil in sowing boxes, and transferred to the greenhouse under long day conditions (day: 16 hours at 21°C/night: 8 hours at 16°C). After two weeks, seedlings were transferred to bigger pots. After five weeks, plants were transferred to higher ambient temperature (day/night: 27°C /22°C) and after 24 hours, meristem-enriched tissue was harvested. Each sample contained tissue from five plants and two biological replicates were collected for each sample.

For flowering time analysis of Gy-0 (CS76139, ecotype id HapMap: 8214) and *atu2af65a* (SALK_144790, this mutant has the T-DNA inserted in the first intron, upstream of the splice sites causing the differentially alternative transcripts.), seeds were sown on soil, cold-imbibed for 4 days at 4° C and then transferred to LD conditions (16h light, 8h dark) at 22°C or 27°C continuous (n=10 per treatment). Light conditions were standard white light (Sylvania, Luxline Plus, Cool white; 150 µmol m^{-2} s^{-1}). We determined flowering time by counting the number of rosette leaves produced at the moment the plants started to flower. For *atu2af65a* morphology analysis, SALK_144790 seeds were sown on rockwool, cold-imbibed for 2 days at 4° C and then transferred to SD conditions (8h light, 16 h dark) under LED lights. Seedlings were grown for 5 weeks at 16° C, after which half of the plants were transferred to 25°C.

RNA extraction

For *A. thaliana*, samples were ground in liquid nitrogen, and total RNA was isolated from seedlings using the InviTrap Spin Plant RNA Mini Kit (Catalog No. 1064100300, Stratec Molecular) and treated with TURBO DNA-*free*™ Kit (Catalog No. AM1907, Ambion) in solution/DNAse I on column (Catalog No. 18068-015, Invitrogen) to remove DNA contaminations.
For cauliflower, RNA was isolated using the Qiagen RNeasy Mini Kit (catalog No. 74104, Qiagen) and treated with DNAse I (Catalog No. 18068-015, Invitrogen) to remove DNA contaminations. Samples were consequently purified with the above-mentioned Qiagen Mini Kit.

RNA sequencing

Paired-end libraries were constructed using the Illumina Paired-End DNA Sample Kit (Catalog No. PE-102-1001, PE-102-1002, Illumina) according to the manufacturer's protocol (in case of the Col-0 cool treatment, Gy-0 and cauliflower) or the modified Fasteris protocol (Fasteris SA) (in case of the Col-0 warm treatment) and paired-end 100 bp reads were generated using Illumina HiSeq 2000. All data were submitted to the National Centre for Biology Institute SRA database (BioProject PRJNA328771).

RNAseq analysis

RNA-seq reads of Arabidopsis Col-0 and Gy-0 plants were mapped against the *Arabidopsis thaliana* genome version TAIR10 (www.arabidopsis.org) using TopHat (Trapnell et al., 2009). *B.oleracea* reads were aligned against the *B.oleracea* genome version 1.0 (www.ocri-genomics.org/bolbase/index.html). Reference based full length transcript isoform reconstruction was performed for each sample separately using Cufflinks (Trapnell et al., 2010). Cuffmerge, which is part of the cufflinks package, was finally used for merging the individual cufflinks results into an overall set of full- length transcripts. For prediction of homologous *A. thaliana* genes in *B.oleracea*, The predicted proteins from *A.thaliana* were searched against the predicted proteome of *B.oleracaea* using blastp (Altschul et al., 1997). Blast alignments were discarded if the aligned sequence fragments were less than 40% identical. All alignments that included less than 75% of the *B.oleracaea* protein were also removed.

Custom python scripts were used for detecting the following type of alternative splicing events (Figure 1): retained intron (IR), skipped exon (ES), alternative 5'donor site (A5), alternative 3' acceptor site (A3) and mutually exclusive exons (MXE). MISO (Katz et al., 2010) was used to quantify AS-events (PSI values) in each individual sample and in pooled samples that were generated by merging the replicates of each condition.

MISO, which was also used for the differential splicing analyses, does not have built-in methods for analysing experimental replicates. As suggested by the authors of MISO, we used the pooled samples for the actual differential splicing test and the individual replicates for filtering AS events based on the following 2 rules: 1. Only those AS events supported by at least 20 isoform-specific reads in all the replicates of the conditions under comparison were considered. 2, the within condition PSI differences were required to be smaller than the between condition PSI differences. Finally, AS events that met the criteria and for which the compare-sample module of MISO returned a Bayes factor of at least 5 were considered significant.

RT-qPCR and RT-PCR

Col-0 RNA samples from the 23°C to 16°C temperature experiment were used for cDNA synthesis using the Bio-Rad iScript™ cDNA Synthesis kit (Catalog No. 1708891, Bio-Rad) or

Invitrogen SuperScript® II Reverse Transcriptase (Catalog number 18064-014, Thermo Fisher Scientific) according to the manufacturer's protocol.

qPCR was performed in 20ul reaction volume using the iQ™ SYBR® Green Supermix (Catalog No. 170-8885, Bio-Rad) according to the manufacturer's protocol. qPCR primers were designed to amplify the splicing event or a region included in all isoforms (Table S1). The latter were used as an internal reference in order to be able to calculate the ratio change between transcripts containing a specific splicing event versus all transcripts of a selected gene. Primers were tested for efficiency and the Pfaffl method (Pfaffl, 2001) was used to calculate the ratio changes.

RT-PCR on *MAF3* was executed using primers on the start and stop codon (Table S1), using Q5® high-fidelity polymerase (Catalog No. M0491S, NEB) and the manufacturer's protocol with an annealing temperature of 67°C and 35 PCR cycles. Samples were run on a 2% agarose gel. Fragments were cut out and extracted from gel using the nucleospin gel and PCR clean-up kit (Catalog No. 740609.250, Macherey-Nagel) and cloned into a pGEM®-T vector using the pGEM®-T Vector System I (Catalog No. A3600, Promega) according to the manufacturer's manual. Vectors were introduced into DH5α competent cells by electroporation. Plasmids were extracted using the NucleoSpin® Plasmid kit (catalog No. 740588.250, Macherey-Nagel) and sequences were obtained using the Macrogen EZ-seq service (for primer sequence, see Table S1).

Statistical methods and GO analysis

For splicing event distribution of differential events upon warm or cold treatment, significant difference of event frequency between total events and differential events was tested using the Chi-square test. For data used for testing, see Table S2.
GO analysis was performed using the Cytoscape plugin BiNGO (Maere et al., 2005) using the most recent go-basic ontology file and *A. thaliana* gene association file from The Gene Ontology Consortium (Consortium, 2015). A custom reference list was created containing intron-containing genes that showed expression in our dataset (≥ 1 read). Genes containing only cryptic introns or genes belonging to the chloroplast were no taken into account in this analysis. Overrepresentation was tested using a hypergeometric statistical test and the Benjamini & Hochberg False Discovery Rate correction was used to correct for multiple testing.
Enrichment analysis for splicing-related genes was conducted using Chi-square testing, with a P-value of <0.01. The same set of reference genes that was used for GO analysis was used for these calculations.

Supporting information

a
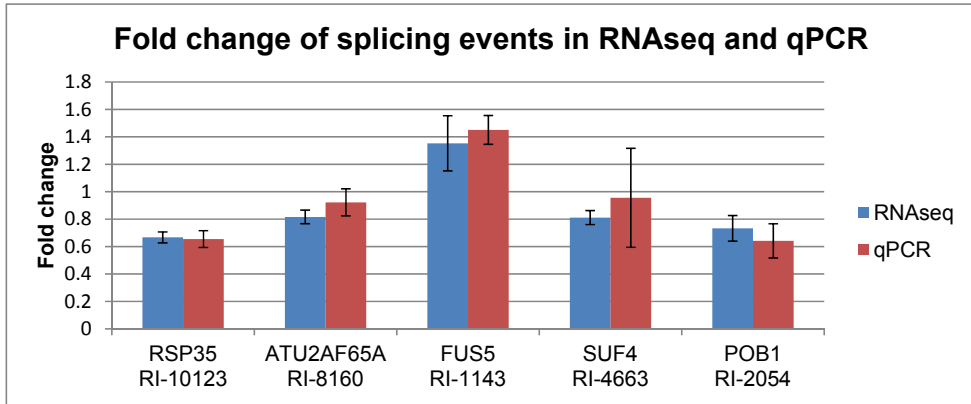
b
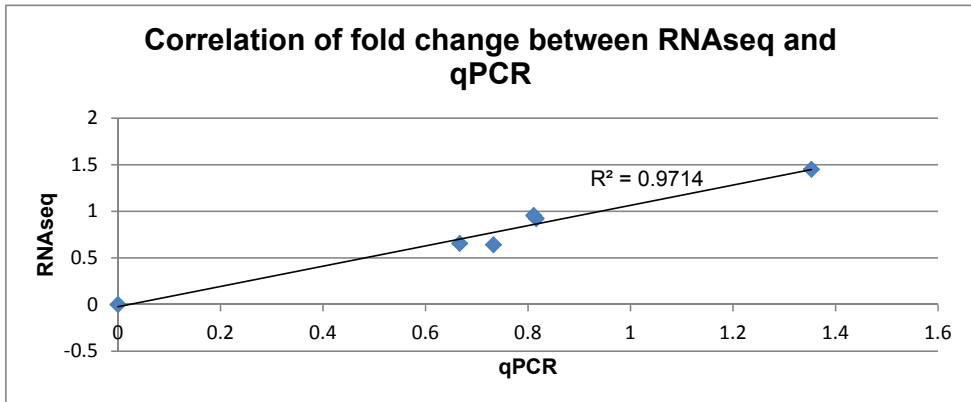
Figure S1. Validation of RNAseq results by qPCR on 5 different genes.
For each gene, we amplified a region only present in transcripts undergoing a splicing event, and a region included in all isoforms. We calculated the fold change in transcripts undergoing the splicing event relative to the total amount of transcripts, and compared this to the RNAseq results. **(a)** Comparison of RNAseq and qPCR fold change (N=2 (16 plants per sample), mean ±SD). **(b)** Correlation of fold change in RNAseq and qPCR.

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Band1    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT
Band2    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT
Band3    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT
Band4    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT
Band5    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT
Band6    1 ATGGGAAGAAGAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGCAGTCGACAAGTCACT


Band1   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA
Band2   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA
Band3   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA
Band4   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA
Band5   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA
Band6   61 TTCTCCAAACGACGCAAAGGTCTCATCGAAAAAGCTCGACAACTTTCAATTCTCTGTGAA


Band1  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT
Band2  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT
Band3  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT
Band4  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT
Band5  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT
Band6  121 TCTTCCATCGCTGTTGTCGCCGTCTCCGGTTCCGGAAAACTCTACGACTCTGCCTCCGGT


Band1  181 GACAACATGTCAAAGATCATTGATCGTTATGAAATACATCATGCTGATGAACTTAAAGCC
Band2  181 GACAA-------------------------------------------------------
Band3  181 GACAACATGTCAAAGATCATTGATCGTTATGAAATACATCATGCTGATGAACTTAAAGCC
Band4  181 GACAACATGTCAAAGATCATTGATCGTTATGAAATACATCATGCTGATGAACTTAAAGCC
Band5  181 GACAACATGTCAAAGATCATTGATCGTTATGAAATACATCATGCTGATGAACTTAAAGCC
Band6  181 GACAA-------------------------------------------------------


Band1  241 TTAGATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC
Band2  186 ---GATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC
Band3  241 TTAGATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC
Band4  241 TTAGATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC
Band5  241 TTAGATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC
Band6  186 ---GATCTTGCAGAAAAAATTCGGAATTATCTTCCACACAAGGAGTTACTAGAAATAGTC


Band1  301 CAAAGGTTAGCACTAAGACACCTTTTATCTCCCTCTTCTTCTGATAAAACATACTTCTTT
Band2  243 CAAAGGTTAGCACTAAGACACCTTTTATCTCCCTCTTCTTCTGATAAAACATACTTCTTT
Band3  301 CAAAG-------------------------------------------------------
Band4  301 CAAAG-------------------------------------------------------
Band5  301 CAAAG-------------------------------------------------------
Band6  243 CAAAG-------------------------------------------------------


Band1  361 TTCTTTTGGCGACTTATGAATACAGCAAGCTTGAAGAATCAAATGTCGATAATGTAAGTG
Band2  303 TTCTTTTGGCGACTTATGAATACAGCAAGCTTGAAGAATCAAATGTCGATAATGTAAGTG
Band3  306 -----------------------CAAGCTTGAAGAATCAAATGTCGATAATGTAAGTG
Band4  306 ----------------------------------------------------------TG
Band5  306 ------------------------------------------------------------
Band6  248 ------------------------------------------------------------


Band1  421 TAGATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA
Band2  363 TAGATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA
Band3  341 TAGATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA
Band4  308 TAGATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA
Band5  306 ---ATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA
Band6  248 ---ATTCTCTAATATCTATGGAGGAACAGCTCGAGACTGCTCTGTCAGTAATTAGAGCTA


Band1  481 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
Band2  423 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
Band3  401 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
Band4  368 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
Band5  363 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
Band6  305 AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGA
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Band1  541  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG
Band2  483  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG
Band3  461  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG
Band4  428  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG
Band5  423  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG
Band6  365  TAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAG

Band1  601  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC
Band2  543  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC
Band3  521  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC
Band4  488  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC
Band5  483  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC
Band6  425  GTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGC

Band1  661  TGCTCAAGTAA
Band2  603  TGCTCAAGTAA
Band3  581  TGCTCAAGTAA
Band4  548  TGCTCAAGTAA
Band5  543  TGCTCAAGTAA
Band6  485  TGCTCAAGTAA
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Figure S2. Sequence alignment of MAF3 isoforms detected by RT-PCR. Sequences obtained by Sanger sequencing after RT-PCR on total RNA with *MAF3* primers at the start and stop codons.
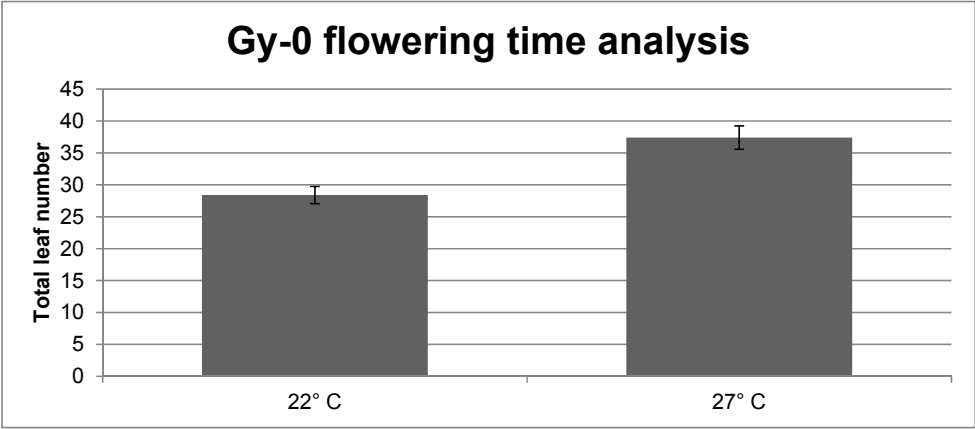

Figure S3. Flowering time analysis of *A. thaliana* Gy-0. Plants were grown under 22°C and 27°C. Flowering time was determined by counting rosette leafs at the moment of flower induction. N=10, mean ±SD.

Supporting information – Table S1: Oligo's used in this study

qPCR primers for RNAseq verification			
Locus/ description	**Splicing event**	**Primers on event**	**Primers on all isoforms**
AT4G25500/ *ATRSP35*	RI-AT4G25500-XLOC_021789-10123	FW: TCTCGTCTTCATTCCATCCA	FW: TCTCGGTGGAGTATGCTGTG
		RV: CCAGGTGAATGTGGTCAATG	RV: GTGACCTATCACGGCGTCTT
AT4G36690/ *ATU2AF65A*	RI-AT4G36690-XLOC_024897-8160	FW: GCGCAATATGGTCTATTTTGAA	FW: TACAGTGAGACGTGCCAACC
		RV: AGGCTGTAAAACAGCACAACC	RV: GCTGCTGTGCATGCAATAAT
AT1G02090/ *FUS5*	RI-AT1G02090-XLOC_004204-1143	FW: GGGGATGGGAGAGACTTGAT	FW: TGAATGCATGTATGCGGGTA
		RV: GGCTGATCGATTCGTTTGTT	RV: AGATCCCTACCAGCTGCAAA
AT1G30970/ *SUF4*	RI-AT1G30970-XLOC_005753-4663	FW: TGGTGATTGGTTCCACAAGA	FW: CGGATGGCGTTTTAGAATTT
		RV: GCAGTGAGATTGTGCAAAGG	RV: TGTTGTAACAAAGCCTTCATCC
AT3G61600/ *POB1*	RI-AT3G61600-XLOC_016687-2054	FW: CCGTTTACTTCTCAATTGTCACTG	FW: GTCTGCCCTGCTCTATCTCG
Primers on start and stop of *MAF3*			
FW *MAF3* on start		ATGGGAAGAAGAAAAGTCGAGATCA	
RV *MAF3* on stop		TGATTACTTGAGCAGCGAAAGAGTCTCC	

Supporting information - Table S2: Detected splicing event counts

	Col-0 cold total	%	Col-0 cold differential	%
A3	4276	40,6	134	37,0
A5	1979	18,8	53	14,6
MXE	25	0,2	1	0,3
RI	3487	33,1	147	40,6
SE	760	7,2	27	7,5
Total	10527	100,0	362	100,0

	Col-0 warm total	%	Col-0 warm differential	%
A3	3767	40,1	41	27,7
A5	1743	18,6	28	18,9
MXE	23	0,2	1	0,7
RI	3184	33,9	66	44,6
SE	668	7,1	12	8,1
Total	9385	100.0	148	100,0

	Col-0 Overall (warm+cold) total	%		
A3	4621	40,8		
A5	2153	19,0		
MXE	26	0,2		
RI	3715	32,8		
SE	820	7,2		
Total	11335	100,0		

	Gy-0 total	%	Gy-0 differential	%
A3	2101	36,4	53	42,1
A5	1136	19,7	18	14,3
MXE	21	0,4	0	0,0
RI	1885	32,7	43	34,1
SE	630	10,9	12	9,5
Total	5773	100.0	126	100.0

	B.ol total	%	B.ol differential	%
A3	4946	37,2	53	29,4
A5	3004	22,6	40	22,2
MXE	61	0,5	1	0,6
RI	3753	28,2	72	40,0
SE	1537	11,6	14	7,8
Total	13301	100.0	180	100.0

Note: Supplementary table S3-7 could not be included due to size limitations, and are available upon request.

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Chapter 4

Temperature-dependent regulation of flowering by antagonistic FLM variants

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Abstract

The appropriate timing of flowering is crucial for plant reproductive success. It is therefore not surprising that intricate genetic networks have evolved to perceive and integrate both endogenous and environmental signals, such as carbohydrate and hormonal status, photoperiod and temperature (Pose et al., 2012; Srikanth and Schmid, 2011). In contrast to our detailed understanding of the vernalization pathway, little is known about how flowering time is controlled in response to changes in the ambient growth temperature. In *Arabidopsis thaliana*, the MADS-box transcription factor genes *FLOWERING LOCUS M* (*FLM*) and *SHORT VEGETATIVE PHASE* (*SVP*) have key roles in this process (Balasubramanian et al., 2006; Lee, 2007). *FLM* is subject to temperature-dependent alternative splicing (Balasubramanian et al., 2006). Here we report that the two main FLM protein splice variants, FLM-β and FLM-δ, compete for interaction with the floral repressor SVP. The SVP–FLM-β complex is predominately formed at low temperatures and prevents precocious flowering. By contrast, the competing SVP–FLM-δ complex is impaired in DNA binding and acts as a dominant-negative activator of flowering at higher temperatures. Our results show a new mechanism that controls the timing of the floral transition in response to changes in ambient temperature. A better understanding of how temperature controls the molecular mechanisms of flowering will be important to cope with current changes in global climate (Mann, 2008; Marcott et al., 2013).

Introduction, Results & Discussion

Distinct aspects of temperature contribute to flowering time control. The vernalization pathway controls flowering of winter-annual *Arabidopsis* accessions in response to prolonged periods of cold by the epigenetic silencing of the potent floral repressor *FLOWERING LOCUS C* (*FLC*) (Chouard, 1960; Michaels and Amasino, 1999; Sheldon, 1999). Ambient temperature also has an essential role, inducing flowering in *Arabidopsis* at warmer temperatures under otherwise non-inductive short-day photoperiods (Balasubramanian et al., 2006). The identification of mutants affected in the ambient temperature response indicates that there is a strong genetic contribution to the regulation of flowering in response to temperature changes (Srikanth and Schmid, 2011). Most recently, H2A.Z and PHYTOCROME INTERACTING FACTOR4 (PIF4) have emerged as important positive regulators of flowering in response to temperature, the latter being essential for the induction of flowering in response to warmer temperatures in short-day conditions (Franklin, 2011; Kumar et al., 2012; Kumar and Wigge, 2010). By contrast, the MIKC-type MADS-domain transcription factors SVP (Lee, 2007), FLM (Balasubramanian et al., 2006) and MADS AFFECTING FLOWERING 2, 3 and 4 (MAF2–4; also known as AGL31, AGL70 and AGL69, respectively)(Gu, 2013; Rosloski et al., 2013) have been described as negative regulators of flowering. Both *SVP* and *FLM* contribute to flowering-time variation between natural accessions of *Arabidopsis* (Mendez-Vigo et al., 2013; Werner, 2005), and mutations in these

genes lead to decreased thermosensitivity (Balasubramanian et al., 2006; Gu, 2013; Lee, 2007) (Table 1, experiment 1). Moreover, SVP and FLM have been shown to interact genetically (Scortecci et al., 2003). All together, these findings suggest a role for these two MADS transcription factors in the ambient temperature pathway. The *FLM* transcript is subject to alternative splicing, with four splice variants (α, β, γ and δ) expressed in the Wassilewskija accession (Scortecci et al., 2001). The *Arabidopsis* Columbia (Col-0) accession primarily transcribes two splice variants, *FLM-β* and *FLM-δ* (Extended Data Fig. 1a, b), which are both translated (Jiao and Meyerowitz, 2010). They incorporate either the second or third exon, respectively, which encodes part of the MIKC 'intervening' (I) region that is thought to contribute to protein–protein interaction properties (Riechmann et al., 1996). Interestingly, *FLM* splicing changes in response to ambient temperature variation (Balasubramanian et al., 2006), suggesting that the proteins encoded might affect flowering in different ways.

To understand the effect of temperature-dependent alternative splicing of *FLM* on flowering we analysed the effect of ambient temperature fluctuation on *FLM* splicing. *FLM-β* and *FLM-δ* were detected at similar ratios in all tissues examined (Fig. 1a, b). By contrast, expression of the two transcripts was different in plants that had been grown at different temperatures (Fig. 1c). *FLM-β* was the prevalent splice variant at 16 °C, whereas *FLM-δ* dominated at 27 °C (Fig. 1c). In addition, after a shift from 16 °C to 27 °C, expression of *FLM-β* decreased within 24 h, whereas *FLM-δ* levels increased (Fig. 1d). The opposite was the case when plants were transferred from 27 °C to 16 °C (Fig. 1d). By contrast, *SVP* expression was only moderately induced with increasing temperature (Fig. 1e). Because SVP regulates flowering in response to ambient temperature, this suggests that SVP might be regulated at the post-transcriptional or protein–protein interaction level (Lee, 2007).

Table 1. Flowering times of mutants and transgenic plants.

	RLN	CLN	TLN	SD	Range	*n*
Experiment 1 (16ºC LD)						
Col-0 BAR	18.1	3.9	21.9	±2.9	15 - 27	29
flm-3 BAR	10.7	3.5	14.2	±1.7	11 - 17	27
35S:FLM-β #11	23.8	9.7	33.4	±5.9	23 - 40	12
35S:FLM-β #21	26.9	11.0	37.9	±3.1	33 - 42	9
35S:FLM-δ #1	12.0	3.6	15.6	±2.2	13 - 20	13
35S:FLM-δ #4	12.9	3.4	16.4	±1.5	14 - 19	14
flm-3 35S:FLM-β #39	17.5	5.2	22.8	±2.5	19 - 28	21
flm-3 35S:FLM-β #54	17.4	5.2	22.6	±3.6	17 - 29	15
flm-3 35S:FLM-δ #3	8.9	2.8	11.7	±1.4	8 - 14	28
flm-3 35S:FLM-δ #43	8.8	2.4	11.3	±2.1	7 - 14	28
Experiment 2 (16ºC LD)						
Col-0 BAR	18.2	5.5	23.7	±1.8	20 - 28	30
flm-3 BAR	11.3	3.5	14.7	±1.3	11 - 17	28
35S:FLM-β #21	27.5	10.2	37.6	±2.8	32 - 43	13
35S:FLM-δ #4	10.5	2.6	13.1	±1.5	9 - 16	38
35S:FLM-β #21 x Col-0 (F1)	24.6	7.1	31.7	±2.7	26 - 39	21
35S:FLM-β #21 x *35S:FLM-δ* #4 (F1)	16.9	4.3	21.2	±2.7	16 - 26	19
flm-3 35S:FLM-β #39	18.0	5.1	23.1	±2.5	19 - 27	24
flm-3 35S:FLM-δ #43	9.1	2.5	11.6	±2.1	7 - 14	26
flm-3 35S:FLM-β #39 x *flm-3* (F1)	17.4	4.9	22.3	±4.2	16 - 30	24
flm-3 35S:FLM-β #39 x *flm-3 35S:FLM-δ* #43 (F1)	13.2	4.0	17.2	±2.6	13 - 23	27
Experiment 3 (16ºC LD)						
Col-0 BAR	19.9	5.8	25.7	±2.9	20 - 30	24
flm-3 BAR	8.9	3.2	12.1	±1.6	9 - 14	14
flm-3 pFLM:gFLM #2	21.2	7.0	28.2	±3.5	21 - 32	13
flm-3 pFLM:gFLM #3	21.0	6.8	27.8	±2.4	25 - 31	5
flm-3 pFLM:gFLM-GFP #2	19.4	7.6	27.0	±2.2	22 - 30	14
flm-3 pFLM:gFLM-GFP #4	19.9	7.4	27.3	±2.2	25 - 32	11
Experiment 4 (16ºC LD)						
Col-0 BAR	22.0	4.8	26.8	±2.7	21 - 31	26
flm-3 BAR	13.7	3.7	17.4	±1.5	14 - 20	29
flm-3 pFLM:iFLM-β #24	26.0	6.8	32.8	±3.2	28 - 39	18
flm-3 pFLM:iFLM-δ #17	11.9	3.4	15.4	±1.6	12 - 19	29
flm-3 pFLM:iFLM-β-GFP #10	20.6	5.6	26.2	±1.9	20 - 32	40
flm-3 pFLM:iFLM-δ-GFP #8	12.5	3.6	16.1	±1.3	13 - 19	40
Experiment 5 (16ºC LD)						
Col-0 BAR	20.6	5.8	26.4	±2.1	21 - 30	43
35S:FLM-β #21	30.5	10.2	40.7	±2.2	37 - 44	15
35S:FLM-β-VP16 (T1)	15.2	4.6	19.8	±4.5	9 - 27	104

n denotes the number of individuals; # denotes the identifier of individual transgenic lines. cauline leaf number; LD, long-day; RLN, rosette leaf number; s.d., total leaf number standard deviation; TLN, total leaf number.
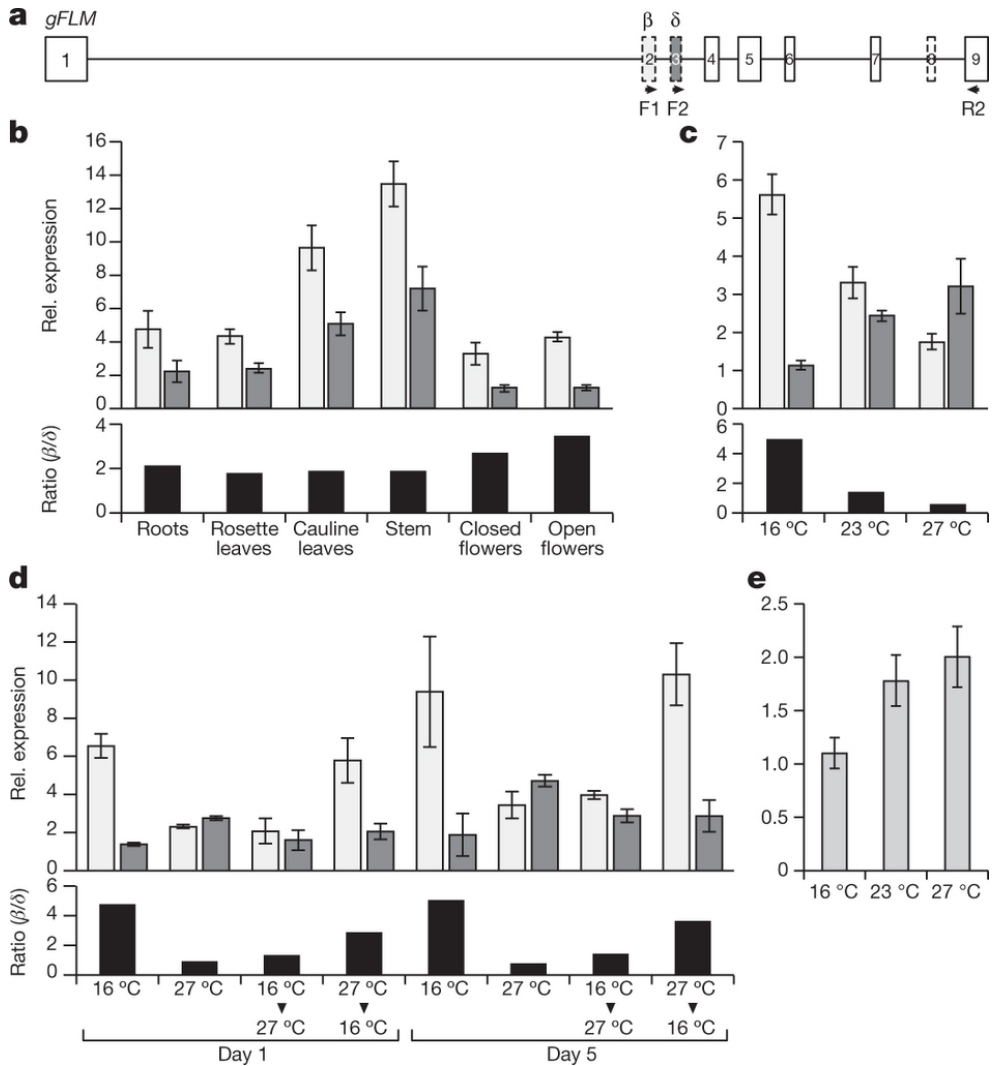
Figure 1: Temperature-dependent expression of *FLM-β*, *FLM-δ* and *SVP*. a, *FLM* locus, including exons (boxes) and introns (lines). Primers used for qRT–PCR are indicated. **b–d**, Relative (rel.) expression of *FLM-β* (light grey) and *FLM-δ* (dark grey) in different tissues in 10-day-old Col-0 seedlings grown at 23 °C long-day (**b**), in whole seedlings at 16 °C, 23 °C and 27 °C long-day (**c**) and days 1 and 5 after a shift between temperatures (**d**). The ratio of *FLM-β*/*FLM-δ* expression is shown in black. **e**, *SVP* expression in 10-day-old Col-0 seedlings grown at 16 °C, 23 °C and 27 °C. Error bars denote s.d. of three biological replicates with three technical repetitions each.

To investigate the effect of the *FLM* splice variants on flowering time, we expressed the *FLM-β* and *FLM-δ* open-reading frames (ORFs) from the *35S* promoter in wild-type and *flm-3* plants. As expected for a floral repressor, expression of *FLM-β* strongly delayed flowering in Col-0, and complemented the early flowering of *flm-3* (Table 1, experiment 2, and Extended

Data Fig. 2a). *FLM-δ* expression had the opposite effect and induced early flowering (Table 1, experiment 2, and Extended Data Fig. 2a). Expression analysis confirmed that endogenous *FLM-β* and *MAF2*, *MAF3*, *MAF5* (also known as *AGL68*) and *FLC*, as well as *SVP*, were expressed normally in *35S:FLM-δ* Col-0 plants, indicating that the early flowering phenotype was not caused by co-suppression (Extended Data Fig. 3a, b). In addition, crosses between *35S:FLM-β* and *35S:FLM-δ* plants displayed an intermediate phenotype (Extended Data Fig. 3c–e), suggesting that FLM-δ is responsible for the acceleration of flowering of these lines.

FLM-β, but not FLM-δ, was able to form homodimers, and the two proteins were able to heterodimerize, according to yeast two-hybrid analyses (Fig. 2a and Supplementary Table 1). Furthermore, both FLM isoforms interacted with fully spliced SVP (At2g22540.1) but not with any other variant tested (Supplementary Table 1). All interactions were confirmed by transient bimolecular complementation (BiFC) assays (Fig. 2b and Extended Data Fig. 4). In addition, both FLM isoforms also interacted in yeast with the type-I MADS-domain protein AGL74N (At1g48150; Supplementary Table 1). However, an *AGL74N* T-DNA insertion allele flowered normally (Extended Data Fig. 5). The finding that both FLM-β and FLM-δ were able to interact with the floral repressor SVP, but had opposite effects on flowering time, suggests a model in which the incorporation of a particular FLM isoform determines the activity of the resulting SVP–FLM heterocomplex.
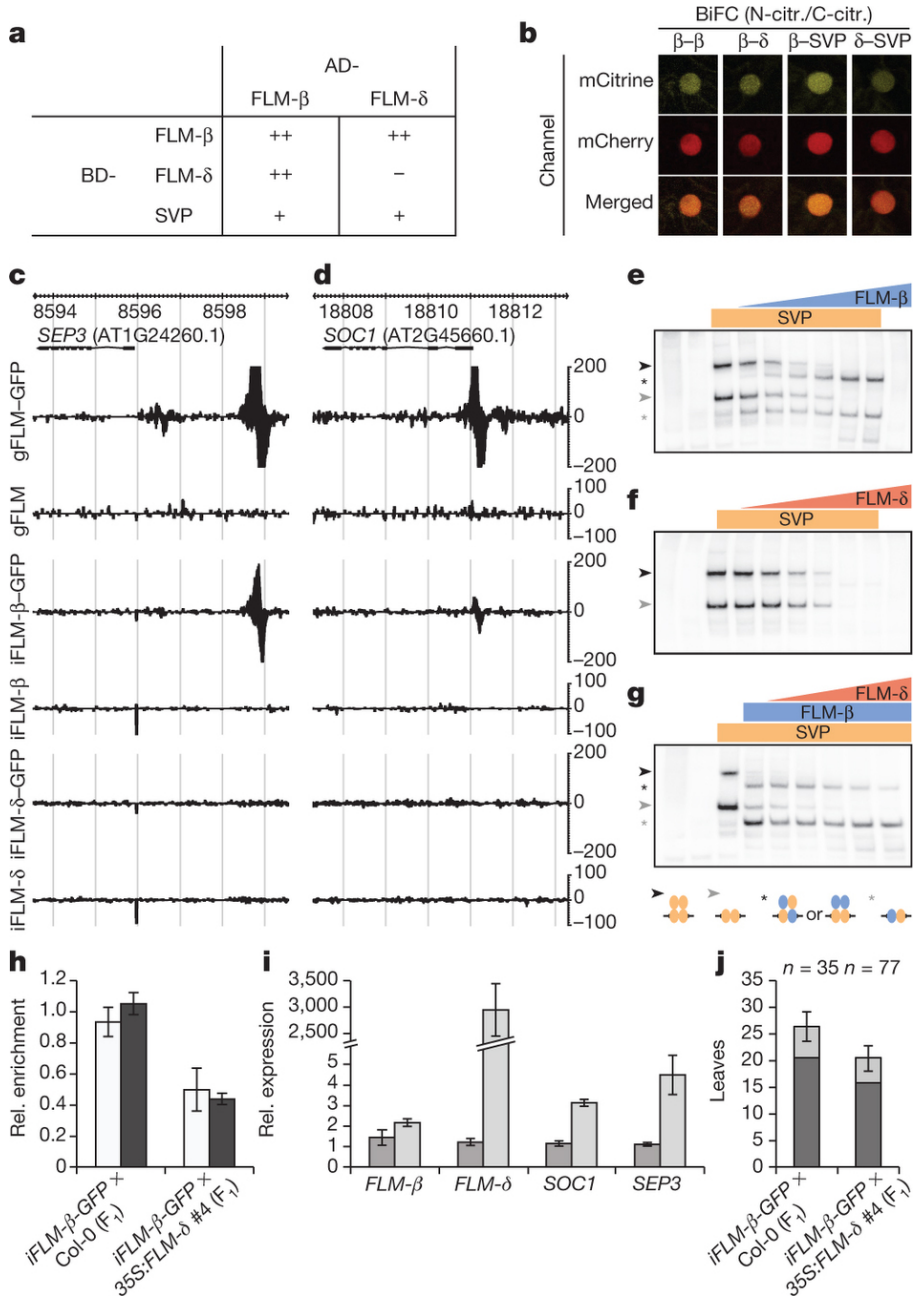
Figure 2: FLM-SVP protein–protein interactions and DNA binding assays. a, Yeast two-hybrid. AD, activation domain; BD, DNA-binding domain. **b**, BiFC FLM-β, FLM-δ and SVP interaction assays. C-citr., C-terminal half of haemagglutinin (HA)-tagged mCitrine; N-citr., N-terminal half of Myc-tagged mCitrine. **c**, **d**, Local enrichment of GFP-tagged FLM, iFLM-β and iFLM-δ bound to the *SEP3* (**c**) and *SOC1* (**d**) regulatory regions assayed by ChIP-seq. Each panel shows a 6-kilobase (kb) window. **e–g**, EMSA competition assays using a *SEP3* promoter probe containing two CArG motifs. Lanes 1 and 2

correspond to 'no protein' and 'shuffled-SVP' controls, respectively. Increasing concentrations of FLM-β (**e**) and FLM-δ (**f**) were added to a constant amount of SVP. **g**, Titration of FLM-δ to constant amounts of SVP and FLM-β. Orange and blue ellipses represent SVP and FLM-β proteins, respectively. **h**, Relative enrichment of binding of *iFLM-β-GFP* to the promoters of *SOC1* (open bars) and *SEP3* (filled bars) in *iFLM-β-GFP × 35S:FLM-δ* F_1 and control plants. **i**, Expression of *SOC1* and *SEP3* in *iFLM-β-GFP × 35S:FLM-δ* F_1 (light grey) and control (dark grey) plants. Error bars denote the s.d. of three biological replicates with three technical repetitions each. **j**, Flowering time of the F_1 plants. Rosette and cauline leaf number are represented in dark and light grey.

To test this model and identify FLM direct targets we constructed a genomic *FLM* (*gFLM*) fragment that rescued *flm-3*, independently of whether a carboxy-terminal enhanced variant green fluorescent protein tag (mGFP6) is present (Table 1, experiment 3, and Extended Data Fig. 2b). Chromatin immunoprecipitation and massively parallel sequencing (ChIP-seq) on a rescued gFLM-GFP line revealed binding of FLM to the regulatory regions of several flowering time related genes, including *SUPRESSOR OF CONSTANS OVEREXPRESSION 1* (*SOC1*; also known as *AGL20*), *Arabidopsis thaliana CENTRORADIALIS homologue* (*ATC*), *TEMPRANILLO 2* (*TEM2*; also known as *RAV2*) and *SCHLAFMUTZE* (*SMZ*), and floral homeotic genes such as *SEPALATA 3* (*SEP3*), *APETALA 3* (*AP3*) and *PISTILLATA* (*PI*) (Fig. 2c, d, Extended Data Fig. 6b–e and Supplementary Table 2).

To identify splice-variant-specific targets of FLM, we established transgenic lines that expressed *FLM-β* (*pFLM:iFLM-β*) or *FLM-δ* (*pFLM:iFLM-δ*), with or without the C-terminal mGFP tag, in *flm-3* (Table 1, experiment 4, and Extended Data Figs 2c and 6a–e). ChIP-seq performed using an *iFLM-β-GFP* rescue line revealed that most of the targets (67%) identified in the *gFLM-GFP* line were bound by iFLM-β-GFP (Supplementary Tables 3 and 4 and Extended Data Fig. 7a). Quantitative reverse transcriptase PCR (qRT–PCR) analysis of *SEP3*, *SOC1* and *TEM2* confirmed that these genes were also regulated in their expression by FLM-β (Extended Data Fig. 6f–h). In addition, expression of an FLM-β-VP16 fusion protein (containing the VP16 transcriptional activation domain) resulted in early flowering (Extended Data Fig. 2d), indicating that FLM-β delays flowering mainly through transcriptional repression. *SEP3*, *SOC1* and *TEM2* were also found among 61 genes that were shared between our iFLM-β ChIP-seq data and SVP targets identified by ChIP coupled to DNA microarray (ChIP-chip) (Tao, 2012) (Supplementary Table 4 and Extended Data Fig. 7b), suggesting that these genes could be regulated by an SVP–FLM-β heterocomplex. By contrast, iFLM-δ–GFP ChIP resulted in only minor enrichment at few loci (Supplementary Table 3), suggesting that FLM-δ does not bind to DNA efficiently.

To analyse the *in vitro* DNA-binding properties of SVP, FLM-β and FLM-δ in detail, we performed electrophoretic mobility shift assays (EMSAs). We observed strong binding of SVP to CArG boxes in the same regions of the *SEP3*, *SOC1* and *ATC* promoters that had shown enrichment for FLM and SVP binding in ChIP-seq and ChIP-chip analyses, respectively (Extended Data Fig. 8a–c). By contrast, no changes in DNA mobility were observed for FLM-δ and FLM-β (Extended Data Fig. 8a–c), indicating that these two proteins alone do not bind DNA *in vitro*. However, simultaneous *in vitro* transcription/translation of SVP and FLM-β, but

not of SVP and FLM-δ, resulted in additional bands, corresponding to different SVP–FLM-β heterocomplexes (Fig. 2e and Extended Data Fig. 8a–c). These findings indicate that FLM-β requires SVP for DNA binding *in vitro*. In agreement with this, we found that the late flowering of *35S:FLM-β* plants was abolished in the *svp-32* mutant (Fig. 3b), suggesting that FLM-β depends on SVP to repress flowering. This dependency seems to be bidirectional, as the late flowering and the homeotic transformation of sepals and petals characteristic of an *SVP* mis-expression line are suppressed in *flm-3* (Fig. 3a, b). Moreover, a double *flm-3 svp-32* mutant line did not display any additional phenotype to that of the single *svp-32* single mutant (Scortecci et al., 2003) (Fig. 3b), and a *35S:FLM-β 35S:SVP* double-transgenic line flowered much later than the individual mis-expression lines (Fig. 3d).
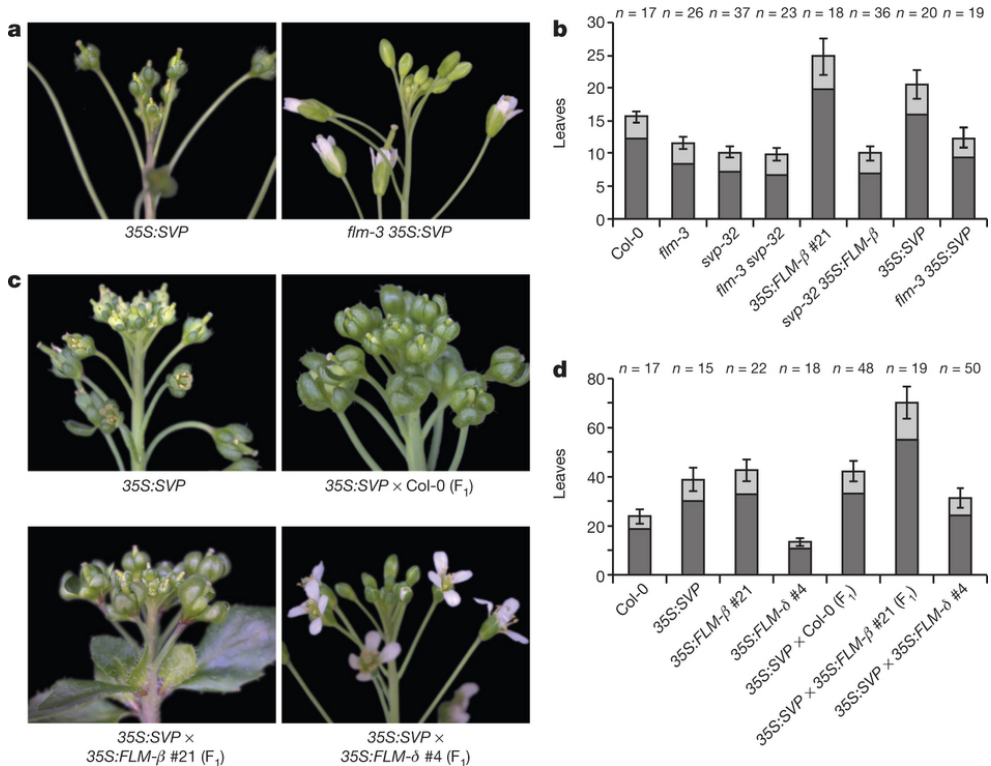

Figure 3: FLM and SVP are interdependent and regulate flowering time and flower morphology. a, *flm-3* suppresses the green and leaf-like sepal and petal phenotype of *35S:SVP* flowers. **b**, Flowering time of *flm-*3 and *svp-32* single and double mutants, and FLM-β and SVP mis-expression lines in wild-type (Col-0), *svp-32* or *flm-3*. **c**, *35S:FLM-δ* suppresses the *35S:SVP* flower phenotype. **d**, *35S:FLM-β* enhances the late flowering of *35S:SVP*, whereas *35S:FLM-δ* has the opposite effect. Plants were grown at 23 °C (**a**, **b**) and 16 °C (**c**, **d**). Rosette and cauline leaf number are represented in dark and light grey, respectively.

To identify the molecular mechanism underlying the flowering-promoting effect of FLM-δ, we performed EMSA competition experiments in which increasing amounts of FLM-δ were added to either SVP (Fig. 2f) or SVP plus FLM-β (Fig. 2g). We observed an FLM-δ

concentration-dependent reduction in DNA binding for both SVP and SVP–FLM-β (Fig. 2f, g and Extended Data Fig. 8d, e), suggesting that FLM-δ functions as a dominant-negative version of FLM that alleviates SVP–SVP and SVP–FLM-β mediated repression by replacing one of the interaction partners in the complex, thereby rendering it inactive. As predicted by this model, constitutive expression of *FLM-δ* suppressed the late flowering and flower phenotypes of *35S:SVP* plants (Fig. 3c, d). Furthermore, binding of iFLM-β-GFP to the promoters of *SEP3* and *SOC1* was reduced in *pFLM:iFLM-β-GFP × 35S:FLM-δ* plants, indicating that FLM-δ negatively affects the ability of FLM-β to bind to its target (Fig. 2h). In addition, expression of these two genes was increased (Fig. 2i) in the double transgenic line, which also flowered considerably earlier than control plants (Fig. 2j).

Our results demonstrate that the protein isoforms encoded by two splice variants of *FLM*, *FLM-β* and *FLM-δ*, compete for interaction with SVP to regulate flowering in opposition. Low ambient temperature favours the formation of SVP–SVP and SVP–FLM-β complexes that actively repress flowering. However, as temperatures increase, not only is the amount of the FLM-β spliceform downregulated, but the flower-repressive function of SVP and the remaining FLM-β proteins are counteracted by a relative increase in the dominant-negative regulator of flowering, FLM-δ (Extended Data Fig. 9). We propose that the opposing activities of two different splice variants of *FLM*, and possibly other transcription factors that are also subject to (temperature-dependent) alternative splicing (James, 2012; Jones, 2012; Rosloski et al., 2013; Wang, 2012), constitute a distinct regulatory pathway that acts in parallel to PIF4 to reinforce the transition from vegetative growth to reproductive development in response to changes in ambient temperature. It will be interesting to see how additional regulatory mechanisms such as posttranslational modification or targeted degradation of FLM or SVP contribute to the regulation of flowering in response to changes in ambient temperature. Furthermore, given that natural variation in *FLM* contributes substantially to flowering time differences among *Arabidopsis* accessions (Balasubramanian et al., 2006; Werner, 2005), the role of temperature-dependent mRNA splicing in adaptation to climate change is worthy of special focus.

Methods

Plant material and growth conditions

All plants used in this work are of the Columbia (Col-0) accession. The *flm-3* and *svp-32* lines have been described previously (Extended Data Table 1). Genotypes were confirmed by PCR using oligonucleotides listed in Supplementary Table 5. Plants were grown in chambers in a long-day condition (16 h light/8 h dark) controlled photoperiod at 16 °C, 23 °C and 27 °C, 65% humidity and a mixture of Cool White and Gro-Lux Wide Spectrum fluorescent lights, with a fluence rate of 125–175 µmol m^{-2} s^{-1}.

Plasmid constructions and plant transformation

All oligonucleotides used in this work are listed in Supplementary Table 5. All PCR reactions were performed using Phusion polymerase (New England Biolabs) and the constructs were verified by Sanger sequencing after cloning.

The ORFs of *FLM* (At1g77080; different splice forms) and *SVP* (At2g22540.1) were amplified from cDNA prepared from Col-0 seedlings grown at 23 °C using primers G-2196 and G-1978, and primers G-28863 and G-28864, respectively. The PCR product was purified and ligated into the pJLSmart Gateway entry vector, generating the constructs *pJM313* (*FLM-β*), *pLY224* (*FLM-δ*) and *pDP57* (*SVP*). pJLSmart is a modified pENTR1A vector (Invitrogen, Life Technologies) with the polylinker and *ccdB* gene replaced by a minimal MCS, flanked by attL1 and attL2 sites, to allow for blunt-end cloning using a SmaI site and subsequent Gateway recombination. For mis-expression experiments, the ORFs were introduced into the Gateway-compatible pGREEN-IIS binary destination vectors *pFK210* (*FLM-β* and *FLM-δ*) and *pFK209* (*SVP*), which provide resistance to BASTA and kanamycin, respectively, for selection in plants, by Gateway recombination, resulting in *pJM356* (*35S:FLM-β*), *pLY225* (*35S:FLM-δ*) and *pDP58* (*35S:SVP*). For the yeast two-hybrid assays the ORFs were recombined into the destination vectors *pDEST22* (AD) and *pDEST32* (BD), creating *pDP10* (AD-FLM-β), *pDP11* (BD-FLM-β), *pDP14* (AD-FLM-δ) and *pDP15* (BD-FLM-δ).

For the BiFC assays, the ORFs were amplified without the stop codon using the oligonucleotides G-2196 and G-31569 for the *FLM-β* and *FLM-δ* splice variants, and G-28863 and G-31886 for *SVP*. The products were cloned into the pCR8/GW/TOPO Gateway entry vector (Invitrogen, Life Technologies) to create *pDP105* (*FLM-β* Δstop), *pDP106* (*FLM-δ* Δstop) and *pDP129* (*SVP* Δstop). The ORFs were recombined into destination vectors *pAS057* (Myc-mCitrine N-terminal) and *pAS061* (HA-mCitrine C-terminal) to generate the split-mCitrine-tagged constructs for *FLM-β* (*pDP157* and *pDP158*), *FLM-δ* (*pDP159* and *pDP160*) and *SVP* (*pDP161* and *pDP162*).

For the *35S:FLM-β-VP16* construct, we recombined the FLM-β ORF from *pDP105* into the destination vector *pFK250*, which provides an in-frame VP16 tag, resulting in the construct *pDP175.*

The 6,876-bp genomic *FLM* (At1g77080; TAIR10, chr1: 28953510..28960386) rescue fragment, which includes approximately 2.1 kb upstream sequence, exons, introns, the 3' untranslated region and approximately 0.3 kb downstream sequence, was amplified by PCR from genomic DNA isolated from Col-0 using the primers G-26819 and G-26820 (Supplementary Table 5). The resulting PCR product was purified and cloned into the pCR8/GW/TOPO vector to create *pDP22*. Subsequently, the *FLM* genomic fragment was recombined from *pDP22* into the pGREEN-IIS binary destination vector pFK387, which provides resistance to BASTA for selection in plants, resulting in *pDP34* (*pFLM:gFLM*). To facilitate ChIP, *FLM* was tagged with mGFP6-6×His. For this purpose, we amplified a genomic

sub-fragment of *FLM* ranging from exon 7, which contains a unique SexAI restriction site, to the last coding triplet before the stop codon of *FLM* using the primers G-22798/G-26831, and the *FLM* 3' region, which contains a unique SacI restriction site, starting with the stop codon using the primers G-26335 and G-26820. The sequence encoding mGFP6-6×His was amplified from plasmid pMD107 (Curtis and Grossniklaus, 2003) using the primers G-26832 and G-26334. Next, the three fragments were combined in an overlapping fusion PCR using primers G-22798 and G-26820. The resulting PCR product (5'-*FLM-mGFP6*-3'; Supplementary Table 5) was cloned into the pGEM-T Easy vector (Promega) to create *pDP23* and subsequently cut with SexAI and SacI and cloned into the corresponding sites of *pDP22* to generate *pDP24*. Finally, the *FLM-mGFP6* genomic fragment was recombined into *pFK387* to create *pDP28* (*pFLM:gFLM-GFP*).

The ORFs of the *FLM-β* and *FLM-δ* splice variants under control of the *FLM* 5' and 3' regions present in the *gFLM* construct were created by overlapping PCR. First, two halves were amplified separately with the oligonucleotides G-29612 and G-28155 using *pJM313* (*FLM-β*) and *pLY224* (*FLM-δ*) as templates, and with G-26118 and G-26820 using *pDP22* as template. The two halves were fused in a PCR using the oligonucleotides G-29612 and G-26820, purified and cloned into pGEM-T Easy to create *pDP67* (*FLM-β*-3') and *pDP68* (*FLM-δ*-3'). These constructs were subsequently cut with NcoI and SacI and cloned into the corresponding sites of *pDP22* to generate *pDP75* (*pFLMp:FLM-β*-3') and *pDP76* (*pFLMp:FLM-δ*-3'), which were recombined into *pFK387* to create *pDP79* and *pDP80*, respectively.

The first intron of *FLM* was introduced into the ORFs of *FLM-β* and *FLM-δ* by overlapping PCR. The 5' halves of the insert were amplified using the oligonucleotides G-29612 and G-30980 for *FLM-β*, and G-29612 and G-30982 for *FLM-δ*, using *pDP22* as template. The 3' halves were amplified using the oligonucleotides G-28150 and G-28154 for *FLM-β* using *pDP75* as a template, and with G-30981 and G-28154 for *FLM-δ* using *pDP76* as a template. The two fragments were fused in a PCR using the oligonucleotides G-29612 and G-28154, and the PCR product was purified and cloned into pGEM-T Easy to create *pDP92* (*FLM-β* fragment with first intron) and *pDP93* (*FLM-δ* fragment with first intron). These constructs were subsequently cut with NcoI and SexAI and cloned into the corresponding sites of *pDP75* and *pDP76* to generate *pDP94* (*pFLM:iFLM-β*) and *pDP95* (*pFLM:iFLM-δ*), respectively. These constructs were subsequently recombined into *pFK387* to create *pDP96* and *pDP97*, respectively. To perform ChIP, *pFLM:iFLM-β/δ* constructs were tagged with mGFP6-6×His by overlapping PCR. We first amplified with the oligonucleotides G-29345 and G-26831 a fragment of the *FLM* cDNA and with G-26832 and G-26820 the mGFP6-6×His plus the 3' region of *FLM* sequence. The two fragments were combined using the oligonucleotides G-29345 and G-26820 and cloned into pGEM-T Easy generating *pDP87* (*FLM* (7th–9th exon)-*mGFP6-FLM* 3'). *pDP87* was cut with SexAI and SacI and cloned into *pDP94* and *pDP95* creating *pDP101* (*pFLM:iFLM-β-GFP*) and *pDP102* (*pFLM:iFLM-δ-GFP*), respectively. The latter were recombined into the destination vector *pFK387* generating *pDP103* and *pDP104*, respectively.

All generated constructs were transformed into Col-0 and/or *flm-3* plants making use of *Agrobacterium tumefaciens* strain ASE and the floral dip method (Clough and Bent, 1998). Transgenic plants were identified by selective germination on soil watered with 0.1% glufosinate (BASTA).

RNA extraction, cDNA synthesis and expression analysis

For gene expression analysis, total RNA was isolated using TRIzol Reagent (Ambion, Life Technologies) according to manufacturer's instructions. One microgram of total RNA was DNase I-treated and single-stranded cDNA was synthesized using oligo(dT) and the RevertAid first-strand cDNA synthesis kit (Fermentas, Thermo Scientific). The resulting single-strand cDNA was diluted 25-fold and 4 µl was used as a template. Quantitative PCR (qPCR) was performed using the Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Life Technologies) and specific oligonucleotides (Supplementary Table 5) on an MJR Opticon Continuous Florescence Detection System. Relative expression values were calculated by the $\Delta\Delta C_t$ method using β-TUB2 (At5g62690) as a control. For each sample, material from a minimum of five seedlings was pooled. Error bars reported in Figs 1 and 2 and Extended Data Figs 3 and 6 denote the s.d. of three biological replicates with three technical repetitions each.

ChIP, library preparation and high throughput sequencing

ChIP was performed using 1 g of tissue collected at zeitgeber (ZT) 8 from homozygous *flm-3 pFLM:gFLM* #2 and *flm-3 pFLM:gFLM-GFP* #2 lines as well as from F_1 plants obtained from crosses of *flm-3 pFLM:iFLM-β-GFP* #10 to Col-0 and *35S:FLM-δ* #4, respectively, that were grown for 15 days under long days at 16 °C. ChIP on *flm-3 pFLM:iFLM-β* #24, *flm-3 pFLM:iFLM-β-GFP* #10, *flm-3 pFLM:iFLM-δ* #17 and *flm-3 pFLM:iFLM-δ-GFP* #8 was performed on seedlings grown for 10 long days at 23 °C.

Two biological replicates were performed for all the ChIP assays except for the *flm-3 pFLM:gFLM* #2 and *flm-3 pFLM:gFLM-GFP* #2 lines, for which three biological replicates were assayed. An anti-GFP antibody (Abcam; ab290) was used for immunoprecipitation. DNA was fragmented and precipitated as previously described (Immink, 2012). The resulting immunoprecipitated DNA was tested for enrichment by qPCR using presumed FLM targets such as *SEP3* and *SOC1* and a negative control locus from *ARR7* using primers described in the Supplementary Table 5. Libraries for high throughput sequencing were prepared as previously described (Yant, 2010) and 40-bp single-end sequencing was performed on an Illumina GAIIx instrument following the manufacturer's instructions.

ChIP-seq analyses

ChIP-seq peak calling was performed as previously described (Moyroud, 2011) using the SHORE software version 0.8 (shore.sf.net). The 40-bp reads were filtered and trimmed using the command 'shore import' with filtering and trimming options '-c -n 10% -k 32'. For

mapping the reads to the TAIR10 reference sequence the 'shore mapflowcell' command was used using the GenomeMapper back end with a 10-mer reference sequence index. Alignment parameters were set as '-n 4–restrict = on', allowing for up to 4 base mismatches and no gaps. Peak calling was then performed on each pair of replicates using 'shore peak' with default peak calling parameters and option '-H 1,1' to exclude reads not assignable to a unique position on the reference sequence.

Matrix-based yeast two-hybrid assays

The pBD-GAL4 plasmids *pDP11* and *pDP15* were transformed into yeast strain PJ69-4A (mating type A (James et al., 1996)) and the pAD-GAL4 *pDP10* and *pDP14* vectors into yeast strain PJ69-4α (mating type α). Three individual colonies from the pBD-GAL4 transformations were suspended in 50 µl sterile MQ water and spotted in 5 µl aliquots on synthetic dropout medium lacking leucine and histidine and supplemented with 1, 3, 5 or 10 mM 3-amino-1,2,4-triazole. Growth of yeast and hence autoactivation, was scored after 5 days incubation at 20 °C, revealing that both FLM-β and FLM-δ do not have any autoactivation capacity. Subsequently, a matrix-based protein–protein interaction assay was performed as described previously (de Folter, 2005), in which FLM-β and FLM-δ were screened against the full collection of *Arabidopsis* MADS domain transcription factors (de Folter, 2005), extended by a collection of potential MADS domain protein isoforms (Severing, 2012) and the SVP protein encoded by the fully spliced *SVP* gene (van Dijk, 2010). After mating the diploid yeast was spotted onto selective medium lacking leucine, tryptophan and histidine, and supplemented with 1 or 5 mM 3-amino-1,2,4-triazole. Growth of yeast was scored after 5 days incubation on the selective media at 20 °C. Three technical replicates were performed.

EMSA assays

ChIP-seq data for FLM (this article) was used to determine the oligonucleotide sequences of the probes. Probes were labelled with 5'-biotin either by cloning into pGEM-T vector and using vector-specific biotinylated primers (in case of the *SEP3* probe) or directly by sequence-specific biotinylated primers (in case of *SOC1* and *ATC* probes).

The coding sequences of *FLM-β*, *FLM-δ* and *SVP* were amplified from cDNA and cloned into the pSPUTK expression vector. All generated constructs were confirmed by sequencing, and proteins were synthesized using TNT SP6 Quick Coupled Transcription/Translation System (Promega) according to the instructions of the manufacturer. To establish protein–protein interactions, protein synthesis was done in a single tube reaction for each protein complex. To ensure equal transcription/translation efficiency for each reaction in the titration experiments, total input of plasmid was kept equal by addition of pSPUTK expression vector containing a synthetically produced gene (GeneScript). This gene was designed by shuffling the *SVP* codon sequence, keeping the start codon at the first position and adding a premature stop codon after 456 bp to be able to distinguish the protein from SVP and FLM isoforms based on size.

The binding reaction was performed in a reaction mixture containing 1.2 mM EDTA, pH 8.0, 0.25 mg ml^{-1} BSA, 7.2 mM HEPES, pH 7.3, 0.7 mM dithiothreitol, 60 µg ml^{-1} salmon sperm DNA, 1.3 mM spermidine, 2.5% HAPS, 8% glycerol, 3.3 nmol ml^{-1} double-labelled double-stranded DNA (in case of SEP3) or 6.6 nm ml^{-1} single-labelled double-stranded DNA (in case of SOC1 and ATC), and 2 µl of *in-vitro*-synthesized proteins. Binding reaction was performed on ice for 45 min and loaded on a 5% polyacrylamide TBE gel. The gel was run at room temperature, followed by 2 h blotting to nylon membrane (Hybond-N+; Amersham, GE Healthcare Life Sciences). DNA shift was detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce) according to the instructions of the manufacturer.

SEP3 probe (pGEM-T sequence underlined):
5'CATGGCCGCGGGATTTTGACGATAACTCCATCTTTCTATTTTGGGTAACGAGGTCCCCTTCCC
ATTACGTCTTGACGTGGACCCTGTCCGTCTATTTTTAGCAGAATCACTAGTGCGGCCGC-3'; *SOC1*
probe: 5'-
CGCTTGAAACCTCATCCTTTACTTATTTTGGAAAAAAGCCTTAAGAAAGACCAAAAATAGCA
TATTTTGATACATATGGACATTTTTACATACACATC-3'; *ATC* probe: 5'-
TGGGTCGCCAACATTAACATTTCCAAAAATGGTAAGTCCAAGAATAATATTAGTTGTTTTGGG
ATATATTCTTTGCAATACATCC-3'; shuffled *SVP*: 5'-
ATGGAAGATAAATCGAAGTGTGCGGACACTAAGGAATTCGAAACGAGGATCGCGGTTGA
AATCGCCGCGCGGCAATCCGAATACTCCTCCAGTGCTATTGAGGCTCTCCATCAAAAGGAGAC
TAACAAGATGGGACTTAGCGTCTTGGCGCACCAGATCTCGGAATCTGAAAGCAGAA
AGTCGCACCAAGAAATTTTAGCACTTCTTAAGATGATTCTAGGATTCCAGCCTAAGA
TCATGAGCCTTCTATGCAAAGAGGTTAAAGACAGACAGCTTCAAGTTAAGCAAGGAACT
TCGGTGGAGGACACGGAAATCAACAAGCTATCTCGAAGGGGACGTGAGAGACTGCGATG
TCCACATCTTAGGGCTGGAGAGAATAAAGATGCTGAACAGCTCACCGAAGGTGGTTCC
GTGTTGACCGGAGAGATTGTCCTTATTAAGAACCGATTTGAAAGGTAGGAGATGT
CTCTCTTCAGCGCCAACAGTACAGAGAACCAGCTAGAACTTGATGGCAACATTATG
CGACAGAGACACTCCTATGAGTCATTAAAACAGAGGCCGACGAACCTTGGTCTCG
GTGCCGGTCAAGGGGATGACGTGCAGAACGCATCCAAGGAGGAGGACAACGGATTC
TTGAGAGAGTCTCTTGTGTTGGAGACGAGTAGCAGTAACATGAAGCTGGACCAGTTG
ATAGGAGACGAGGCTGAGATGCAAACGGCCGGC-3'.

BiFC analyses

The binary vectors were co-infiltrated into 3-week-old tobacco (*Nicotiana benthamiana*) leaves as previously described (de Felippes and Weigel, 2010). The absorbance (*A*) at 600 nm of the *Agrobacterium* cultures transformed with the split-mCitrine-tagged FLM and SVP constructs was adjusted to 0.25 (Fig. 2b and Extended Data Fig. 8d, e) and 0.3 (Extended Data Fig. 4) before the infiltration. To reduce false positive interactions, competition assays were performed by adding increasing concentrations of untagged version of FLM (*pJM356*, *35S:FLM-β*; *pLY225*, *35S:FLM-δ*) or SVP (*pDP58*, *35S:SVP*) to the infiltration. For the nuclear marker, *35S:NLS-mCherry* and the silencing suppressor *35S:p19*, an $A_{600 \, nm}$ of 0.1 was used.
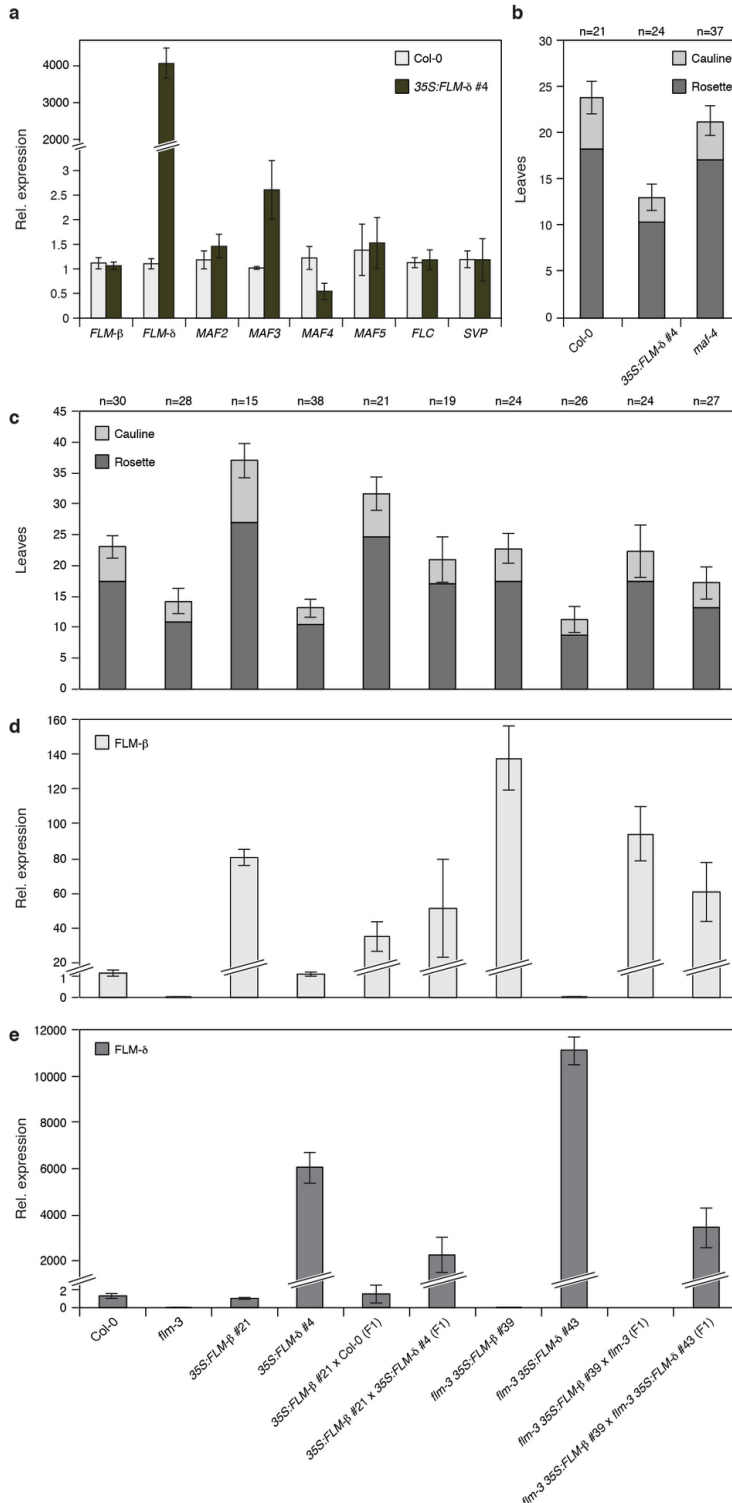
Extended data
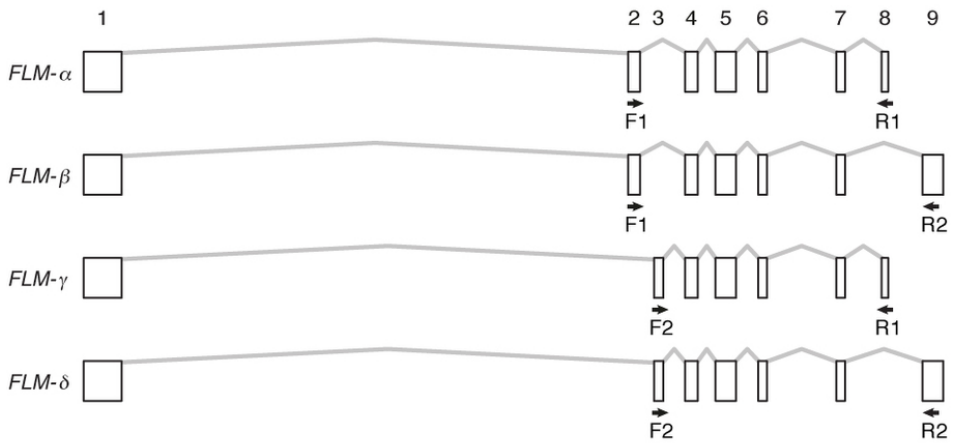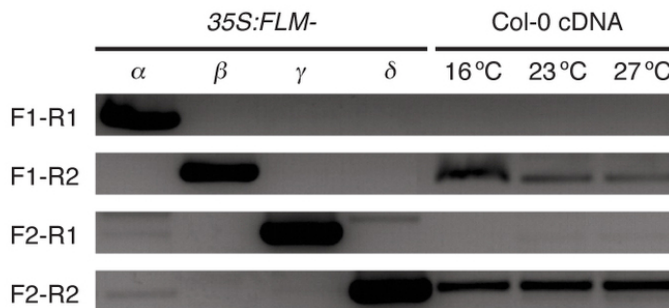
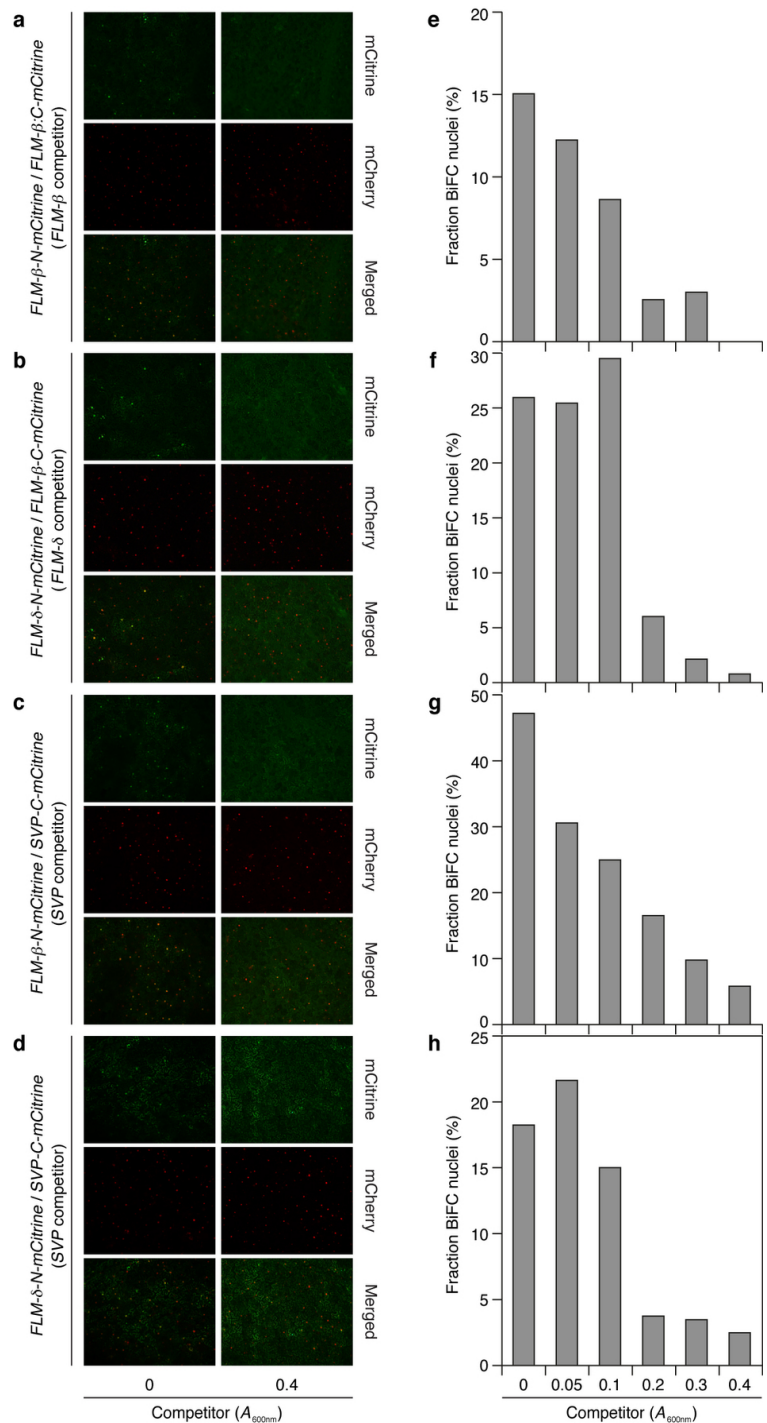**Extended Data Figure 1: Analysis of FLM splice variant expression in Col-0. a**, Graphic representation of the FLM-α, FLM-β, FLM-γ and FLM-δ transcripts, including exons (boxes) and introns (lines). Primers used for FLM-α (F1-R1), FLM-β (F1-R2), FLM-γ (F2-R1) and FLM-δ (F2-R2) amplification are shown. **b**, Semi-quantitative RT–PCR of FLM splice variants in Col-0 cDNA at different temperatures, using plasmids for each splice variant as controls (lanes 1–4).
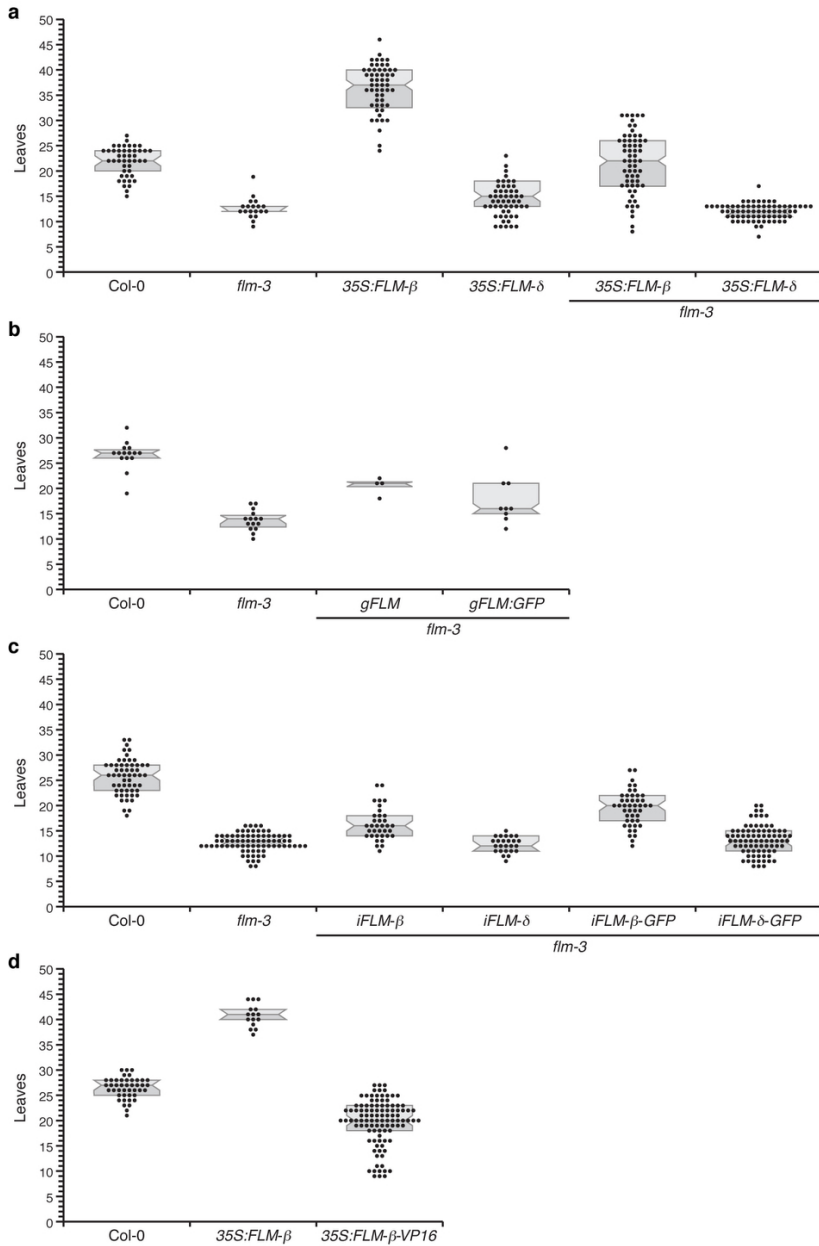
Extended Data Figure 2: Distribution of flowering time of independent transgenic T1 lines established in this study. a–d, Flowering time of 35S:FLM-β and 35S:FLM-δ in Col-0 and flm-3 mutant background **(a)**, flm-3 pFLM:gFLM and flm-3 pFLM:gFLM-GFP **(b)**, flm-3 pFLM:iFLM-β, flm-3 pFLM:iFLM-δ, flm-3 pFLM:iFLM-β-GFP and flm-3 pFLM:iFLM-δ-GFP **(c)** and 35S:FLM-β-VP16 **(d)**, grown under 16 °C, long-day are shown. Shaded areas mark the median and the 25% and 75% percentile of flowering time for a given genotype.

**Extended Data Figure 3: Analysis of *FLM* co-suppression in *35S:FLM-δ*.** **a**, Analysis of *FLM-β*, *FLM-δ*, *MAF2-5*, *FLC* and *SVP* in Col-0 control and *35S:FLM-δ* #4. All genes except *FLM-δ* and *MAF4* were expressed at similar levels in Col-0 and *FLM-δ* overexpression line. **b**, Flowering time of *maf-4* (SALK_028506) is similar to that of Col-0 control plants, indicating that the *MAF4* downregulation observed in **a** cannot explain the early flowering phenotype of the *35S:FLM-δ* line. **c–e**, Flowering time (**c**) and expression (**d**) of *FLM-β*, and expression of *FLM-δ* (**e**), as determined by qRT–PCR analysis in F_1 populations from crosses between *35S:FLM-β* and *35S:FLM-δ* plants in both Col-0 and *flm-3* backgrounds. **d**, *FLM-β* expression is not co-suppressed in response to the *FLM-δ* misexpression (**e**) in both Col-0 and *flm-3* backgrounds. Rosette and cauline leaf numbers after bolting are represented in dark and light grey, respectively, in **b** and **c**. Error bars denote the s.d. of three biological replicates with three technical repetitions each in **a**, **d** and **e**.

a FLM-β-N-mCitrine / FLM-β-C-mCitrine (FLM-β competitor)
mCitrine / mCherry / Merged

b FLM-δ-N-mCitrine / FLM-β-C-mCitrine (FLM-δ competitor)
mCitrine / mCherry / Merged

c FLM-β-N-mCitrine / SVP-C-mCitrine (SVP competitor)
mCitrine / mCherry / Merged

d FLM-δ-N-mCitrine / SVP-C-mCitrine (SVP competitor)
mCitrine / mCherry / Merged

Competitor (A_{600nm}): 0, 0.4

e Fraction BiFC nuclei (%)

f Fraction BiFC nuclei (%)

g Fraction BiFC nuclei (%)

h Fraction BiFC nuclei (%)

Competitor (A_{600nm}): 0, 0.05, 0.1, 0.2, 0.3, 0.4

Extended Data Figure 4: BiFC competition experiment. a–h, Microscope images (**a–d**) and quantification of mCitrine-positive nuclei (**e–h**). Increasing amounts (A600 nm; bottom) of an untagged version of one of the interactors tested were included in the assay. The number of BiFC-positive nuclei decreases with increasing amounts of the specific competitor: FLM-β–FLM-β homodimerization (**a, e**) and FLM-δ–FLM-β (**b, f**), SVP–FLM-β (**c, g**) and FLM-δ–SVP (**d, h**) heterodimerization.
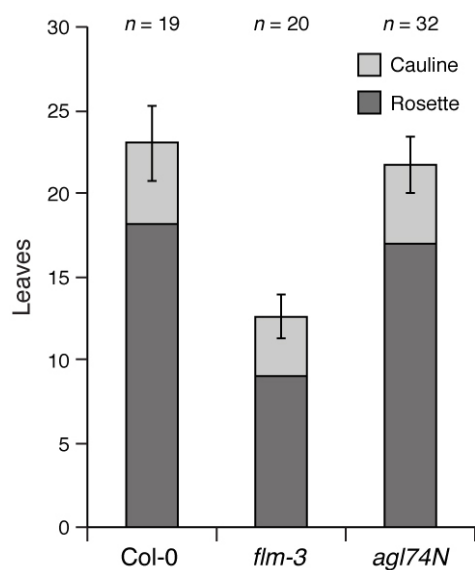
Extended Data Figure 5: Flowering time of the agl74N T-DNA mutant. Flowering time of Col-0, *flm-3*, and a homozygous *agl74N* T-DNA insertion line (SALK_016446).
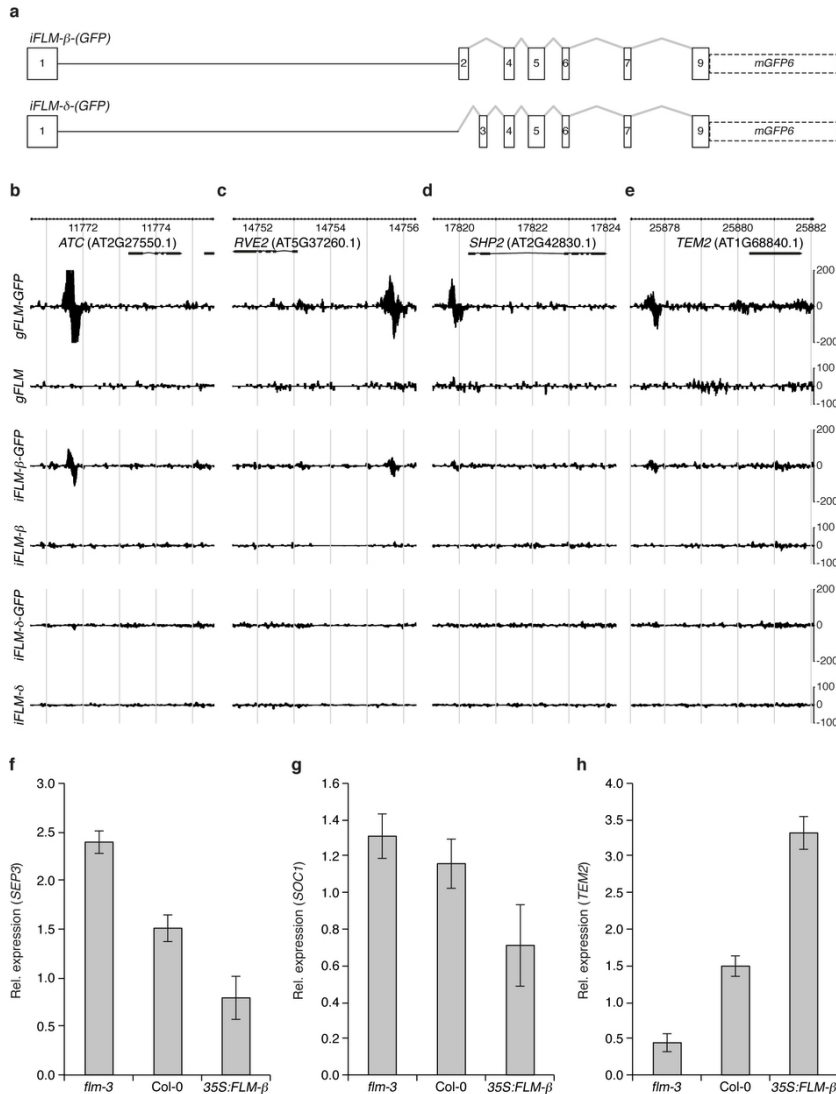
Extended Data Figure 6: Graphic representation of iFLM-β/δ-(GFP) constructs, gBrowse traces of mapped ChIP-seq reads and validation of FLM targets. a, iFLM-β/δ-(GFP) constructs representation including exons (boxes), introns included (black flat line) and introns missing (grey lines). Dashed boxes indicate presence only in the mGFP6-tagged constructs. **b–e**, Local enrichment of FLM, iFLM-β and iFLM-δ binding in ATC **(b)**, RVE2 **(c)**, SHP2 **(d)** and TEM2 (e). Chromosomal position (TAIR10) and models of the genes close to the peaks are given at the top of the panels. Each panel shows a 5-kb window. Forward reads are mapped above each line and reverse reads below. **f–h**, qRT–PCR expression analysis of SEP3 **(f)**, SOC1 **(g)** and TEM2 **(h)** in flm-3 mutant, Col-0 wild-type and a 35S:FLM-β transgenic line show how increasing levels of FLM-β downregulate SEP3 and SOC1 expression, but induce TEM2. Error bars in **f–h** denote the s.d. of three biological replicates with three technical repetitions each.
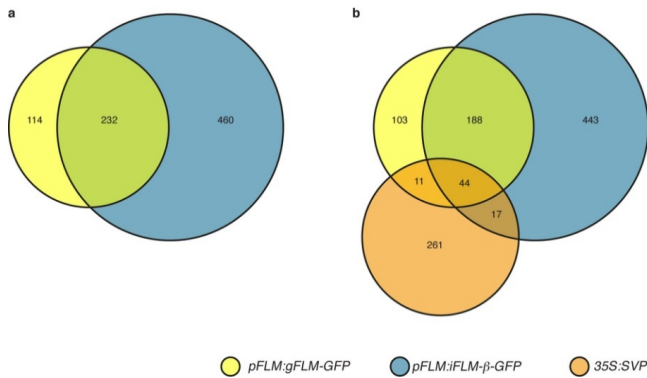
Extended Data Figure 7: Venn diagram showing the number and overlap of FLM and SVP targets. a, Overlap of loci bound in gFLM-GFP and iFLM-β-GFP ChIP-seq experiments with a false discovery rate (FDR) < 0.1 in all biological replicates. At this FDR, the high quality iFLM-β-GFP data set identifies 460 targets that are missing from the gFLM-GFP data set, which includes a replicate (#2) that contains substantial fewer uniquely mappable reads than the other replicates (see Supplementary Table 2). **b**, Overlap of loci bound in gFLM-GFP and iFLM-β-GFP ChIP-seq (FDR < 0.1) and SVP (FDR < 0.05) ChIP-chip assays (Tao, 2012).
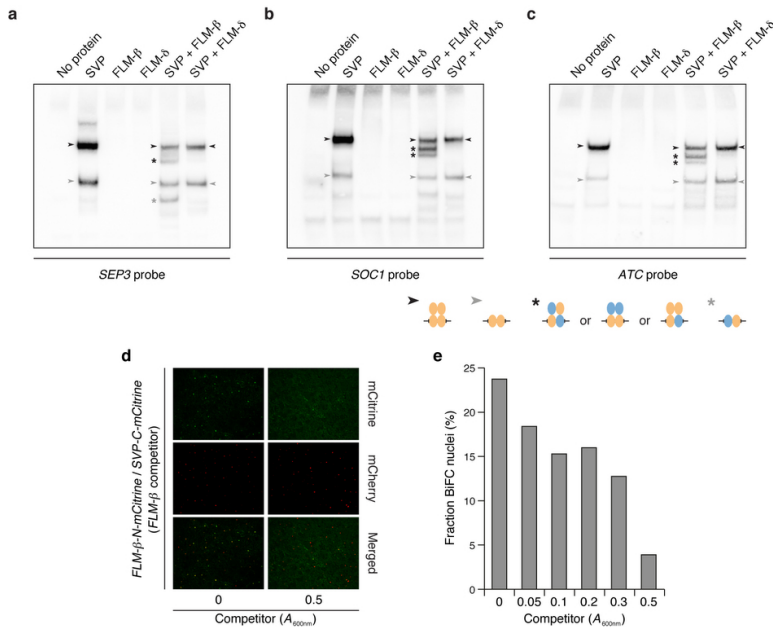
Extended Data Figure 8: EMSA assays and FLM-β/SVP BiFC competition experiment. a–c, EMSA assay with three sequences identified as binding-sites for SVP(Tao, 2012) and FLM (this work) by ChIP-chip and ChIP-seq, respectively. SEP3 **(a)**, SOC1 **(b)** and ATC **(c)** promoter probes that include two **(a, b)** or one **(c)** CArG motif(s) were used in EMSA. Different order complexes are represented by black arrowheads and asterisks for homo- or heterotetramers, respectively, and with grey arrowheads and asterisks for homo- or heterodimers, respectively. Orange and blue ellipses represent SVP and FLM-β proteins, respectively. **d, e**, Microscope images **(d)** and quantification **(e)** of mCitrine-positive nuclei. Increasing amounts (A600 nm; bottom) of untagged 35S:FLM-δ were added to FLM-β and SVP mCitrine-tagged vectors. A reduction in the number of BiFC-positive nuclei is observed with increasing amounts of competitor.
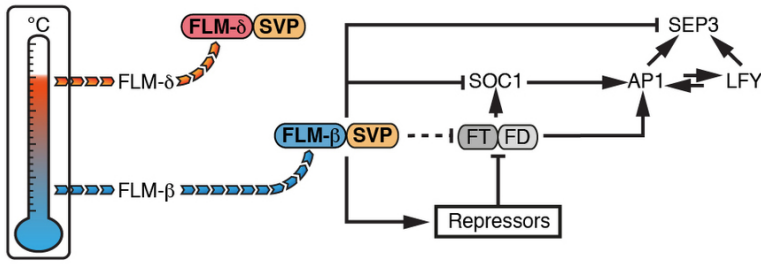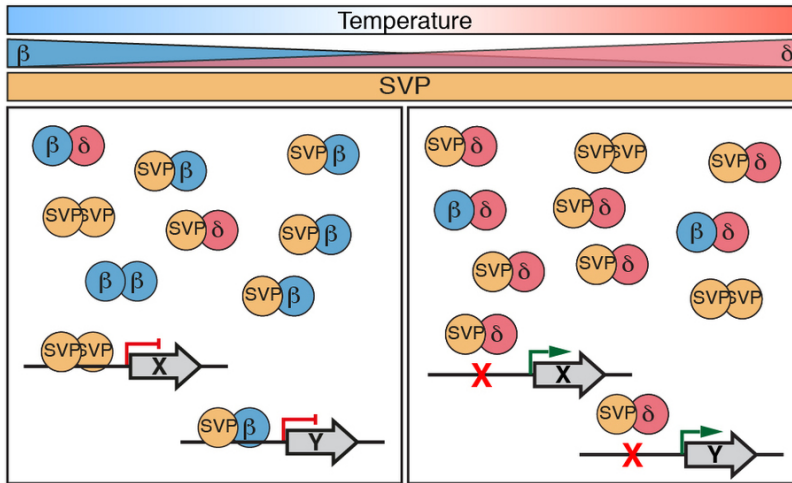
Extended Data Figure 9: Models of SVP–FLM complex function. a, Temperature-dependent FLM splicing and genetic interactions of SVP–FLM-β heterocomplex in the flowering pathway. Strong binding of FLM to *FT* was observed in only one ChIP-seq replicate. Hence we propose that FLM–SVP downregulates *FT* expression in leaves indirectly through the induction of floral repressors transcription factors such as *TEM2* and the AP2-like *TOE3*. The FLM–SVP complex contributes to the repression of floral transition by directly downregulating *SOC1* and *SEP3* expression, where SOC1 is a major floral activator. Arrows and block lines denote activation and repression, respectively. Dotted lines indicate a putative direct regulation. Rounded rectangles indicate proteins. **b**, Model of the temperature-dependent SVP–FLM complex function. Although *SVP* expression level is constant, *FLM-β* and *FLM-δ* levels are regulated in an antagonistic manner, with the former being the prevalent protein at low temperature and the latter dominating at high temperatures. At low temperatures SVP and FLM-β can interact, forming both homo- or heterocomplexes. The SVP-containing complexes are able to bind to the CArG boxes in the *cis* elements of important flowering related genes such as *SEP3*, *SOC1*, *ATC*, *TEM2* and *TOE3* and regulate their expression. When temperature increases, alternative splicing of *FLM* occurs, making *FLM-δ* the predominant splice variant. FLM-δ proteins compete with the remaining FLM-β and SVP proteins for complex formation. This results in the formation of non-functional SVP–FLM-δ complexes, which are impaired in their DNA-binding capability. The temperature-dependent splicing regulation of *FLM* occurs within 24 h, allowing the plant to quickly sense and respond to changes in ambient temperature, ensuring the switch between the non-flowering and flowering phase of development.

Extended Data Table 1: Mutants and transgenic lines used in this study.

Line	Source
flm-3	(ref. 3)
svp-32	(ref. 4)
svp-32/flm-3	this study
maf4	SALK_028506, B. Davies
agl74N	this study; SALK_016446
35S:FLM-β	this study
35S:FLM-δ	this study
flm-3 35S:FLM-β	this study
flm-3 35S:FLM-δ	this study
35S:SVP	P. Huijser (ref. 35)
svp-32 35S:FLM-β	this study
flm-3 35S:SVP	this study
flm-3 pFLM:gFLM	this study
flm-3 pFLM:gFLM-GFP	this study
flm-3 pFLM:FLM-β	this study
flm-3 pFLM:FLM-δ	this study
flm-3 pFLM:iFLM-β	this study
flm-3 pFLM:iFLM-δ	this study
flm-3 pFLM:iFLM-β-GFP	this study
flm-3 pFLM:iFLM-δ-GFP	this study
35S:FLM-β-VP16	this study

Supplementary data

Supplementary table 1

Yeast-two hybrid analysis of FLMβ and FLMδ interactions with a collection of Arabidopsis MADS domain transcription factors.

Gene Name	Gene Locus	MADS Lineage	FLM-β	FLM-δ
AGL23	AT1G65360	Type I	-	-
AGL28	AT1G01530	Type I	-	-
AGL29	AT2G34440	Type I	-	-
AGL34	AT5G26580	Type I	-	-
AGL35	AT5G26630	Type I	-	-
AGL36	AT5G26650	Type I	-	-
PHE1/AGL37	AT1G65330	Type I	-	-
AGL39	AT5G27130	Type I	-	-
AGL40	AT4G36590	Type I	-	-
AGL43	AT5G40220	Type I	-	-
AGL45	AT3G05860	Type I	-	-
AGL46	AT2G28700	Type I	-	-
AGL47	AT5G55690	Type I	-	-
AGL48	AT2G40210	Type I	-	-
AGL49	AT1G60040	Type I	-	-
AGL50	AT1G59810	Type I	-	-
AGL51	AT4G02235	Type I	-	-
AGL52	AT4G11250	Type I	-	-
AGL53	AT5G27070	Type I	-	-
AGL54	AT5G27090	Type I	-	-
AGL55	AT1G60920	Type I	-	-
AGL56	AT1G60880	Type I	-	-
AGL57	AT3G04100	Type I	-	-
AGL58	AT1G28450	Type I	-	-
AGL59	AT1G28460	Type I	-	-
DIANA/AGL61	AT2G24840	Type I	-	-
AGL62	AT5G60440	Type I	-	-
AGL73	AT5G38620	Type I	-	-
AGL74N	AT1G48150	Type I	++	++
AGL75	AT5G41200	Type I	-	-
AGL76	AT5G40120	Type I	-	-
AGL77	AT5G38740	Type I	-	-
AGL78	AT5G65330	Type I	-	-

AGL80	AT5G48670	Type I	-	-
AGL81	AT5G39750	Type I	-	-
AGL82	AT5G58890	Type I	-	-
AGL83	AT5G49490	Type I	-	-
AGL85	AT1G54760	Type I	-	-
AGL86	AT1G31630	Type I	-	-
AGL87	AT1G22590	Type I	-	-
AGL89	AT5G27580	Type I	-	-
AGL90	AT5G27960	Type I	-	-
AGL91	AT3G66656	Type I	-	-
AGL92	AT1G31640	Type I	-	-
AGL93	AT5G26950	Type I	-	-
AGL95	AT2G15660	Type I	-	-
AGL96	AT5G06500	Type I	-	-
AGL97	AT1G46408	Type I	-	-
AGL98	AT5G39810	Type I	-	-
AGL99	AT5G04640	Type I	-	-
AGL102	AT1G47760	Type I	-	-
AGL103	AT3G18650	Type I	-	-
AGL30	AT2G03060	Type II (MIKC*-P)	-	-
AGL65	AT1G18750	Type II (MIKC*-P)	-	-
AGL66	AT1G69540	Type II (MIKC*-P)	-	-
AGL94	AT1G77980	Type II (MIKC*-S)	-	-
AGL104	AT1G22130	Type II (MIKC*-S)	-	-
PI	AT5G20240	Type II (MIKC)	-	-
AP3	AT3G54340	Type II (MIKC)	-	-
ABS/AGL32	AT5G23260	Type II (MIKC)	-	-
GOA/AGL63	AT1G31140	Type II (MIKC)	-	-
FLC/AGL25	AT5G10140	Type II (MIKC)	-	-
MAF1/FLM-α/AGL27	AT1G77080.5	Type II (MIKC)	++	++
MAF1/FLM-β/AGL27	AT1G77080.4	Type II (MIKC)	++	++
MAF1/FLM-δ/AGL27	AT1G77080.2	Type II (MIKC)	++	-
MAF2/AGL31	AT5G65050	Type II (MIKC)	-	-
MAF3/AGL70	AT5G65060	Type II (MIKC)	-	-
MAF4/AGL69	AT5G65070	Type II (MIKC)	-	-
MAF5/AGL68	AT5G65080	Type II (MIKC)	-	-
SEP1/AGL2	AT5G15800	Type II (MIKC)	-	-
SEP2/AGL4	AT3G02310	Type II (MIKC)	-	-
SEP3/AGL9	AT1G24260	Type II (MIKC)	-	-
SEP4/AGL3(I)	AT2G03710	Type II (MIKC)	-	-
SEP4/AGL3(II)	AT2G03710	Type II (MIKC)	-	-
AGL13	AT3G61120	Type II (MIKC)	-	-
AGL6	AT2G45650	Type II (MIKC)	-	-

FUL/AGL8	AT5G60910	Type II (MIKC)	-	-
AP1/AGL7	AT1G69120	Type II (MIKC)	-	-
CAL/AGL10	AT1G26310	Type II (MIKC)	-	-
AGL79	AT3G30260	Type II (MIKC)	-	-
XAN/AGL12	AT1G71692	Type II (MIKC)	-	-
AGL14	AT4G11880	Type II (MIKC)	-	-
AGL19	AT4G22950	Type II (MIKC)	-	-
SOC1	AT2G45660	Type II (MIKC)	-	-
AGL71	AT5G51870	Type II (MIKC)	-	-
AGL72	AT5G51860	Type II (MIKC)	-	-
AGL42	AT5G62165	Type II (MIKC)	-	-
AGL16	AT3G57230	Type II (MIKC)	-	-
AGL17	AT2G22630	Type II (MIKC)	-	-
AGL21	AT4G37940	Type II (MIKC)	-	-
AGL44/ANR1	AT2G14210	Type II (MIKC)	-	-
AGL15	AT5G13790	Type II (MIKC)	-	-
AGL18	AT3G57390	Type II (MIKC)	-	-
AGL24	AT4G24540	Type II (MIKC)	-	-
SVP.1/AGL22	AT2G22540.1	Type II (MIKC)	+	+
SVP.2/AGL22*(1)	AT2G22540.X*(2)	Type II (MIKC)	-	-
SVP.3/AGL22*(1)	AT2G22540.2	Type II (MIKC)	-	-
AG	AT4G18960	Type II (MIKC)	-	-
STK/AGL11	AT4G09960	Type II (MIKC)	-	-
SHP1/AGL1	AT3G58780	Type II (MIKC)	-	-
SHP2/AGL5	AT2G42830	Type II (MIKC)	-	-

The matrix-based yeast two-hybrid was performed in three replicates and growth of yeast was scored after five days incubation on selective Synthetic Dropout

Notes: *(1) Numbering of SVP isoforms according to Severing et al, 2012.

*(2) The SVP2 splicing variant is due to retention of the 6th intron. This isoform is not present in TAIR

Supplementary Table 2: read numbers, peak number fdr<0.1 and flower related targets for pFLM:gFLM-GFP

Read numbers pFLM:gFLM-GFP

Biological Replicate	Sample	Raw reads	Reads aligned in *A. thaliana* genome	Unique reads (align in an unique position of the genome)	Unique and duplicate filtered reads
#1	*pFLM:gFLM* (negative ctrl)	35656497	26436356	22489310	2547569
	pFLM:gFLM-GFP	35762800	30462662	26737207	4223770
#2	*pFLM:gFLM* (negative ctrl)	33324597	1549082	1180735	479897
	pFLM:gFLM-GFP	32982912	939842	807157	634339
#3	*pFLM:gFLM* (negative ctrl)	30213334	20055716	16209965	5465299
	pFLM:gFLM-GFP	36719133	19176515	15959439	2530609

Peak number fdr<0.1 pFLM:gFLM-GFP

fdr<0.1 in all 3 replicates: 236	
genic:	60
intergenic, distance <3kb:	169
intergenic, distance >=3kb:	7
fdr<0.1 in at least 2 replicates: 785	
genic:	232
intergenic, distance <3kb:	534
intergenic, distance >=3kb:	18

Flower related targets pFLM:gFLM-GFP

Orp_rank	Up/Down AGI	Gene Model Name	Up/Down_dist	Up/Down_strands
3	AT1G24260	SEP3	2712	-
11	AT2G27550	ATC	1552	+
22	AT2G45660	SOC1	114	-
58	AT5G37260	RVE2	2599	-
60	AT3G54990	SMZ	2596	-
77	AT5G20240	PI	1573	+
90	AT3G54340	AP3	120	-
97	AT2G21070	FIO1	2875	+
115	AT2G42830	SHP2	360	+
146	AT5G67180	TOE3	1403	-
168	AT1G68840	TEM2	2654	+
179	AT5G11977	MIR156E	1499	+
180	AT4G36920	AP2	2835	+
203	AT2G28056	MIR172A	419	-
264	AT1G69120	AP1	86	-

Note: for the complete list with peak positions, we refer to Supplemental Table 2, which can be found online (http://www.nature.com/nature/journal/v503/n7476/abs/nature12633.html).

Supplementary Table 3: read numbers, peak number fdr<0.1 and flower related targets for pFLM:iFLM-β-GFP

Biological Replicate	Sample	Raw reads	Reads aligned in *A. thaliana* genome	Unique reads (align in an unique position of the genome)	Unique and duplicate filtered reads
#1	*pFLM:iFLM-β* (negative control)	43141125	24083839	19285143	5481775
	pFLM:iFLM-β-GFP	43449131	26512023	22004878	4993662
#2	*pFLM:iFLM-β* (negative control)	42639004	12440740	10220085	2903704
	pFLM:iFLM-β-GFP	42819711	20854327	17587095	4540194
#1	*pFLM:iFLM-δ* (negative control)	42290070	20025758	16228218	7254096
	pFLM:iFLM-δ-GFP	39792113	28484892	23010105	9854569
#2	*pFLM:iFLM-δ* (negative control)	40501286	31676681	26135833	5895535
	pFLM:iFLM-δ-GFP	41240737	27236798	22382053	4338323

Peak number fdr<0.1 for pFLM:iFLM-β-GFP

fdr<0.1 in *pFLM:iFLM-β-GFP*: 495	
genic:	201
intergenic, distance <3kb:	280
intergenic, distance >=3kb:	14
fdr<0.1 in *pFLM:iFLM-δ-GFP*: 309	
genic:	209
intergenic, distance <3kb:	87
intergenic, distance >=3kb:	13

Flower related targets for pFLM:iFLM-β-GFP

Orp_rank	Up/Down AGI	Gene Model Name	Up/Down_dist	Up/Down_strands
2	AT1G24260	SEP3	2720	-
7	AT2G27550	ATC	1546	+
27	AT2G45660	SOC1	117	-
47	AT5G20240	PI	1574	+
50	AT5G37260	RVE2	2603	-
57	AT2G42830	SHP2	369	+
75	AT1G68840	TEM2	2628	+
92	AT3G54990	SMZ	2615	-
95	AT5G11977	MIR156E	1500	+
102	AT3G54340	AP3	80	-
157	AT2G21070	FIO1	2854	+

Note: for the complete list with peak positions, we refer to Supplemental Table 3, which can be found online (http://www.nature.com/nature/journal/v503/n7476/abs/nature12633.html).

A file contains 232 common targets can be found online in Supplemental Table 4 at the above-mentioned location.

Supplemental Table 5

Oligonucleotides used in this study			
Gene (AGI)	Oligo	Sequence (5' -> 3')	Comment
Oligonucleotides used for cloning			
FLM-β/δ (At1g77080.4/.2)	G-2196	ATGGGAAGAAGAAAAATCGAG	589/577 bp
	G-1978	TAATTGAGCAGCGGGAGAGT	
gFLM (At1g77080)	G-26819	GCTTGGGATTTTAATAGAGAGCACGTGGTTATTATATC	6876 bp
	G-26820	ACCAAAACATGAAACAGAGTTCAAAAGCTG	
5'-*FLM-mGFP6*-3'	G-22798	CCAAACAGGAGAAATTGCTGAGAG	549 bp
	G-26831	GCCGCAGAACCGGATCCAGATCCAGATCCATTGAGCAGCGGGAGAG	
	G-26832	GGATCCGGTTCTGCGGCCGACAGTAAAGGAGAAGAAC	766 bp
	G-26334	GTTGATGATGGTGGTCAGTGGTGGTGGTGGTGGTGTTTG	
	G-26335	CAAACACCACCACCACCACCACTGACCACCATCATCAAC	565 bp
	G-26820	ACCAAAACATGAAACAGAGTTCAAAAGCTG	
FLM-β/δ-3'	G-29612	CCATGGGAAGAAGAAAAATCGAG	522/510 bp
	G-28155	TCATCTGTTGCCAGCAACGTA	
	G-26118	GCTGGCAACAGATGATGAGA	625 bp
	G-26820	ACCAAAACATGAAACAGAGTTCAAAAGCTG	
iFLM-β	G-29612	CCATGGGAAGAAGAAAAATCGAG	2711 bp
	G-30980	GGCTCTAAGTTCATCAGCATGTTG	
	G-28150	CATGCTGATGAACTTAGAGCCTTA	291 bp
	G-28154	CCAGCAACGTATTCTTTCCCAT	
iFLM-δ	G-29612	CCATGGGAAGAAGAAAAATCGAG	2666 bp
	G-30982	CGCTTCTATCCTGGAGATAAAACAAAAATGC	
	G-30981	TGTTTTATCTCCAGGATAGAAGCGCTGTTCAAG	327 bp
	G-28154	CCAGCAACGTATTCTTTCCCAT	
FLM (7th-9th *exon*)-*mGFP6-FLM* 3'	G-29345	GAACTGATGATGGAGTATATCG	208 bp
	G-26831	GCCGCAGAACCGGATCCAGATCCAGATCCATTGAGCAGCGGGAGAG	
	G-26832	GGATCCGGTTCTGCGGCCGACAGTAAAGGAGAAGAAC	1293 bp
	G-26820	ACCAAAACATGAAACAGAGTTCAAAAGCTG	
	G-29345	GAACTGATGATGGAGTATATCG	1485 bp

	G-26820	ACCAAAACATGAAACAGAGTTCAAAAGCTG	
FLM-β/δ Δstop	G-2196	ATGGGAAGAAGAAAAATCGAG	588/576 bp
	G-31569	GATTGAGCAGCGGGAGAGTC	
SVP	G-28863	ATGGCGAGAGAAAAGATTCAG	722 bp
	G-28864	CTAACCACCATACGGTAAGCC	
SVP Δstop	G-28863	ATGGCGAGAGAAAAGATTCAG	720 bp
	G-31886	GACCACCATACGGTAAGCC	
Oligonucleotides used for genotyping			
FLM (At1g77080)	G-12063	CGGAGAAACCTCAATGTTTTG	
	G-12064	GGTTTTGTGGAGTAATTGGTTG	
flm-3 (At1g77080)	G-17720	ATTTTGCCGATTTCGGAAC	
	G-12064	GGTTTTGTGGAGTAATTGGTTG	
SVP (At2g22540)	G-28720	TCATCCATATCTTGCAATGCC	
	G-28721	TCAGCGAACTTCAGAAAAAGG	
svp-32 (At2g22540)	G-17720	ATTTTGCCGATTTCGGAAC	
	G-28721	TCAGCGAACTTCAGAAAAAGG	
AGL74N (At1g48150)	G-32372	GAAGAAGACCCACTGTCGTTG	
	G-32373	TTCTCATGCATTGATCCTTCC	
agl74N (At1g48150)	G-17720	ATTTTGCCGATTTCGGAAC	
	G-32373	TTCTCATGCATTGATCCTTCC	
Oligonucleotides used for semiquantitative RT-PCRs			
FLM-α (At1g.77080.5)	G-28150 (F1)	CATGCTGATGAACTTAGAGCCTTAGATC	300 bp
	G-28153 (R1)	ATCGGTGAGACATACCTTTCTTC	
FLM-γ (At1g.77080.x)	G-30796 (F2)	GATAGAAGCGCTGTTCAAGC	322 bp
	G-28153 (R1)	ATCGGTGAGACATACCTTTCTTC	
Oligonucleotides used for qRT-PCRs			
β-TUB2 (At5g62690)	N-0078	GAGCCTTACAACGCTACTCTGTCTGTC	180 bp
	N-0079	ACACCAGACATAGTAGCAGAAATCAAG	E=2.024
FLM-β (At1g77080.4)	G-28150 (F1)	CATGCTGATGAACTTAGAGCCTTAGATC	290 bp
	G-28156 (R2)	CAGCAACGTATTCTTTCCCAT	E=2.006
FLM-δ (At1g77080.2)	G-30796 (F2)	GATAGAAGCGCTGTTCAAGC	312 bp
	G-28156 (R2)	CAGCAACGTATTCTTTCCCAT	E=1.998
SVP (At2g22540)	G-20863	CAAGGACTTGACATTGAAGAGCTTCA	103 bp
	G-20864	CTGATCTCACTCATAATCTTGTCAC	E=2.05

			4
SOC1 (At2g45660)	G-30998	AAACGAGAAGCTCTCTGAAAAG	142 bp
	G-30999	AAGAACAAGGTAACCCAATGAAC	E=2.01
SEP3 (At1g24260)	G-0648	GGGTATCAGATGCCACTCCAGCTGAAC	196 bp
	G-0649	AACCCAACATGTAATTATTCACACTTG	E=1.98
TEM2 (At1g68840)	G-22652	GACTAGAGCGGCAGTTATATATTGAT	148 bp
	G-22653	CTTTCCACCGCAAACGGCCA	E=2.034
FLC (At5g10140)	G-0632	CTTGTGGGATCAAATGTCAAAAATGTG	219 bp
	G-0633	CATCTCAGCTTCTGCTCCCACATGATG	E=2.028
MAF2 (At5g65050)	G-35480	GGCACCAGCCTTATCGGAGG	265 bp
	G-2353	GTAACGATCAATGATCTTTGACATGTTG	E=2.064
MAF3 (At5g65060)	G-35481	GAAGAAAAAAAGCAAACACATTTTGGGTCC	610-786 bp
	G-35482	AAGAACTCTGATATTTGTCTACTAAGGTAC	E=1.996
MAF4 (At5g65070)	G-35483	ATTAGGTCAGAAGAATTAGTCGGAGAAAAC	765 bp
	G-35484	CTTGGATGACTTTTCCGTAGCAGGGGGAAG	E=2.072
MAF5 (At5g65080)	G-35485	CACACAAGGAGTTACTAGAAATAGTTCAAAGAAAGA	264 bp
	G-35486	TTACTGCTCTTTCATCCCCTGTTTCC	E=1.998
Oligonucleotides used for ChIP validation			
ARR7 (At1g19050)	G-4417	GGCTGTTGTCCTGGTATTATTTCTC	130 bp
	G-15952	GAGGACTAAGGCAATAGTACATGTT	E=1.974
SEP3 (At1g24260)	G-31798	TTTGAGGCAATGTCGTGAAG	102 bp
	G-31799	CCCTTCCCATTACGTCTTGA	E=1.976
SOC1 (At2g45660)	G-31800	ATGATGGACGCTTGAAACCT	149 bp
	G-31801	GACAGGCATTTCCATCCAAC	E=1.918

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Chapter 5

Plasticity versus Adaptation of Ambient–Temperature Flowering Response

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It is challenging to understand how plants adapt flowering time to novel environmental conditions, such as global warming, while maintaining plasticity in response to daily fluctuating temperatures. A recent study shows a role for transposons and highlights the need to investigate how these different responses evolved.

Plants optimize their reproductive success adjusting flowering time to environmental conditions, such as day length and temperature. Historically, scientists have focussed their flowering time studies on understanding the role of the photoperiod (day length) and vernalization (prolonged exposure to winter cold). But lately, in light of the climate change, investigation of the ambient temperature response came into the spotlight. These efforts resulted in major breakthroughs in our understanding of the molecular mechanisms underlying ambient temperature-dependent flowering in Arabidopsis. *SHORT VEGETATIVE PHASE* (*SVP*) and the *FLOWERING LOCUS C (FLC)* clade MADS-box genes – *FLOWERING LOCUS M (FLM)*, and to a lesser extend *FLC, MADS AFFECTING FLOWERING2 (MAF2), MAF3, MAF4 and MAF5* – have shown to be key players (Gu, 2013). At the molecular level, the response appears to be mediated by temperature-dependent degradation of SVP (Lee et al., 2013) and alternative splicing of *FLM* (Airoldi et al., 2015; Posé et al., 2013) and *MAF2*. These post-transcriptional mechanisms provide plants with the essential flowering time plasticity in response to transient ambient temperature fluctuations. However it remains unknown, whether these molecular mechanisms are conserved in other plant species or in Arabidopsis accessions other than Col-0. This interesting and important research question has now been addressed by Lutz and colleagues (Lutz et al., 2015).

To interpret the novel findings and postulated hypothesis of this study, it is essential to distinguish between thermoplasticity within a plants life cycle and long-term adaptation to different ambient temperature environments over thousands of years. Arabidopsis Col-0 shows a high thermoplasticity: it accelerates flowering upon a transient increase in ambient temperature and this response can be quantified as the ratio in number of days to bolting at low ambient temperatures over the number of days to bolting at high ambient temperatures. For example, when a plant would not adapt flowering time under different ambient temperatures, this ratio would be 1, whereas for a plant that accelerates flowering in higher ambient temperatures, this ratio is higher than 1. For Arabidopsis Col-0, this ratio is much higher than 1, while the *flm-3* null mutant shows a highly attenuated response with a ratio close to 1, indicating lack of thermoplasticity (Balasubramanian et al., 2006) and (Severing, 2012). A ratio of 1 is observed also for Cvi-0 from Cape Verde island (Balasubramanian et al., 2006). This Arabidopsis accession grows at relative constant temperatures throughout the year and therefore may have lost its thermoplasticity. In other cases of adaptation to a changed environment, it is also possible that a population adapts by becoming either early or late flowering, but keeps its thermoplasticity. All these different short and long-term ambient temperature adaptation scenarios are summarized in Figure 1A.

(A)

(B)
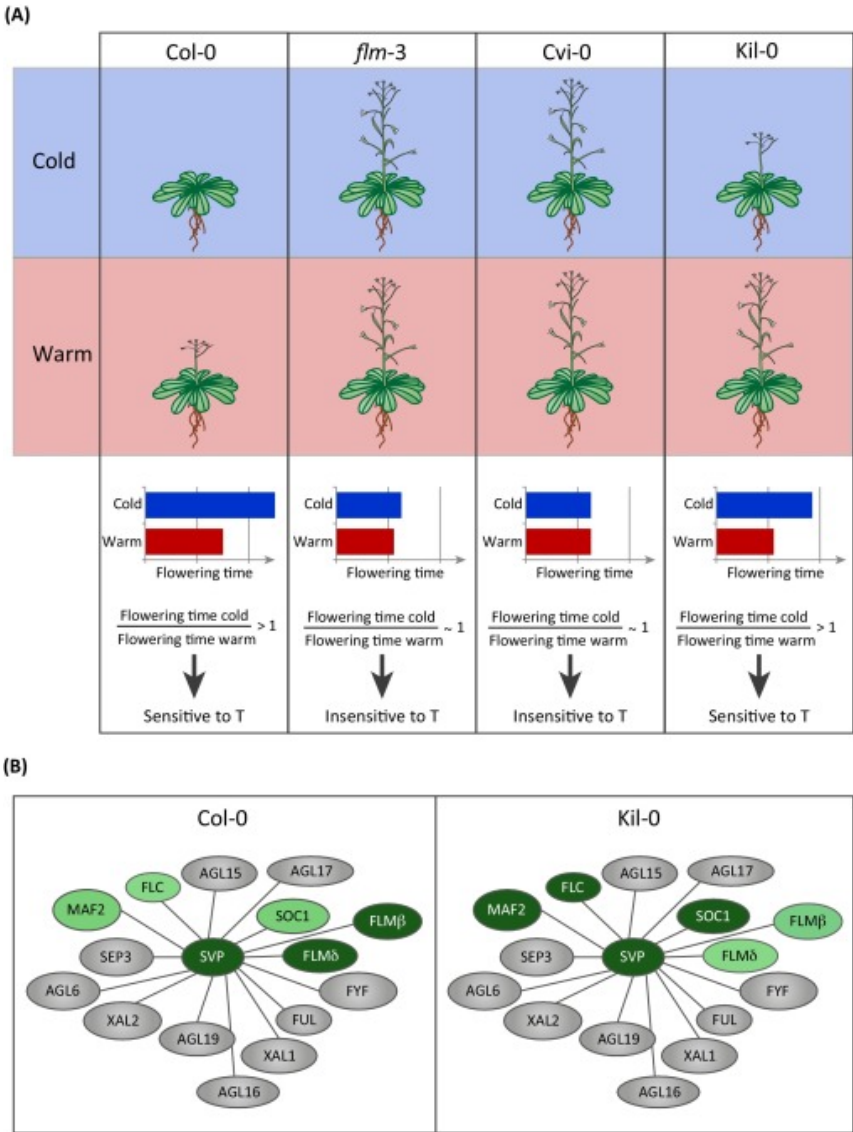
Trends in Plant Science

Figure 1. Long- and Short-Term (Plasticity) Adaptation of Flowering Time to Changing Ambient Temperature Conditions and a Possible Underlying Molecular Mechanism. (A) Flowering time of Arabidopsis accessions (Col-0, Cvi-0, and Kil-0) and the flowering locus m (flm-3) mutant under cold and warm ambient temperatures. Ambient temperature mediated plasticity in flowering is quantified by the flowering time ration at low vs. high ambient temperatures. Both Kil-0 and Cvi-0 adapted to local temperature conditions and are early flowering in comparison to Col-0, but these two accessions differ substantially in their ability to respond to transient temperature changes within a life-cycle, which we refer to as thermoplasticity. **(B)** Part of the interactome of the MADS-domain transcription factor SHORT VEGETATIVE PROTEIN (SVP) (van Dijk, 2010). Protein–protein interactions that have shown to be involved in the ambient temperature flowering time response in the Col-0

accession are marked in green. Interacting proteins that are supposed to be the most important related to this function are colored dark green. In Kil-0, FLOWERING LOCUS M (FLM) isoforms are strongly reduced, probably changing the balance in the SVP network. Hypothetical new key interactors of SVP in relation to thermoplasticity are marked in dark green. Light green colored proteins interact with SVP, but are supposed to have less impact on ambient temperature-mediated flowering time control.

In the recent study by Lutz and colleagues, the authors search for accessions with an attenuated ambient temperature flowering time response and identified the accession Kil-0 from Scotland as early flowering. Plants from this accession bolt two weeks earlier than Col-0 at 15 °C and 1 week earlier at 21 °C. Although this accession has clearly adapted its flowering time to the Scottish climate, its thermoplasticity is hardly affected with a ratio in days to bolting at low compared to high temperatures of 2.19, reflecting a decrease in thermoplasticity of ~3.5% in comparison to Col-0 (ratio of 2.27). Hence, Kil-0 shows a clear altered ambient flowering time response, but only a mild altered thermoplasticity (Figure 1A). Thorough investigation of the cause of the attenuated flowering time response in Kil-0 revealed the presence of a LINE transposon into the first intron of the FLM locus. FLM encodes different transcript isoforms and in Col-0, FLMβ is the predominant transcript at low ambient temperatures. The encoded FLMβ protein forms a complex with SVP, which represses key flowering integrator genes. When temperatures rise, the FLMδ transcript accumulates at the expense of FLMβ and SVP protein levels drop. The decreased amount of SVP and the inability of the FLMδ–SVP complex to bind DNA release the floral integrators from their transcriptional repressed state and thereby accelerates flowering. RNA-seq experiments in Kil-0 revealed that the LINE transposon insertion causes a 6-fold reduction in FLMβ transcript and a 27-fold decrease in FLMδ. Still, upon changing ambient temperatures, FLMδ transcript levels increased and FLMβ decreased with relative similar dynamics as in Col-0. However, due to the very strong reduction in absolute amount of FLMδ transcript, this increase upon rising temperatures has most likely not any biological effect. Consequently, and as suggested by the authors, FLMβ becomes the major player in this situation; but how then to explain the almost similar thermoplasticity between Col-0 and Kil-0? In this respect it is important to keep in mind that Kil-0, with strong reduction in FLM, might have evolved additional compensatory mutations and therefore can act different from the flm-3 mutant, which is a single mutation in the Col-0 background lacking thermoplasticity (Figure 1A). FLM isoforms act in a network of which SVP is a central component, because it forms complexes with both FLMβ and FLMδ (Severing, 2012). Besides that, SVP interacts with other ambient temperature flowering time regulators, such as SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), FLC, and a MAF2 isoform 1, 4 and 7. In Kil-0, reduction in FLM isoforms may be compensated by increased levels of other SVP interactors and establishment of a new balance in this complex interaction network (Figure 1B). However, it can also not be ruled out that thermoplasticity is taken care of by other genes in Kil-0, or by pathways acting in parallel. Depending on the day length conditions, PHYTOCHROME INTERACTING FACTOR4

(PIF4) has been suggested to play a role (Kumar et al., 2012). However, its importance in relation to ambient temperature mediated flowering time regulation has recently been questioned and the plant hormone gibberellic acid was put forward as possible alternative mediator (Galvão et al., 2015). Considering this complexity, it is currently impossible to point to one single, most likely cause of flowering plasticity in Kil-0.

Concluding Remarks

Kil-0 adapted to the Scottish climate by a strong reduction in FLM isoforms due to a transposon insertion, but the molecular mechanism that regulates thermoplasticity in this accession still needs further investigation. In general, it is definitely too early to conclude that temperature dependent alternative splicing of FLM and, especially, the concomitant increased production of FLMδ at higher ambient temperatures, is of less importance for the ambient temperature flowering time response. The current climate change goes along with two main temperature effects: global warming and stronger temperature fluctuations with more extreme outliers. Plants need to adapt to both situations, occurring at different time scales. Alternative splicing of FLM is a fast, transient, and reversible process, and therefore represents an ideal mechanism for thermoplasticity in the timespan of a plant's life cycle. In contrast, the reported LINE transposon insertion in Kil-0 and nine other accessions provide these plants with a more drastic way to adapt their flowering time to long-term temperature effects. The work done by Lutz and colleagues is a first step towards the understanding of the way the ambient temperature flowering time response has evolved. Though, more studies are needed to reveal whether underlying molecular mechanisms identified in Col-0 are conserved and play a role in other Arabidopsis accession and most importantly in main food and feed crops.

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Chapter 6

A putative role for Polymerase II elongation rate in alternative splicing of *FLM*

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Abstract

In the ambient temperature-directed flowering time pathway, thermosensitive alternative splicing of *FLM* is a key mechanism. The mode of action of the differential splice forms was recently unravelled. However, how differential splicing of *FLM* at different ambient temperatures is established, has remained completely enigmatic. In this study, we investigated the possibility that splicing is altered by the direct effect of temperature on transcription elongation rate. We mimicked a situation in which *FLM* is transcribed at a higher rate, by expressing the genomic *FLM* gene under a strong artificial promoter. Preliminary results show altered splicing ratios and altered flowering time in transgenic plants harbouring the construct. These results give a first insight into the possible regulatory mechanism behind temperature dependent alternative splicing.

Introduction

We unravelled the mechanism by which *FLM* controls temperature-directed floral timing through alternative splicing (Chapter 4, (Posé et al., 2013)). However, how temperature is able to influence alternative splicing of *FLM* remains completely enigmatic.
An important factor in splicing regulation is the fact that splicing is not an isolated process, but is actually coupled to transcription, and occurs when the transcript is in contact with RNA polII. Due to "kinetic coupling" the splicing outcome is dependent on the elongation rate of RNA polII. The idea behind this is that when two splice sites compete, the event more upstream will have an advantage over the more downstream event because the upstream position is earlier reached by RNA polII. This advantage for the upstream event is bigger when RNA polII speed is high, and smaller when RNA polII speed is low (as reviewed by (Saldi et al., 2016)). Since temperature is well known to influence enzyme-catalysed reactions (Chapter 2), it is a conceivable idea that fluctuating ambient temperature can influence RNA polII speed, and consequently alter alternative splicing. In 2005, Abbondanzieri and colleagues performed *in vitro* experiments using optical trapping to characterize transcription at the single-molecule level at different temperatures (Abbondanzieri et al., 2005). Using *E. coli*-derived RNA polymerase and temperatures in the ambient range for *e. coli*, they showed a significant change by a factor of more than two when temperature increased from 23° C to 34° C. More recently, it was also shown that *in vivo*, alterations in growth temperature change RNA polII elongation speed (Miguel et al., 2013). In this study, performed in the yeast *Saccharomyces cerevisiae*, the authors showed that within an ambient temperature growth range of yeast (23° C-37° C), RNA polII speed increased linearly with temperature with about 0.5 kb/min every 10° C.

To find out whether an altered transcription speed can affect alternative splicing of *FLM*, we mimicked a situation in which *FLM* is transcribed at a higher rate, by expressing the genomic *FLM* gene under a strong artificial promoter. Strong promoters exhibit high transcription initiation rates, due to high efficiency of recognition of the promoter sequence by RNA polII. As a result of the cooperation between RNA polymerase molecules moving one after

another on the same DNA molecule, a higher transcription initiation rate – an thus more polymerase molecules on the DNA – results in a higher elongation rate (Epshtein and Nudler, 2003).

Introduction of an *FLM* construct under a strong promoter by agrobacterium-mediated plant transformation involves random introduction in the chromatin. Since the surrounding chromatin might also affect alternative splicing (as described in Chapter 1 and 2), we decided to compare the effect of introducing the *FLM* gene under control of a strong artificial promoter and its native promoter. Preliminary results show that random introduction of *FLM* under its own promoter in an *flm-3* mutant background does not influence alternative splicing, and flowering time is complemented in a dose-dependent manner. Introduction of *FLM* under a strong promoter however, altered the *FLMβ*/*FLMδ* splicing ratio significantly, and caused an early flowering phenotype due to relatively increased *FLMδ* abundance. These results give a first insight into how different ambient temperatures may cause differential splicing of *FLM*, and consequently, adjust floral timing.

Results

Random introduction of FLM in the chromatin leads to a range of flowering time phenotypes

We cloned the genomic *FLM* sequence including the intergenic regions up- and downstream of the gene to assure all *cis*-regulatory regions were included (Figure 1A), and introduced the sequence in the *flm-3* knockout mutant background (Balasubramanian et al., 2006), using a binary vector carrying the phosphinothricin (PPT) resistance gene (*bar*) as a selective marker. After selecting and growing transgenic T1 plants, T2 plants were tested for a mendelian 1:3 segregation ratio of the *bar* gene to eliminate multi-chromosomal insertion lines. Subsequently, eight correctly segregating T2 lines were selected and re-sown for flowering time experiments, together with the *flm-3* mutant and Col-0 wild type (WT) to compare the degree of complementation for each transgenic line. Seeds were germinated and grown under long day (LD) conditions at a constant temperature of 21° C until the moment of flowering.
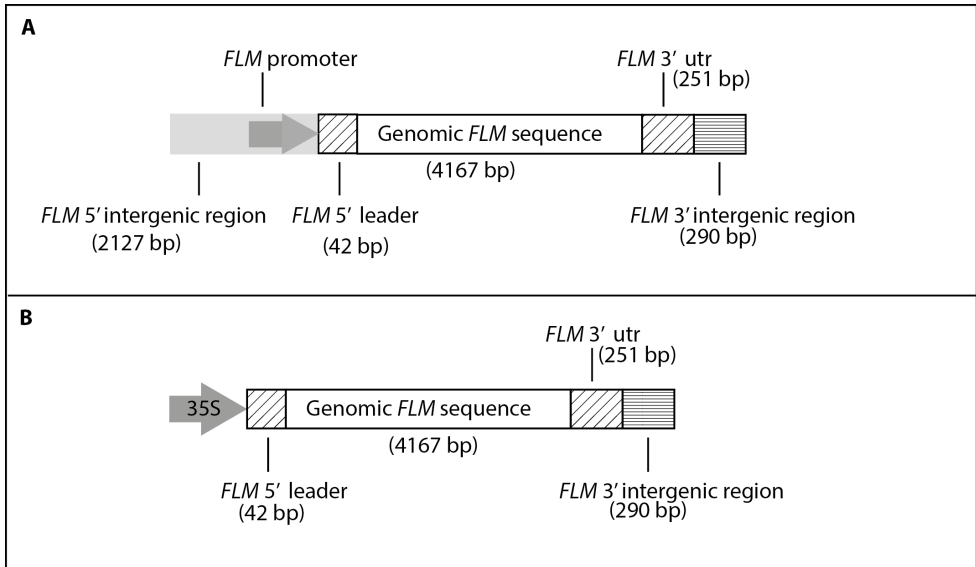
Figure 1: Constructs used in this study. (A) Genomic *FLM* sequence under the native promoter, including the 5' and 3' intergenic region and the 5' leader sequence. **(B)** Genomic *FLM* sequence under the *35S* promoter, including the *FLM* 5' leader and the 3' intergenic region.

Flowering time was scored as Days to Flowering (DtF) and Rosette Leaves Number (RLN). The two screening methods gave similar results. Figures 2 (RLN) and S1 (DtF) show the population distribution graphs for each of the transgenic lines. Several lines showed a clear distribution of flowering time in three segregating groups, suggesting a dose effect of the transgene due to segregation of the T2 population. When we projected the flowering time of *flm-3* mutant and Col-0 wild type (WT) (Figure S2) onto the flowering time distribution graphs of each line, it became clear that the transgenic lines demonstrated a range of flowering times, including early (lines 46, 63 and 74), mid (lines 43 and 45) and late (lines 18, 20 and 72) flowering, when compared to WT.
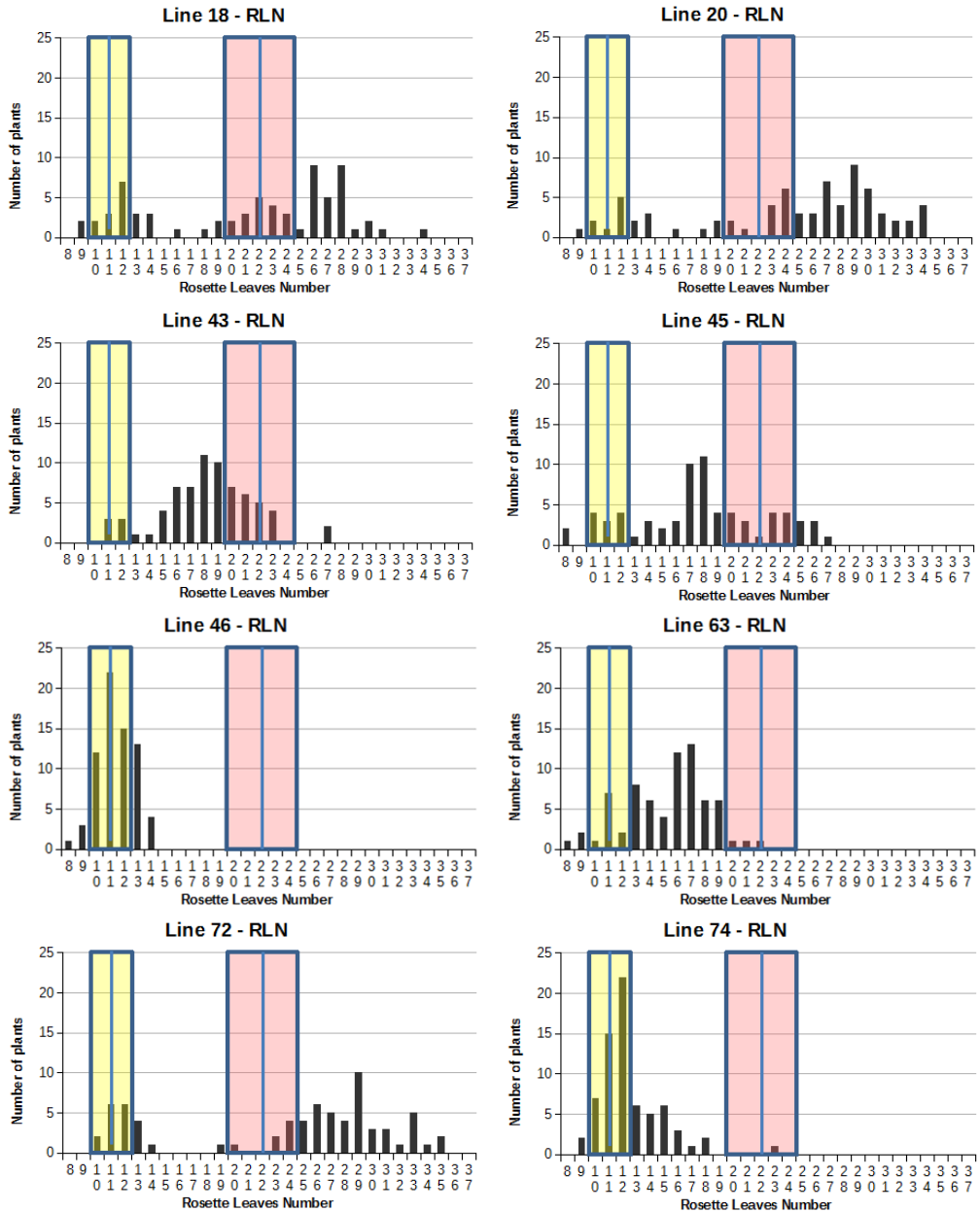
Figure 2: Flowering time distribution of T2 lines with randomly inserted genomic *FLM* expressed from its endogenous promoter. Eight T2 lines that showed 1:3 segregation for the inserted transgene in the *flm-3* knockout background were tested for flowering time under constant temperature (21° C) and LD (16h light/8h dark) conditions. Flowering time was measured in Rosette Leaves Number (RLN). Mean flowering time and standard deviation of the *flm-3* knockout and the Col-0 wild type (WT) was projected on each graph with a blue line (mean) and yellow and red shaded boxes (SD) respectively. The lines showed a range of flowering times, including early (line 46, 63, 74), mid (line 43, 45) and late (line 18, 20, 72) flowering time. Note: since lines are segregating 1:3, the

effect of the transgene on flowering time is best assessed by comparing the latest ¼ of plants (putative homozygous) to WT and *flm-3* flowering time.

In order to explain the observed flowering time phenotypes, we analysed expression of *FLMβ* and *FLMδ* by performing RT-qPCR on two of the early flowering lines (line 46 and 74). We generated homozygous T3 lines and selected individual plants for expression analysis. For line 46, which was the most early line, flowering at the same time as the *flm-3* mutant, *FLM* expression turned out to be extremely low (Figure 4A), indicating that the early flowering phenotype was due to unsuccessful complementation. For line 74, a mid-early flowering line, total expression of *FLM* was close to WT expression in one sample, and slightly (but significant) lower in the other sample (Figure 4A). In both cases, the ratio between *FLMβ* and *FLMδ* was not significantly different from WT (Figure 4B). Hence, the early flowering time might be due to slightly lower total expression of *FLM*.

Introduction of FLM under a strong constitutive promoter induces early flowering

To investigate the effect of transcription initiation speed on alternative splicing, we cloned *FLM* under the Cauliflower mosaic virus (CaMV) *35S* promoter (Odell et al., 1985). Primers were designed to clone the genomic *FLM* sequence starting with the 5' leader sequence and ending with the 3' intergenic region until the UTR of the downstream gene (Figure 1B). After successful transformation in the *flm-3* mutant background, T2 plants were tested for 1:3 segregation and positive lines were re-sown for flowering time examination. Flowering time was screened by RLN and DtF, as explained above. In contrast to the lines expressing *FLM* under the native promotor, flowering time variation due to segregation of the transgene was not clearly visible, indicating that dose effect plays a smaller role in these lines. Moreover, seven out of eight lines showed a clear early to mid-early flowering phenotype, both in RLN and DtF (Figure 3, Supplemental Figure S3).
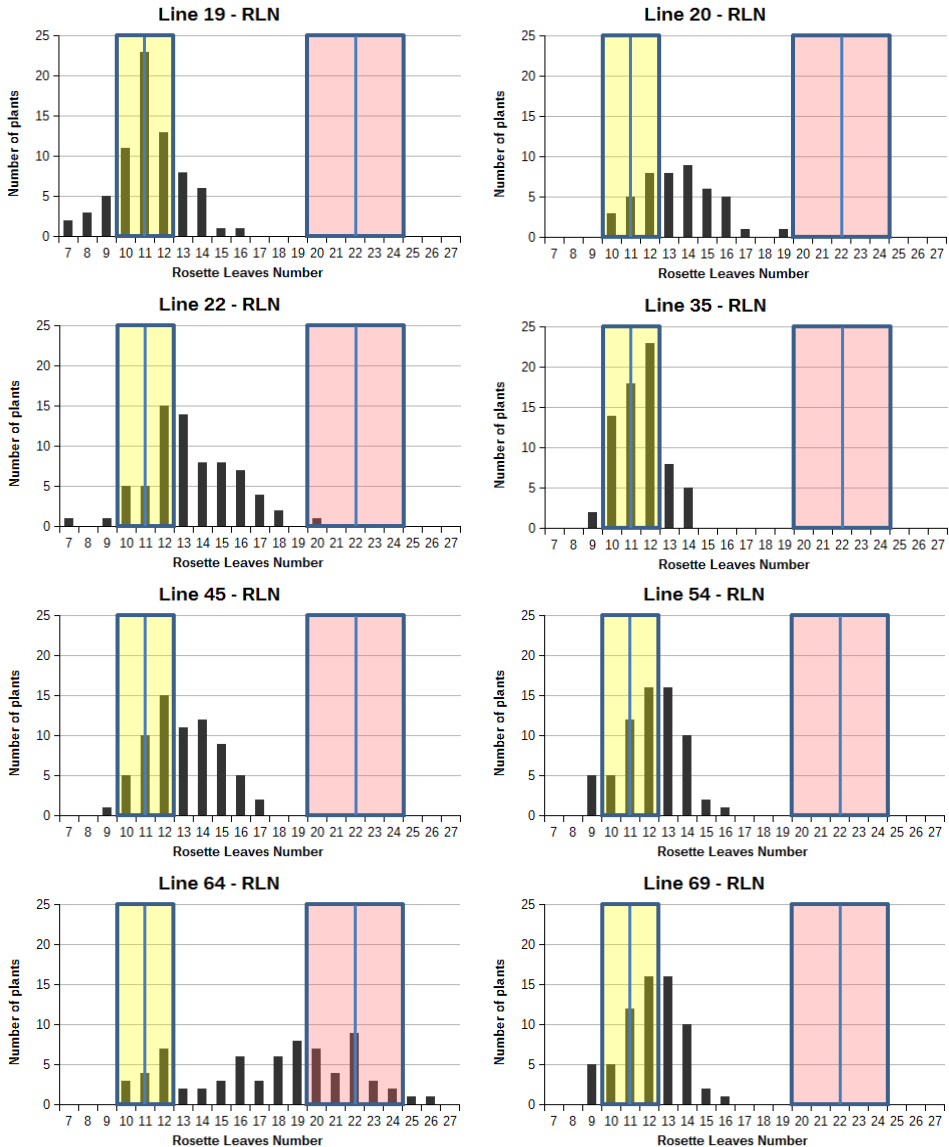
Figure 3: Flowering time distribution of T2 lines expressing FLM under the *35S* promoter. Eight T2 lines that showed 1:3 segregation for *35S::FLM* inserted in the *flm-3* knockout background were tested for flowering time under constant temperature (21° C) and LD conditions (16h light/8h dark). Flowering time was measured in Rosette Leaves Number (RLN). Mean flowering time and standard deviation of the *flm-3* knockout and the Col-0 WT was projected on each graph with a blue line (mean) and yellow and red shaded boxes (SD) respectively. All but one line (line 64) showed early flowering. Note: since lines are segregating 1:3, the effect of the transgene on flowering time is best assessed by comparing the latest ¼ of plants (putative homozygous) to WT and *flm-3* flowering time.

Expression of FLM under the 35S promoter induces different FLMβ/FLMδ splicing ratio's

In order to determine why the *35S*::*FLM* shows early flowering in seven out of the eight screened lines, we performed qPCR on homozygous T3 plants selected from three of the segregating T2 lines (line 20, 45 and 54). In all tested samples, *FLM* showed significant overexpression, eliminating the possibility of silencing of the *FLM* transgene or *FLM*-related genes, such as *FLC* or *MAF2,* as the cause for early flowering of the *35S*::*FLM* lines (Figure 4A). Moreover, we showed that, compared to WT plants, these lines show an altered, significantly lower *FLMβ*/*FLMδ* spicing ratio (Figure 4B).
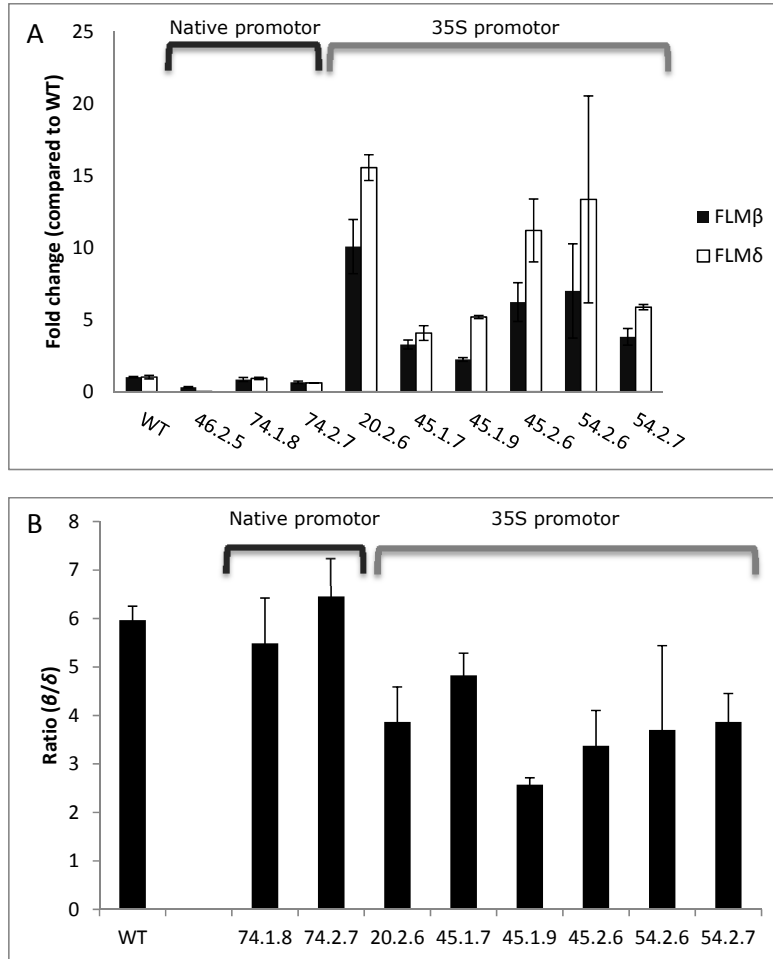
Figure 4: Expression analysis of *FLMβ* and *FLMδ* in random T-DNA insertion lines expressing genomic *FLM* from its native promoter or from the CaMV35S promoter (generation T3). qRT-PCR on 11-d old seedlings grown under LD (16h light/8h dark), 21° C. **(A)** Expression fold change of *FLMβ* and *FLMδ* compared to WT expression (Note that WT *FLMβ* and *FLMδ* expression values were set to 1.0). Plants from lines containing *35S*::*FLM* showed clear overexpression of both *FLMβ* and *FLMδ*.**(B)** Ratio of *FLMβ*/*FLMδ* expression. In plants from lines expressing *35S*::*FLM*, the *FLMβ*/*FLMδ* ratio is

generally lower than in WT and native promoter plants. The data set was normalized against *YLS8* (internal control) and SDs resulted from 1 biological sample with 2 technical replicates for each sample. Samples are annotated as follows: T1.T2.T3. (each sample is derived from one T3 plant).

Plants expressing FLM under the 35S promoter show reduced ambient temperature sensitivity

To investigate the temperature-sensitivity of *FLM* splicing in the lines expressing *FLM* under the strong 35S promoter, we performed temperature experiments in three of these lines. Plants were grown under short day (SD) conditions at 16° C, after which half of the plants were transferred to 25°C. Flowering time was determined by screening RLN (Figure 5). In general, *35S* plants flowered early under both low and high ambient temperature. Like WT plants, all tested *35S::FLM* lines showed earlier flowering at 25° C compared to 16° C. However, the effect of the higher ambient temperature on flowering time in these lines was much smaller than for WT plants.
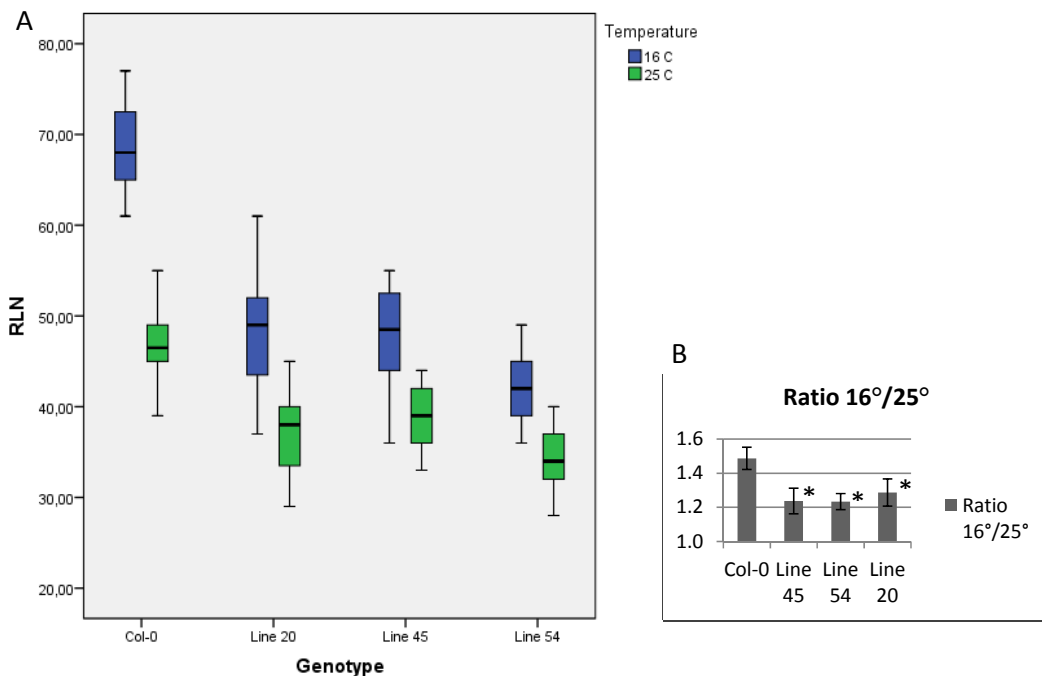
Figure 5: Plants expressing *35S::FLM* show reduced temperature sensitivity. (A) Box-whisker plot representation of flowering time (RLN) of Col-0 WT and *35S::FLM*-expressing plants. All three transgenic *35S* lines show highly reduced floral thermosensitivity, compared to Col-0 WT plants. **(B)** Flowering time ratios of mean flowering time at 16° C to 25° C. * means significantly different compared to Col-0. Error bars represent upper and lower bounds in a 95% confidence interval for mean. Col-0: N= 36/26 (16°/25°), Line 20:N=39/35,Line 45: N=28/26, Line 54: N=39/34. 2 outliers were removed.

Discussion

Chromatin position does not influence alternative splicing of FLM

When we randomly introduced genomic *FLM* under its native promotor in the *flm-3* knockout background, we observed a segregation of flowering time consistent with a dose effect of the transgene, where the most early group probably represent the 25% non-transgene-carrying plants, the mid-flowering group the 50% hemizygous plants and the late group the 25% homozygous plants. Moreover, the eight different lines showed a range in flowering time, with early, mid and late flowering responses, when compared to WT. Based on the expression analysis on two of these lines, early flowering in comparison to WT plants seems to be caused by partial complementation and not due to altered splicing ratios between *FLMβ* and *FLMδ*. Since we observed a similar ratio of the two splicing variants, it is likely that the mid- and late flowering responses are due to differences in total *FLM* expression, although more expression studies on these lines are needed to ensure this. When total *FLM* expression increases while *FLMβ*/*FLMδ* ratio remains unaltered, flowering time will be delayed, because the level of the flowering suppressor *FLMβ* becomes several times higher expressed than *FLMδ* (Figure 4b). When we assume that one FLMδ molecule can "neutralize" one FLMβ molecule by titrating out the required interaction partner SVP (see Chapter 4), higher total expression of *FLM* will have a net effect of more active (non-neutralized) FLM molecules (see figure 6). Thus, our preliminary results indicate that chromatin context surrounding the *FLM* locus is not affecting alternative splicing of *FLM*. Nevertheless, more expression analyses are needed to completely rule out that different flowering times are caused by a positional effect. Moreover, temperature experiments should be performed to test whether the randomly inserted *FLM* genes splice like WT when ambient temperature changes.
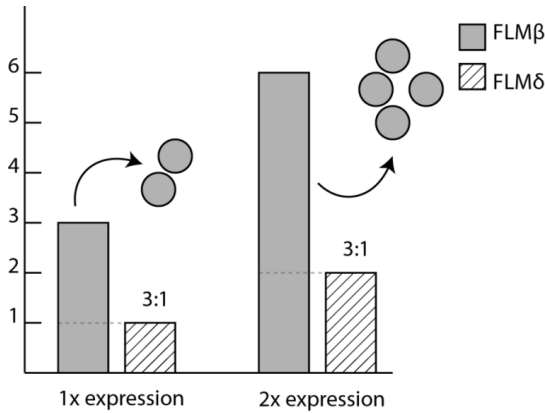
Figure 6: Simplified explanation why higher overall expression of *FLM* can lead to more repressing FLMβ molecules when the FLMβ/FLMδ ratio remains unchanged. With a hypothetical ratio of FLMβ/FLMδ of 3:1, at 1x expression, 3 FLMβ molecules and 1 FLMδ molecule will be produced. The δ molecule will "neutralize" 1 β molecule, and 2 β molecules will be able to exert their repressive function. When total *FLM* expression is doubled, 6 β molecules and 2 δ molecules will be produced. The 2 δ molecules will "neutralize" 2 β molecules, and 4β molecules will be able to exert their repressive function. This rule is applicable as long as the FLMβ/FLMδ ratio is higher than 1.

Transcription initiation rate appears to affect splicing of FLM

When we introduced *FLM* under the strong constitutive *35S* promoter, 7 out of 8 transgenic T2 lines were early flowering. This is remarkable, because based on the explanation in Figure 6, showing that higher overall expression of *FLM* will lead to more active FLMβ molecules, we expected to find a late flowering phenotype upon overexpression of *FLM*. Also, previous research showed that overexpression of *FLM* under the 35S promoter resulted in later flowering (Scortecci et al., 2001), although the origin and selected *FLM* gene or genomic region that this study used cannot be determined, which makes it difficult to compare this study to ours. Nevertheless, we considered the possibility of silencing due to high expression of the transgene under the *35S* promoter as possible explanation for the discrepancy between our results and what has been published. When we analysed expression by RT-qPCR in three of the early flowering lines, we observed a significantly higher *FLM* expression in all cases. Thus, at least on the transcriptional level, silencing is not the case. However, we did observe that *FLMβ*/*FLMδ* ratios were lower in all tested lines (Figure 4B), which could explain the flowering promoting effect observed in the *35S::FLM* lines.

It should be mentioned that this does not fully explain the early flowering time phenotype we observed, since *FLMβ* expression is still higher than *FLMδ* expression in all cases, and hence, the 'higher expression-more FLMβ molecules' theory still holds true (Figure 6). Although it is highly speculative, this is not necessarily a contradiction. If we assume that the pool of SVP molecules is limited, which is a prerequisite for the model presented in chapter

4, there is also a limit for FLMβ molecules to be active as a repressor, because SVP is an obligate interaction partner. When overall expression of *FLM* becomes so high that the number of FLMβ molecules exceeds the number of SVP molecules, the excess FLMβ molecules will not be active as repressors. At the same time, due to the increased expression of FLMδ, as shown in figure 4B, more FLMβ molecules will be neutralized, and the plant will flower earlier (Figure 7).
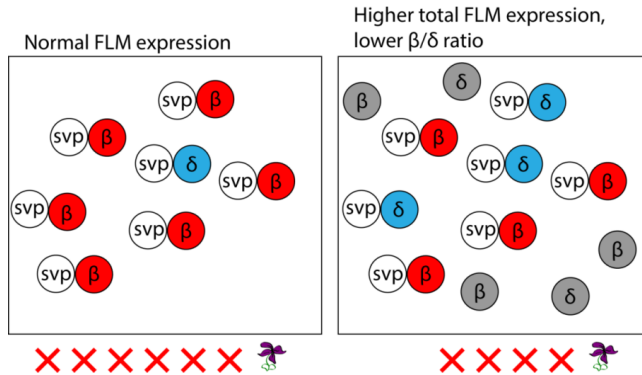

Figure 7: simplified explanation why higher total *FLM* expression and a lower β/δ ratio can lead to earlier flowering even when expression of *FLMβ* is higher than *FLMδ*. At high expression of *FLM* , the amount of FLMβ exceeds the amount of SVP molecules, and the excess FLMβ molecules will not be active as repressors. At the same time, due to the increased expression of FLMδ, more FLMβ molecules will be outcompeted, and the plant will flower earlier.

Altered FLM splicing using the 35S promotor might be due to higher transcription elongation rate

It was shown that a higher transcription initiation rate leads to a higher transcription elongation rate (Epshtein and Nudler, 2003). Our preliminary results indicate that *FLM* splicing might be sensitive to the elongation rate, since we found splicing to be affected in plant overexpressing *FLM.* However, we cannot exclude that other factors caused the observed altered splicing ratio, for example when the promoter sequence itself is recruiting splicing factors (as reviewed by (KORNBLIHTT et al., 2004). Additional experiments are needed to prove that the altered splicing is caused by enhanced PolII elongation rate and that this higher PolII speed is affected by changing temperatures. Nevertheless, our results give a first insight into the possible regulatory mechanism behind temperature dependent alternative splicing.

Materials and methods

Constructs

For the construct under the native promoter, the full genomic *FLM* sequence, including the intergenic regions up- and downstream of the gene, was amplified by PCR using Q5® High-Fidelity DNA Polymerase (Catalog No. M0491S, NEB) according to the manufacturer's protocol (for primer sequences, see Table S1). For the construct under the*35S* promoter, the genomic sequence was amplified starting from the 5' leader sequence and ending with the 3' intergenic region until the UTR of the downstream gene. The products were run on a 1% agarose gel and the fragments were cut out using the nucleospin gel and PCR clean-up kit (Catalog No. 740609.250, Macherey-Nagel). The fragments were A-tailed using TAQ-polymerase with a final concentration of 100 µM dATP (Catalog No. 10297018, Invitrogen) and cloned into pCR™8/GW/TOPO® (Catalog No. K250020). The plasmids were introduced into electrocompetent E.coli DH5α and grown on spectinomycin LB-agar plates. Plasmids was purified by miniprep (Catalog No. 740727, Macherey-Nagel) and the fragment was transferred to the binary vector pBGW (native promoter) or pB7WG2 (*35S* promoter) (Karimi et al., 2002) by a Gateway LR reaction according to the manufacturer's protocol (Catalog No. 11791100, Invitrogen). pBGW is a minimal vector that does not add any unnecessary sequences (like an AMV leader) to the transcript. The sequence of the final construct was checked using the Macrogen EZ-seq service.

Plant transformation

Above-mentioned plasmids were purified by miniprep and introduced in *A. tumefaciens* by electroporation. Bacteria were grown on plate after which a 10 ml liquid culture was produced overnight (o/n) at 28°C, 300 rpm. 5 ml of this culture was used to inoculate 250 ml LB and grown o/n. Next day, cells were harvested by centrifuging at 5000 rpm and resuspended in infiltration medium (0.5 MS + vitamins, 5% sucrose, 0.02% Silwet-L77). Flowering *flm-3* plants (SALK_141971,(Balasubramanian et al., 2006)) were submerged in the medium and put in a closed bag o/n in a horizontal position. Next day, plants were put upright and the bag was opened. Seeds were harvested when they were ripe and transgenic plants were selected for the *bar* resistance gene on PPT-containing plates (0,5X MS agar media +2,3g vitamins+0,5%sucrose+ 15mg/L PPT). Surviving transgenic plants were transferred to rockwool and grown up for seed production. Subsequently, T2 lines were selected for 1:3 segregation ratio of the *bar* gene on PPT containing plates. Only correctly segregating lines were selected for further research. T3 plants were selected on phenotype and progeny of putative homozygous plants was tested for a 100% segregation of the *bar* gene on PPT containing plates.

Flowering time screens

For flowering time screens of T2 plants, 8 lines for each construct were sown on rockwool blocks. WT (Col-0) and *flm-3* seeds were used as controls. Seeds were stratified at 4° C for 2 days/3 nights and then moved into a LD (16h light/8h dark), 21°C growth chamber. Plants were regularly watered once every 4-5 days with nutrient solution (HYPONeX). Each plant was scored for two different parameters as soon as it started bolting: Days to Flowering (DtF), based on how many days the plant took to bolt since it was moved into the growth chamber, and Rosette Leaves Number (RLN),based on how many rosette leaves the plant had at the time of bolting.

For flowering time screens of T3 plants under different ambient temperatures, seeds were sown on rockwool and after stratification transferred to SD conditions (8h light/16h dark) at 16° C. After 5 weeks, half of the plants were transferred to 25° C. A mixed-plot set-up was used to randomize plants with the different genetic backgrounds and to rule out effects of position in the growth chamber. Flowering was screened by scoring RLN.

qPCR

Homozygous T3 lines were sown on rockwool and grown in the same conditions under LD conditions at 21°C. At 11d after germination, 10 to 12 T4 seedlings were harvested (all above-ground tissue) and flash frozen in liquid nitrogen before performing RNA extraction. RNA extraction was performed using Invitrap® Spin Universal RNA Mini kit(Stratec) accordingly to manufacturer's instructions and treated with TURBO DNA-free™ kit (Ambion) in order to degrade residual genomic DNA. 1µg of RNA for every sample was treated using iScript™ cDNA Synthesis kit (BIO-RAD) to obtain a corresponding cDNA sample by reverse transcription. Samples were diluted 5 times before loading 5µL into 96-wells plate with iQ™ SYBR® Green supermix (BIO-RAD) and 10pmol primers for *FLMβ*, *FLMδ* and *YLS8* (At5g08290; temperature-stable internal control). For primer sequences see Table S1.
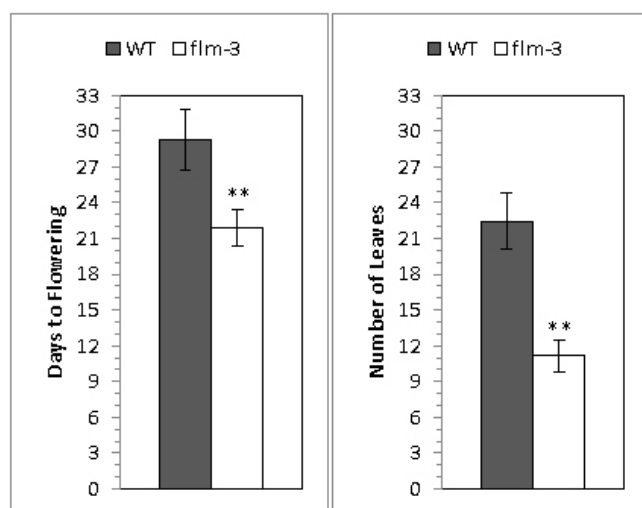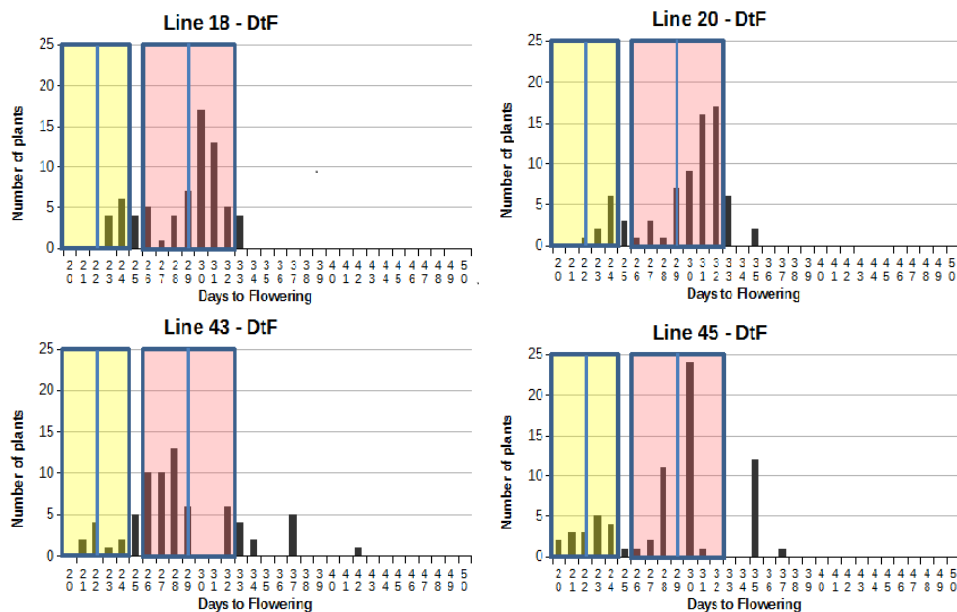
Supplemental material

Figure S1: Average flowering time in DtF and RLN for WT (Col-0) and *flm-3*. The *flm-3* mutant flowers significantly earlier than WT. Plants were grown under constant 21°C and LD conditions. Bars represent SD (n=98 for WT; n=68 for flm-3; **=2-tailed Student t-test, p<0,01).
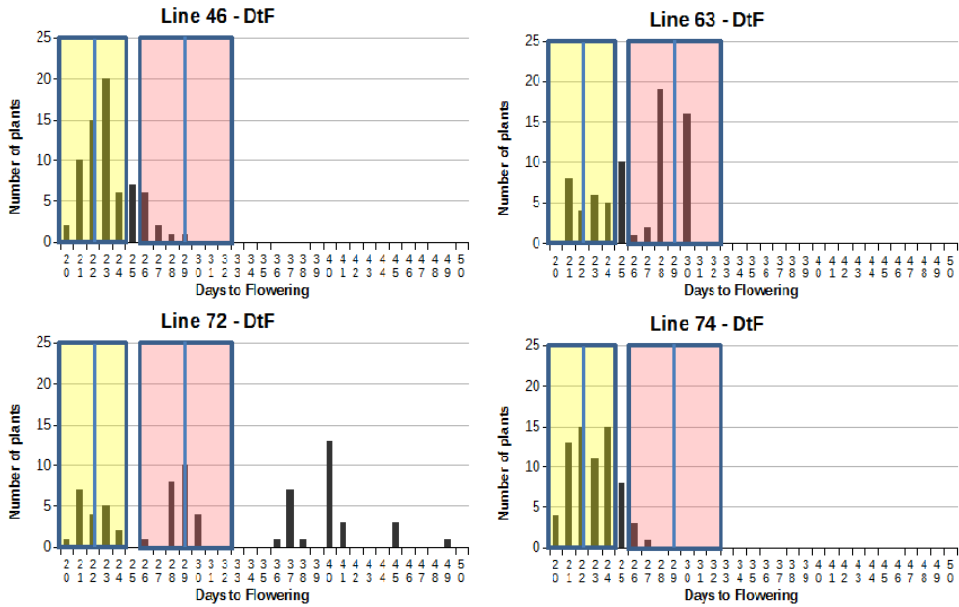
Figure S2: Flowering time distribution of T2 lines with randomly inserted genomic *FLM* in DtF. Eight T2 lines that showed a 1:3 segregation for the transgene inserted in the *flm-3* knockout background were tested for flowering time under constant temperature (21° C) and LD (16h light/8h dark) conditions. Flowering time was measured in Days to Flower (DtF). Mean flowering time and standard deviation of the *flm-3* knockout and the Col-0 WT was projected on each graph with a blue line (mean) and yellow and red shaded boxes (SD) respectively. The lines showed a range of flowering times, including early (line 46, 63, 74), mid (line 43, 45) and late (line 18, 20, 72) flowering time.
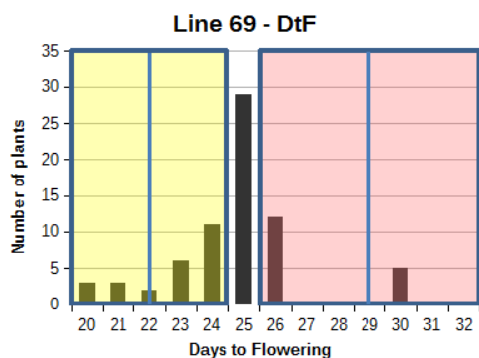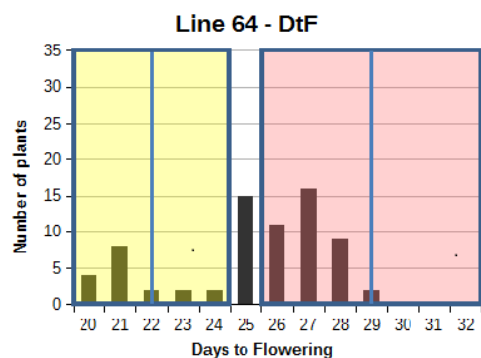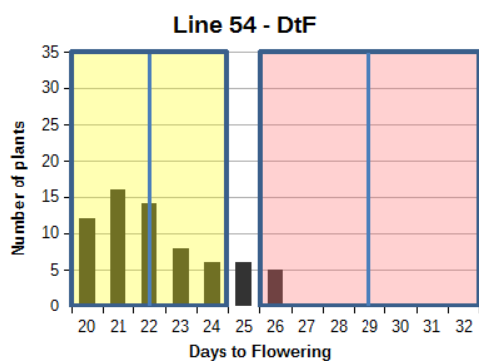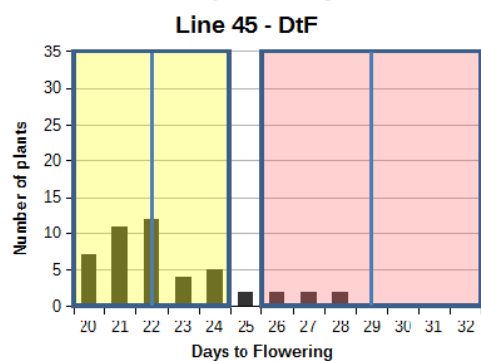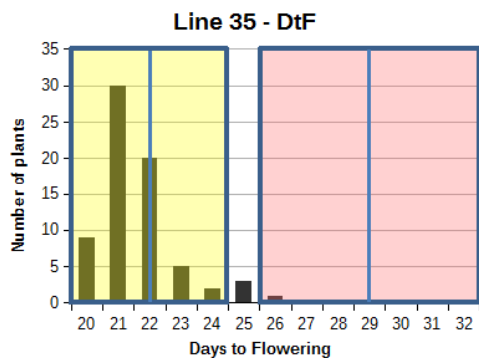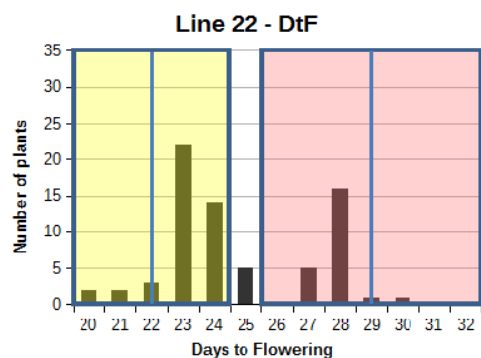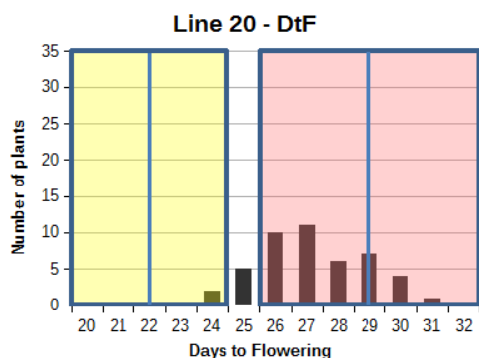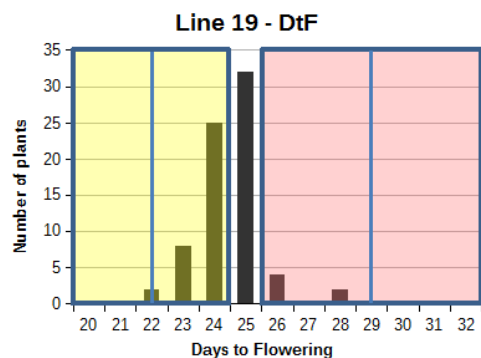
Figure S3: Flowering time distribution of T2 lines expressing FLM under the *35S* promoter in DtF.
Eight T2 lines that showed a 1:3 segregation for *35S*::*FLM* inserted in the *flm-3* knockout background were tested for flowering time under constant temperature (21° C) and LD conditions (16h light/8h dark). Flowering time was measured in Days to Flowering (DtF). Mean flowering time and standard deviation of the flm-3 knockout and the Col-0 WT was projected on each graph with a blue line (mean) and yellow and red shaded boxes (SD) respectively. All but one line (line 64) showed early flowering.

Table S1. Primers used in this study	
FW *FLM* 5' intergenic region	GCTTGGGATTTTAATAGAGAGCAC
FW *FLM* 5' leader	AGGATTAAATTAGGGCATAACCCTT
RV *FLM* 3' intergenic region	ACCAAAACATGAAACAGAGTTCAAA
FW qPCR *FLMβ*	GATCGTTATGAAATACAACATGC
RV qPCR *FLMβ*	GTATTCTTTCCCATCTGGCTAGC
FW qPCR *FLMδ*	CCTCAATGTTTTGAACTCGATC
RV qPCR *FLMδ*	TCGACATTTGGTTCTTCAAGCTTGC
FW qPCR *YLS8*	TTACTGTTTCGGTTGTTCTCCATTT
RV qPCR *YLS8*	CACTGAATCATGTTCGAAGCAAGT

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Chapter 7

It's all in the family: conservation of thermosensitive floral timing by alternative splicing of *FLC*-like genes

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Abstract

In many plants, flowering time is affected by ambient temperature. In *Arabidopsis thaliana*, key players in this process are *FLM*, and the closely related *MAF2* gene. *FLM* and *MAF2* are MADS domain transcription factors that are members of the *FLC*-clade family, existing of *FLC*, *FLM* and *MAF2-5*. *FLM* and the *MAF*s all have the potency to regulate floral timing, and all of them undergo alternative splicing. This evokes the question what distinguishes these genes. We showed that one of the differences between *MAF2* and *FLM* is the production of a dominant-negative isoform. However, overall, *FLM* and the *MAF* genes show more resemblances than differences. We investigated the conservation of the *A. thaliana FLC*-clade members in other species, and showed that *FLM* and the *MAF*-genes are exclusive to the Brassicaceae family. However, many plant species in other plant families have *FLC*-like genes, raising the question what function these genes fulfil. We demonstrate that *MBP25* and *MBP8*, two *FLC*-like genes from tomato, undergo ambient temperature-directed alternative splicing. Moreover, a binding assay for MBP8 reveals a high similarity between FLM/MAF isoforms and MBP8 isoforms. This study shows for the first time that thermosensitive floral timing by alternative splicing of *FLC*-like genes might be a conserved mechanism in flowering plants.

Introduction

Previously, we showed that *FLOWERING LOCUS M* (*FLM*), also called *MADS AFFECTING FLOWERING 1* (*MAF1*), is a key player in the ambient temperature directed flowering time pathway, which regulates flowering time through temperature-sensitive alternative splicing (Posé et al., 2013). The closely related gene *MAF2* has shown to be involved in the ambient temperature-directed floral transition as well. Like *FLM*, this gene regulates flowering time through thermosensitive alternative splicing (Airoldi et al., 2015). *FLM* and *MAF2* are members of the *FLOWERING LOCUS C* (*FLC*)-clade, which consists of the highly related *FLC*, *FLM*, and *MAF2-5* genes, of which the latter are arranged in a tandem repeat at the extremity of chromosome 5 (Figure 1). These genes are all members of the MIKC-type MADS box transcription factor gene family, which are widely recognized for their role in plant development (Smaczniak et al., 2012). Overexpression studies showed that all six *FLC*-clade genes have the potential to alter flowering time (Ratcliffe et al., 2003), but their role in response to temperature seems to be different. *FLC*, the supposed ancestral gene and namesake of the clade, is the most intensively studied member, and is broadly accepted to be the key regulator of the vernalization response: the process by which plants acquire the ability to flower after exposure to a prolonged period of cold, winter-like temperatures (Sung and Amasino, 2004). Before the cold period, *FLC* is highly expressed, and inhibits the transition from the vegetative to the generative phase by repressing floral integrator genes. During cold periods, the gene becomes epigenetically silenced in a mitotically stable fashion, removing the strong repression on flowering and allowing floral activators to initiate the

floral transition (Berry and Dean, 2015). Despite the high similarity between *FLC*, *FLM* and the *MAF* genes, *FLC* is the only member of the *FLC*-clade that shows such a strong reduction in expression after vernalization. *FLM* and *MAF2-4* are downregulated upon vernalization, but the repression is less strong and the mechanism responsible for *FLC* silencing contributes only partly to silencing of the other *FLC*-clade members (Sheldon et al., 2009). *MAF5* has even been reported to respond in an opposite way to vernalization, showing an increase in expression (Kim and Sung, 2010). Moreover, this *MAF* gene has also been shown to be repressed under non-inductive photoperiods, suggesting a different biological functionality. Therefore, one of the main differences between *FLC* and *FLM/MAFs* is the responsiveness to vernalization. Amongst *FLM* and *MAF2-4*, differences in response and function are less clear. Recently, we showed that *MAF3* is also alternatively spliced upon altering ambient temperatures (Chapter 3, this thesis), and therefore it is possible that *MAF3* is another *FLC*-clade gene that regulates the transition to flowering in response to ambient temperature. Nevertheless, mutational analyses have shown that *FLM* and *MAF2-4* do not act redundantly, but in an additive manner (Gu et al., 2013). Moreover, *FLM* appears to be the strongest floral regulator in this pathway, showing the most severe effect on the ambient temperature response when mutated.

As for *FLM* and *MAF2/3*, several splice forms are annotated for *MAF4* and *MAF5* in the current version of The Arabidopsis Information Resource (TAIR). Due to all the similarities between the *MAF* genes, including *FLM*, it is ambiguous which factor makes these genes different from each other.
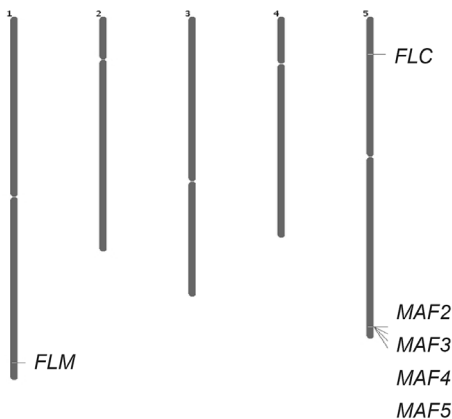

Figure 1: position of the *FLC*-clade genes on the A. thaliana chromosomes. *MAF2-5* is located in a ~22kb tandem repeat at the extremity of chromosome 5.

FLC, the supposed ancestor of the *FLC*-clade, is a relatively ancient gene, as a recent study showed that its origin can be found in a common ancestor of the seed plants (Ruelens et al., 2013). However, the origin of the other clade members has remained enigmatic. Mining

literature shows that many plant species harbour more than one *FLC*-like gene. For example, apple (*Malus domesticus*), a member of the Rosaceae, has been reported to contain at least three *FLC*-like genes (Porto et al., 2015). Also for chicory (*Cichorum intybus*), belonging to the family of the Asteraceae, several *FLC*-like genes seem to be present (Locascio et al., 2009). However, nothing is known about the evolutionary relationships between these genes. In tomato (S. lycopersicum), which belongs to the Solanaceae family, three *FLC*-like genes have been identified (Hileman et al., 2006). *MBP25*, the *FLC*-like gene on chromosome 5, has shown to exhibit conserved synteny with *FLC* (Ruelens et al., 2013), whereas the origin of the other two genes, *MBP8* and *MBP15*, which are arranged as a tandem on chromosome 12, has remained enigmatic, but are most likely tomato-specific paralogs.

In this study, we assessed conservation of the *FLC*-paralogues in *A.thaliana* by comparing the DNA binding properties of different splicing isoforms. Consecutively, we evaluated the presence of these genes in other species, and found that orthologues of *FLM* and the *MAF* genes cannot be detected outside the Brassicaceae family. This brings up the question what the function is of multiple *FLC*-like genes in other plant species. We decided to focus on two of the *FLC*-like genes from tomato. This species is unresponsive to vernalization, but does respond to different ambient growth temperatures, i.e. flowers later when temperature rises (Calvert, 1957; Wittwer and Teubner, 1957). Our preliminary results show that *MBP8* and *MBP25* produce various splice forms that are differentially produced under different ambient temperatures. Additional DNA-binding analyses for MPB8 revealed its potency to function as a flowering time regulator. This is the first indication that alternative splicing of *FLC*-like genes by ambient temperature is more widely conserved than *FLM* and the *MAF*-genes from Arabidopsis, supporting a hypothesis that alternative splicing of *FLC*-clade MADS domain transcription factors is a key mechanism underlying the ambient temperature flowering time response.

Results

To better understand the difference between *FLM* and *MAF2-5* in *A. thaliana*, we performed electrophoresis mobility shift essays (EMSAs) with MAF isoforms (Figure 3), in as similar way as described for FLM ((Posé et al., 2013), Chapter 4)). We used different isoforms of which the transcript could be detected in Col-0 plant material, and a fragment of the *SOC1* promoter was used as a DNA probe in these EMSAs (as described in (Posé et al., 2013). Previously, we showed that FLM proteins form heterodimers with SVP, and genetic experiments have shown that *MAF2* functioning is dependent on *SVP*. Therefore, we performed the EMSAs for all MAFs with- and without addition of SVP.

MAF2 is different from FLM due to the lack of dominant-negative isoforms

As mentioned above, *MAF2* regulates flowering time by ambient temperature through differential splicing. At low ambient temperatures, there is predominant production of the repressive isoform *MAF2var1*, whereas at higher ambient temperatures, the *MAF2var2* variant becomes more abundant (Airoldi et al., 2015). In the EMSA experiment, we used four different isoforms, which included the two major splice forms that showed differential expression. Figure 2 shows that three of the four isoforms, including the repressive MAF2var1, are able to bind the DNA probe in the presence of SVP. The fourth isoform, representing MAF2var2, is not able to bind DNA, since no shift in SVP binding pattern can be observed. As was shown in chapter 4, a dominant-negative splice form can be recognized by the reduction in SVP-binding signal upon addition of the respective FLM isoform, and a 1:1 SVP:FLMδ ratio showed a strong reduction in SVP-DNA binding. For MAF2var2, we did not observe any decrease in SVP intensity, indicating that this isoform does not bind and titrate out SVP. Thus, MAF2var2 seems to lack a dominant-negative mode of action.

MAF3-5: SVP-independent DNA binding and potential dominant negative splice forms

For MAF3, we tested four different isoforms (Figure 2). The isoforms we tested showed no binding to DNA, and no reduction in SVP signal could be observed, indicating that none of these forms has dominant-negative functionality and are most likely non-functional proteins. However, previously we showed the existence of six *MAF3* transcripts (see Chapter 3), and therefore, further research is necessary to test the remaining two splice forms. Nevertheless, the splice forms that showed a thermosensitive response (*MAF3.2* and *MAF3-5*), were included in this EMSA.

The splice forms of *MAF4* showed a surprising pattern: MAF4.1 was able to bind to the *SOC1* DNA probe without addition of SVP, but is outcompeted by the SVP-MAF4.1 heterodimer. MAF4.2 did not bind to the DNA probe, but addition of MAF4.2 reduced the signal of SVP-DNA binding, suggesting that MAF4.2-SVP dimers are formed that are not able to bind to DNA. MAF4.3 and MAF4.III isoforms were not able to bind to DNA, neither as homodimer nor in a complex with SVP.

For MAF5, we could detect two isoforms that showed SVP-independent DNA binding, MAF5.1 and MAF5.I. However, when SVP was added, a gel shift was observed, and the bands representing the MAF homodimer-DNA interaction completely disappeared. This means that when SVP is present, the DNA probe is preferentially bound by a MAF5.1/5.I-SVP heterodimer, or that the formation of heterodimers is favoured over the formation of homodimers. Since SVP is co-expressed with MAFs in leaves, SVP-independent binding may not occur under native conditions.
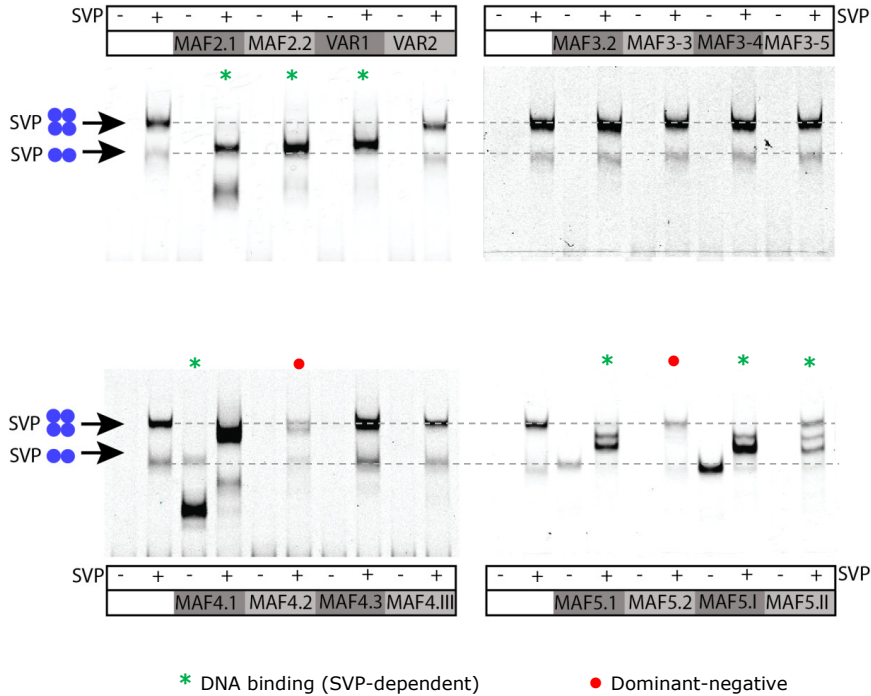
Figure 2: Specific DNA binding capacity of MAF isoforms. Each protein isoform was tested by EMSA for DNA binding with- and without addition of SVP, using part of the promotor sequence of *SOC1* as a DNA probe. The SVP-binding pattern is indicated by the dashed line and proposed stoichiometry of binding complexes (homodimers and homo-tetramers) is schematically indicated by the blue balls. Each lane that contains a MAF isoform that shows a different pattern from SVP, and thus binds to the DNA in an SVP-dependent fashion, is indicated by a green asterisk (*). The two lanes that contain a MAF isoform that decreases SVP-binding, indicative for a dominant negative functioning, are labelled by a red dot (•). For MAF2var2 and all MAF3 isoforms, no strong reduction in SVP pattern could be observed, indicating that *MAF2* and *MAF3* do not produce dominant-negative isoforms. MAF4.1, MAF5.1 and MAF5.I showed binding independent of SVP.

MAF4.2 and MAF5.2 caused a strong reduction in SVP signal, suggesting maintenance of heterodimerization capacity, but lack of DNA binding of the formed complexes. Hence, these two isoforms appear to have the capacity to act in a dominant-negative manner to SVP. We compared the protein sequences of each putative dominant-negative isoform to an isoform from the same gene that binds DNA in an SVP-dependent fashion (Figure 3). For each DNA-binding isoform, we predicted the position of conserved protein domains using Prosite (http://prosite.expasy.org). Remarkably, all three dominant-negative isoforms showed a difference in the I region of the MADS domain protein, which is an indication that alterations

in this domain of the protein can disrupt the DNA-binding capacity, while it does not interfere with the protein-protein interaction capacity with SVP.
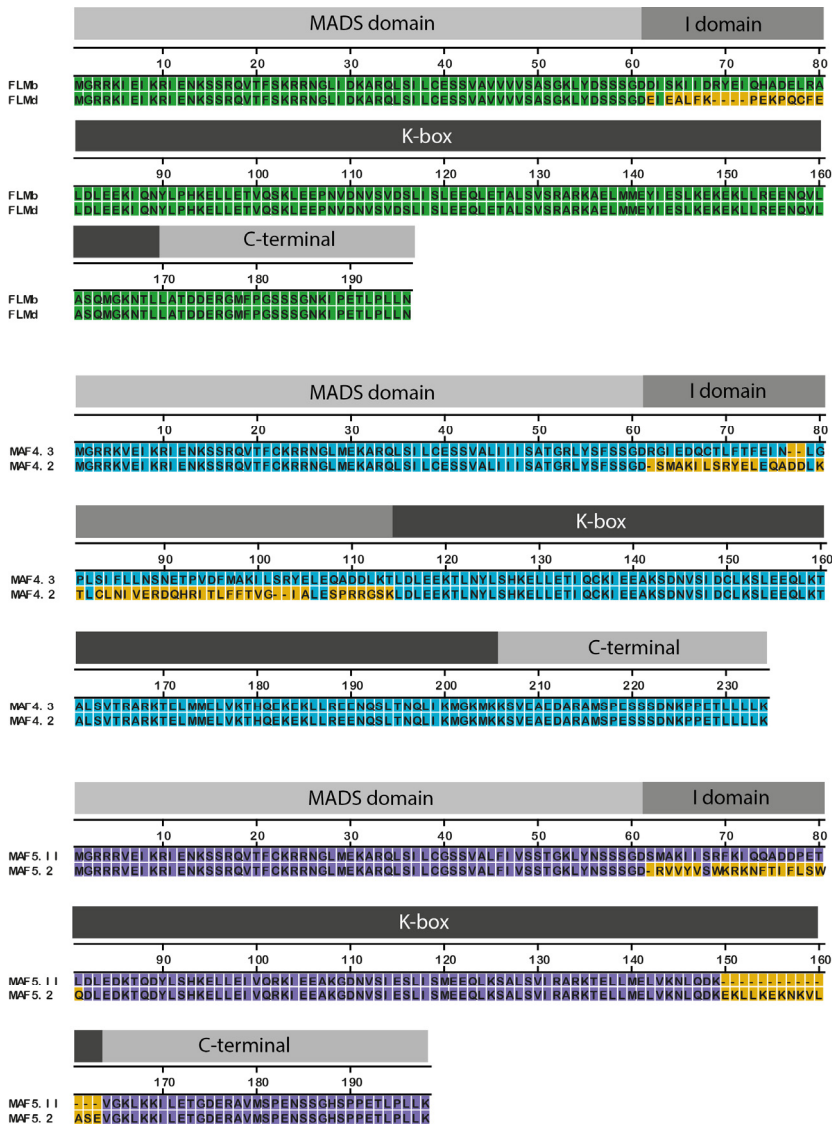

Figure 3: Protein-sequences of dominant-negative isoforms aligned to DNA-binding isoforms derived from the same MAF genes. For each MAF MADS domain transcription factor, the upper sequence represents the DNA-binding isoform, and the lower sequence the dominant-negative isoform. The non-homologous amino acids are shown in orange. For each MAF protein, conserved protein domain predictions were performed for the DNA-binding isoform using Prosite (http://prosite.expasy.org). The three dominant-negative MAF protein variants have in common that

they all differ from the DNA-binding form in the I-domain. MADS = Conserved DNA binding motif. I = less conserved intervening region. K-box = keratin-like conserved coiled-coil domain. C-terminus = remaining of protein without any recognisable conserved Prosite domain.

As shown above, *A. thaliana FLM* and the *MAF*-genes show a high degree of intraspecific functional conservation. To find out how these genes are conserved in other species, we assessed the evolutionary relationships of these genes.

FLM and MAF2-5 are recent paralogs that originated at different moments

Based on sequence homology, we cannot distinguish the different functions of the six *FLC*-clade genes in *A. thaliana*. Therefore, we performed synteny analysis using the GenomicusPlants browser (Louis et al., 2015). We could only detect syntenic blocks that contain *FLM* in *Arabidopsis lyrata* and *Capsella rubella*, indicating that *FLM* originated at the Camelineae speciation event (Figure 4). When we performed this search for *MAF2*, for which involvement in ambient temperature regulated floral timing has been shown (Airoldi et al., 2015; Rosloski et al., 2013), no interspecific synteny could be detected. Thus, this gene appears to exist in *Arabidopsis thaliana* only. However, for *MAF3* we could find syntenic blocks in several Brassicaceae, including *Thelungiella halophila* (Eutremeae) and *Capsella rubella* (Camelineae), which represent species from different major clades (Figure 4). This indicates that *MAF3* originated from a common ancestor of the Brassicaceae. The same is true for *MAF4* and *MAF5* (Figure S1). In conclusion, from the *MAF* subclade, *MAF3-5* are the most ancient genes, *MAF2* is the youngest and *FLM* originated in-between.
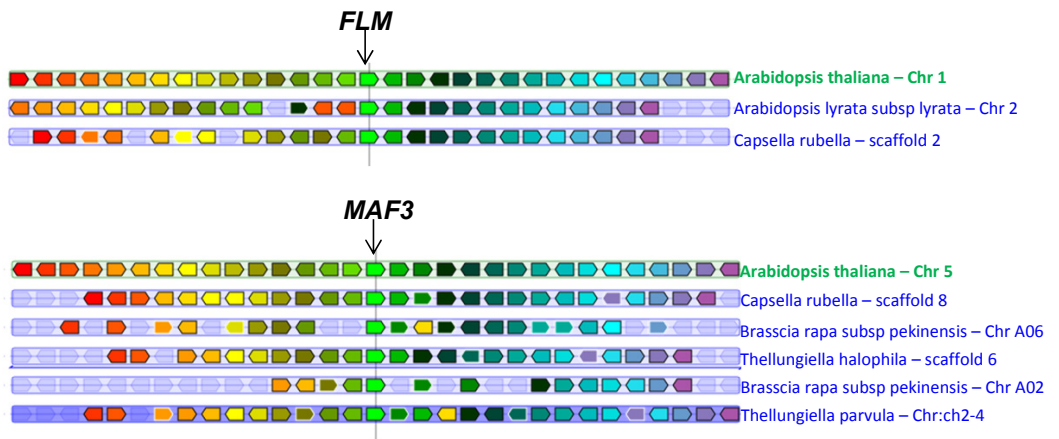

Figure 4: Synteny of *FLM* and *MAF3*. From synteny analysis, we can conclude that *FLM* originates from a recent event that preceded speciation of the Camelineae tribe of the Brassicacea, whereas *MAF3* originates from a common ancestor of the Brassicaceae family. Coloured arrows represent genes, and orthologous genes have the same colour.

We showed that *FLM* and the *MAF*-genes cannot be detected outside the Brassicaceae family. However, as described above, *FLC*-like duplicates have been observed in several other plant families, raising the question what the functions of these genes are.

In tomato, a plant species that is not responsive to vernalization, but that does react to different ambient growth temperatures (Calvert, 1957), three *FLC*-like genes have been identified: *MBP25*, *MBP15* and *MBP8* (Hileman et al., 2006). To find out if these genes produce different splice forms, we designed primers at the start and the stop codon of *MBP25*, which is the orthologue of *A. thaliana FLC*, and *MBP8*, one of the *MBP25* duplicates on chromosome 12. We performed reverse transcriptase (RT)-PCR on tomato RNA, and cloning and sequencing of the products showed that for both *MBP8* and *MBP25*, three different splice forms could be detected, which differ from each other by exon skipping (*MPB8var2/3* and *MPB25var2*), or intron retention (*MPBvar3*) (Figure 5 and S2).
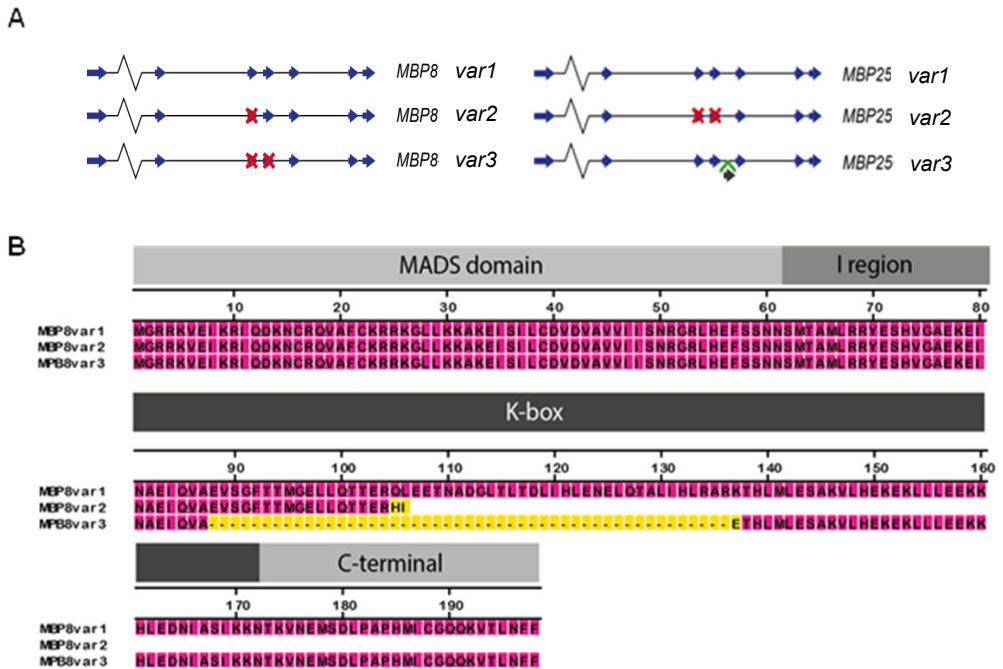

Figure 5: Isoforms encoded by *FLC*-like genes *MBP8* and *MBP25* from tomato. For both *MBP25* and *MBP8*, we could clone 3 different isoforms. **(A)** Model of each detected isoform. Exons are indicated by blue arrows. The isoforms differ from each other by exon skipping (x; *MBP8var2*, *MBP8var3* and *MPB25var2*), or intron retention (black arrow; *MBP25var3*). **(B)** Protein prediction for the 3 splice forms of *MBP8*. MPB8var2 has a premature termination codon, and lacks the C-terminal and part of the K-box. In MBP8var3, part of the K-box is spliced out.

To investigate if the tomato *FLC*-like *MBPs* are differentially spliced upon ambient temperature, like *A. thaliana FLM* and *MAF2/3*, we grew tomato plants at 23° C and

transferred half of the plants to 30° C, 10 days after germination. After 24 hours, whole plantlets were harvested for RNA isolation and cDNA production. We designed primers that were specific for each of the isoforms that we detected, and tested the expression by qPCR (Figure 6). Although we did not test all isoforms yet, preliminary results showed differential alternative splicing of both *MBP8* and *MBP25*, with most distinct effects for *MBP8*. This gene showed a decrease of *var1* at higher ambient temperature, whereas *var3* remained constant*,* resulting in a ratio change between these two isoforms.
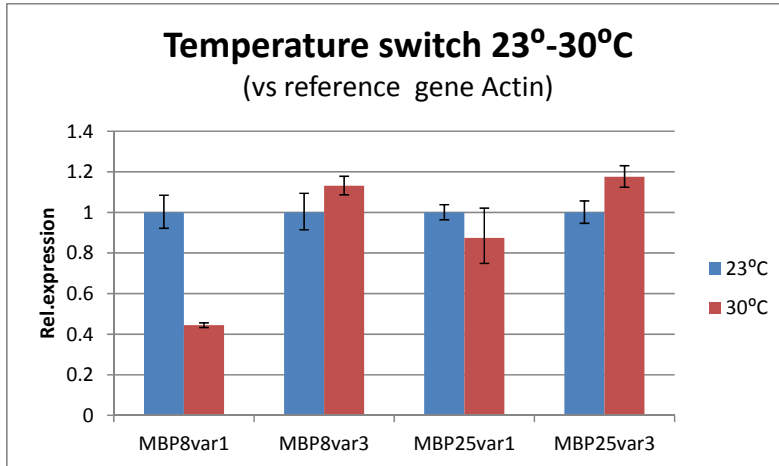

Figure 6: Expression analysis for *MBP8* and *MBP25* splice forms of tomato plants constantly grown at 23° C and plants switched from 23° to 30° C. Expression of each splice variant 24 hours after the temperature switch was studied by qRT-PCR. Detected expression at increased ambient temperatures was normalized against 23° C, and Actin was used as a reference gene. Error bars represent SD for 3 biological replicates, each consisting of a five independent plantlets.

To get more information about the functionality of the different MBP8 isoforms, we performed an EMSA experiment. We used a heterologous system with SVP from *A. thaliana* and a *SEP3* DNA probe, which had previously been used successfully in EMSAs with FLM (Posé et al., 2013). Preliminary results showed that similar to FLM, MBP8 isoforms do not bind to the DNA as a homodimer. However, upon addition of SVP, we observed DNA binding of all three MBP8 isoforms (Figure 7).
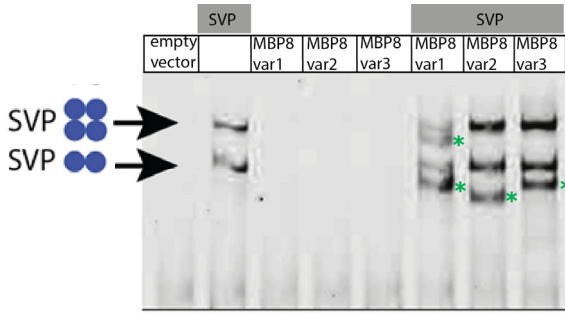
Figure 7: DNA binding capacity of tomato MBP8 isoforms. MBP8 isoforms were produced independently and in combination with *A. thaliana* SVP protein. None of the isoforms showed DNA binding without SVP. When SVP was added, we could observe extra bands besides the SVP bands for all three isoforms (see green asterisks), indicating that all of them can bind to DNA in combination with SVP.

Based on these results, we conclude that MBP8 isoforms show a high resemblance to FLM and MAF isoforms, being able to bind the *SEP3* DNA probe in an SVP-dependent manner. However, MBP8 most likely does not act exactly like FLM or MAF proteins, because in contrast to the Arabidopsis proteins we could not detect differential binding between the tomato isoforms. Further research using an SVP-homologue and a DNA-probe from tomato will hopefully shed light on the exact molecular mode of action of the different isoforms. Nevertheless, our results provide novel information on temperature-sensitive splicing of *FLC*-like genes in tomato.

Discussion

Dominant-negative isoforms

Despite the role of *MAF2* (and possibly *MAF3*) in ambient temperature regulation of flowering in *A. thaliana*, several studies indicate that *FLM* is the most responsive thermosensitive floral regulator in this pathway (Gu et al., 2013). This can partly be due to total expression differences, but here we showed that this may also be explained by the fact that MAF2 lacks a dominant-negative isoform. Such a dominant-negative form is able to interact with the floral suppressor SVP, but does not bind DNA, resulting in an inactive SVP complex. For MAF3, we could also not detect such a splice variant. However, both *MAF4* and *MAF5*, produce a dominant-negative form, although a role of these genes in ambient temperature signalling in *A. thaliana* has not been demonstrated so far. Thus, our results showed that the dominant-negative FLM isoform is not an exception. Other proteins exist that produce isoforms that lost DNA-binding capacity, while the interaction with SVP is still intact. Hence, it is imaginable that in Brassicaceae species that do not contain a copy of *FLM*, one of the other *MAFs* functions like *FLM* (in case of a dominant-negative splice form), or as

MAF2 (when a dominant-negative form is lacking). However, further research is necessary to prove this hypothesis.

In conclusion, the differences between *FLM* and the *MAFs* are minor, with the biggest difference being the presence of a dominant-negative splice form for *FLM*, *MAF4* and *MAF5*.

Alterations in the I-region may disrupt DNA-binding capacity

FLC-like proteins are members of the MIKC-type MADS domain protein family. These proteins have a modular structure consisting of the highly conserved DNA-binding MADS domain, the intervening (I) region that is important for dimerization together with the K-box, and the C-terminal region that plays a role in higher-order complex formation, together with the K-box. Because the dominant-negative isoforms lost DNA-binding capacity whereas interaction with the SVP protein is still intact, the difference between these isoforms and the DNA-binding forms may be expected to be localized in the DNA binding MADS domain. Strikingly, for all three dominant-negative forms, we found divergence in the I-region. The function of the I-region has been assigned by domain swapping experiments and bioinformatic predictions of motifs involved in protein-protein interaction, which revealed that the dimerization partner-specificity is mediated to a large extent by this region (Riechmann et al., 1996; van Dijk, 2010). The finding that the minimal DNA-binding domain includes both the MADS domain and the I-region, as was shown previously by Mizukami and colleagues (Mizukami et al., 1996), has been attributed to the model that MADS domain proteins bind to the DNA as dimers (Riechmann and Meyerowitz, 1997). We showed for the first time that the I-region is also important for DNA binding of FLM and MAF proteins, in addition to its dimerization function. Whether this is a more general feature of the I-region in MADS domain proteins remains to be determined. Strikingly, the I-region is a highly variable region, both in sequence and length, while it is essential for binding of the MADS domain to DNA. Therefore, it is likely that it is not the sequence that contributes to DNA binding, but that the structure of the I-region facilitates DNA binding by the MADS domain. This hypothesis is supported by the fact that in all MADS domain protein 3D models, the I-region does not make direct contact with the DNA (Pellegrini et al., 1995; Santelli and Richmond, 2000; Tan and Richmond, 1998). This suggests that the contribution to DNA binding of the I-region is indirect, possibly by positioning correctly of the MADS domain towards the DNA.

FLM and MAF2-5 are recent genes

Synteny search for *FLM* only returned collinearity in *A. lyrata* and *C. Rubella*, both belonging to the Camelineae tribe of the Brassicaceae, indicating that *FLM* is a very recent paralogue from the *FLC*-clade. *MAF2* even showed no synteny at all, and is most likely an *Arabidopsis-*

specific gene. Thus, for these two particular *FLC*-clade genes in *A. thaliana* that have shown to control floral timing by ambient temperature, hardly any conservation exists. In contrast, *MAF3*, *MAF4* and *MAF5* revealed a broader synteny, and are probably present in the complete Brassicaceae family. Nevertheless, outside the Brassicaceae family, *FLM* and *MAF2-5* cannot be detected.

An FLC-like gene from tomato may function in thermosensitive floral timing through alternative splicing

FLM and the *MAF* genes do not have orthologues outside the Brassicaceae family. However, for several plant species that belong to different families, several *FLC*-like genes have been detected (Hileman et al., 2006; Locascio et al., 2009; Porto et al., 2015).
Tomato, which is a member of the Asterids (whereas Brassicaceae belongs to the Rosid clade) is not sensitive to vernalization, but does react to different ambient temperatures. The fact that we found temperature-dependent alternative splicing of tomato *FLC*-like genes is a first indication that thermosensitive floral timing through alternative splicing of *FLC*-like genes is broader conserved, despite the lack of *FLM/MAF* genes outside the Brassicaceae.

Our preliminary results with MBP8 DNA-binding specificity imply a slightly different functionality compared to FLM or MAF2, since we could not detect differential binding for the three investigated isoforms. This might be explained by the fact that we made use of a heterologous system, using tomato MBP8 isoforms and *A. thaliana* SVP protein and DNA probe. However, *SVP* appears to be a well-conserved gene, as constitutive overexpression of *SVP*-like genes of *Antirrhinum majus* (*INCOMPOSITA*), trifoliate orange (*ptSVP*), and even from the monocot *Oryza sativa* (*OsMADS55*) in *A. thaliana*, caused a late flowering time phenotype, which is a similar phenotype as obtained with overexpression of the native gene (Lee et al., 2012).

Assuming that *MBP8* plays a role in thermosensitive floral timing through alternative splicing, there are several other possible explanations why we do not see differential binding of the MPB8 isoforms, while expression analysis shows differential regulation. One possibility is that part of the transcripts are not translated, and shifting the ratio towards these splice forms will downregulate the net production of the functional protein. In our EMSA experiment, we used an *in vitro* transcription-translation system to produce the different protein isoforms, and we cannot exclude the possibility that these transcripts are not translated *in vivo*, due to targeting of the transcripts for degradation by the nonsense-mediated decay (NMD) pathway. The most likely target for NMD is *MBP8var2*, because of the presence of a premature termination codon (PTC). However, due to the position of the PTC at the end of an exon, NMD is not predicted for this isoform (Nyikó et al., 2013). Another explanation for the observed binding pattern of MBP8 isoforms might be that MBP8 isoforms function differently from the Arabidopsis MAF and FLM splice forms. As can be seen in Figure 5b, the difference between MBP8var1 and MBP8var3 is localised in the K-box of the protein, whereas the dominant-negative FLM and MAF isoforms are different in the I-

region. As described above, the K-box is important for dimerization and higher-order complex formation. Since de isoforms show interaction with SVP and DNA in the EMSA, dimerization and tetramer formation seems not affected, leaving differential complex formation with co-factors open as molecular mode of action. In this scenario, one of the isoforms lost its functionality due to loss of particular higher-order protein-protein interactions.

Of course, all these scenarios are highly speculative, since the true function of MBP8 remains enigmatic. Further research is necessary to find out if, and how, MBP8, or alternatively MBP25 or MBP15, plays a role in thermosensitive floral timing in tomato.

Materials and methods

Synteny determination

Synteny was determined using GenomicusPLants (Louis et al., 2015). Results were inspected by hand and incorrect hits with synteny blocks of only one gene (due to sequence similarity to FLC-like proteins and the tandem repeat of MAF2-5), were removed.

EMSA experiments

MAF isoforms were obtained from cDNA by performing PCR with primers on the start and stop of the coding sequences, except from MAF4.3 and MAF5.1, for which the sequences were synthetically produced (GeneScript) and introduced into the pSputK vector through Gateway cloning (for sequences, see Figure S3). The *SEP3* probe was used from (Posé et al., 2013). The *SOC1* probe was amplified from cDNA using Q5 high-fidelity DNA polymerase (NEB, Catalog nr. M0491S) , according to the manufacturers' recommendations (For primer sequences, see Table S1). The fragment was A-tailed using Taq polymerase and dATP (Invitrogen, Catalog nr. 10297018), and consequently introduced into pGEM-T (Promega, Catalog nr. A3600). Fluorescent probe was produced using fluorescently labelled forward and reverse primers (Dy682, Eurofins), for primer sequence see Table S1. Protein production and EMSAs were performed according to (Posé et al., 2013).

Plant material

Tomato plants were grown at 23° C for 10 days under long day conditions (16h light, 8h dark), after which half of the plants were transferred to 30° C growing conditions. After 24 hours, 5 plants were harvested per sample were harvested and flash-frozen in liquid nitrogen.

Isolation of tomato FLC-like isoforms and qPCR experiments

Whole plantlets were ground and RNA was isolated using the NucleoSpin plant RNA isolation kit (Macherey-Nagel, Catalog nr. 740949.250) according to the manufacturers' manual. cDNA

was produced using Q5 high-fidelity DNA polymerase (NEB, Catalog nr. M0491S). qPCR was performed using iQ 2x SYBR green master mix (Bio-rad, Catalog nr. 1708885), according to the manufacturers' protocol. For primer sequences, see Table S1.
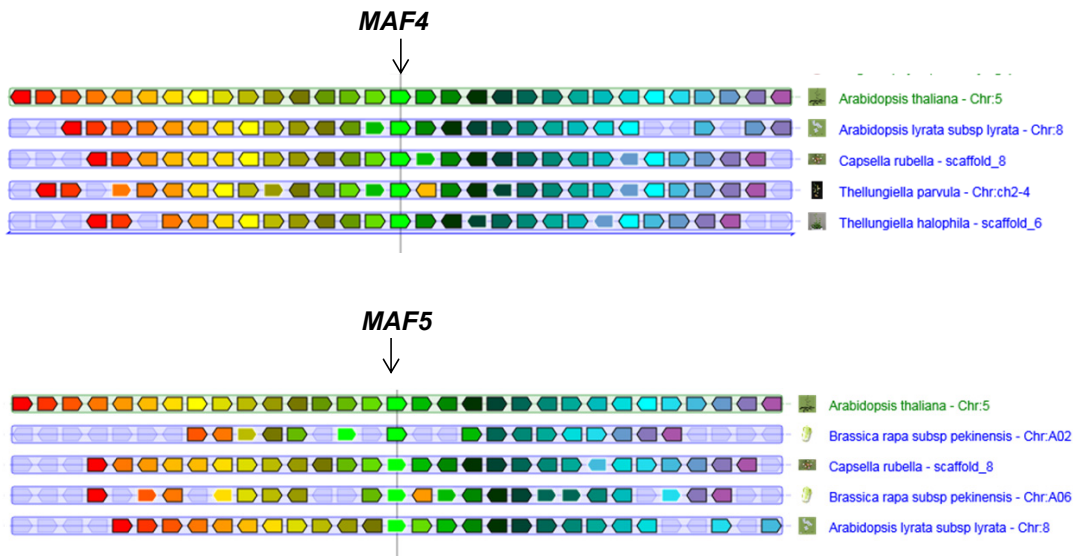
Supplementary data

MAF4

MAF5

Figure S1: Synteny of MAF4 and MAF5. Like MAF3, MAF4 and MAF5 can be found back in two major tribes of the Brassicaceae family, indicating that these genes originate from a common ancestor of the Brassiceae.

Figure S2: Sequences of MAF spice forms used in this study

MAF2.1
ATGGGTAGAAAAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGTAGTCGACAAGTCACTTTCTCCAAACGACGCAATGGTCTCATCGAGAAAGCTCGAC
AACTTTCAATTCTCTGTGAATCTTCCATCGCTGTTCTCGTCGTCTCCGGCTCCGGAAAACTCTACAAGTCTGCCTCCGGTGACAACATGTCAAAGATCATTGA
TCGTTACGAAATACATCATGCTGATGAACTTGAAGCCTTAGATCTTGCAGAAAAAACTCGGAATTATCTGCCACTCAAAGAGTTACTAGAAATAGTCCAAAG
CAAGCTTGAAGAATCAAATGTCGATAATGCAAGTGTGGATACTTTAATTTCTCTGGAGGAACAGCTCGAGACTGCTCTGTCCGTAACTAGAGCTAGGAAGA
CAGAACTAATGATGGGGGAAGTGAAGTCCCTTCAAAAAACGGTGGGGAAGAAGACGTTTCTGGTTATAGAAGGTGACAGAGGAATGTCATGGGAAATG
GCTCCGGCAACAAAGTACGGGAGACTCTTCCGCTGCTCAAGTAA

MAF2.2/MAF2var5
ATGGGTAGAAAAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGTAGTCGACAAGTCACTTTCTCCAAACGACGCAATGGTCTCATCGAGAAAGCTCGAC
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CAAGCTTGAAGAATCAAATGTCGATAATGCAAGTGTGGATACTTTAATTTCTCTGGAGGAACAGCTCGAGACTGCTCTGTCCGTAACTAGAGCTAGGAAGA
CAGAACTAATGATGGGGGAAGTGAAGTCCCTTCAAAAAACGGAGAACTTGCTGAGAGAAGAGAACCAGACTTTGGCTAGCCAGGTGACAAAAACATCTC
TTGAAGCTAATTCATCAGTTGATACACAATAA

MAF2.3/MAF2var1
ATGGGTAGAAAAAAAGTCGAGATCAAGCGAATCGAGAACAAAAGTAGTCGACAAGTCACTTTCTCCAAACGACGCAATGGTCTCATCGAGAAAGCTCGAC

AACTTTCAATTCTCTGTGAATCTTCCATCGCTGTTCTCGTCGTCTCCGGCTCCGGAAAACTCTACAAGTCTGCCTCCGGTGACAACATGTCAAAGATCATTGA
TCGTTACGAAATACATCATGCTGATGAACTTGAAGCCTTAGATCTTGCAGAAAAAACTCGGAATTATCTGCCACTCAAAGAGTTACTAGAAATAGTCCAAAG
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CAGAACTAATGATGGGGGAAGTGAAGTCCCTTCAAAAAACGGAGAACTTGCTGAGAGAAGAGAACCAGACTTTGGCTAGCCAGGTGGGGAAGAAGACG
TTTCTGGTTATAGAAGGTGACAGAGGAATGTCATGGGAAAATGGCTCCGGCAACAAAGTACGGGAGACTCTTCCGCTGCTCAAGTAA

MAF2var2
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TCGTTACGAAATACATCATGCTGATGAACTTGAAGCCTTAGATCTTGCAGAAAAAACTCGGAATTATCTGCCACTCAAAGAGTTACTAGAAATAGTCCAAAG
GTTAGCACAAAGACACTTTTATCTCCCTCTTCTTCTGATGAAAAATACTTTTTTTTTTCTTTTCTTTTGGCGAATTATGAATACAGCAAGCTTGAAGAATCAAA
TGTCGATAATGCAAGTGTGGATACTTTAA

MAF3.2
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ACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGCTGCTCAAGTAA

MAF3-3
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GCCAGGTGGGGAAGAAGACGTTTCTGGTTATAGAAGGTGACAGAGGAATGTCACGGGAAAATGGCTCCGGCAACAAAGTACCGGAGACTCTTTCGCTGC
TCAAGTAA

MAF3-4
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MAF3-5
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AGAAGACAGAACTAATGATGGAGGATATGAAGTCACTTCAAGAAAGGGAGAAGTTGCTGATAGAAGAGAACCAGATTCTGGCTAGCCAGGTGGGGAAG
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MAF4.1
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MAF4.2
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MAF4.3

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MAF4.III

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MAF5.1

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CGCTTCTCAAGTAA

MAF5.2

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MAF5.I

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MAF5.II

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GAAAATAGCTCTGGCCACAGCCCACCGGAGACTCTCCCGCTTCTCAAGTAA

MBP08var1

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MBP8var2

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MBP8var3

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ACCCCACATGATTTGTGGACAACAAAAAGTTACCCTGAATTTCTTCTAG
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MBP25var1

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CACCGATATAACACCCACATGAAAGCAGAAGATAAGGTCTCTGCAGAAGTCGATGACACCGAGGAATCAAAATATGCAAGTTTCATGAGAATGGAAGAAC
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GAATTTTCTCTA
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MBP25var2

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CAAGGAAAAGCAGCTGATTGATGAAACAAAGTTCTGGAGGATGAGATAGGTACCATAAAGAACAGCGAAGAAATGAGATGGCAATGAATCTAAATAA
CATCGCACCAACCCATATGGATTGTGGACAACAGAGGGTGACCCTGAATTTTCTC
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MBP25var3

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ATGGGAAGGAAGAAAGTGGAAATAAAGCGAATTGAAGATAAGAGCAGTAGGCAAGCAACTTTCTCCAAACGGAGAAATGGACTCATGAAGAAAGCTAA
ACAGCTCTCTGTTCTCTGCGATGTTGATGTCGCCGTCCTCGTCTTCTCCAGTCGCGGACGCCTCTTTGAATTCTCCAGTACCAACAGTCTGACAGGAGTTATT
CACCGATATAACAACCACATGAAAGCAGAAGATAAGGCCTCTGCAGAAGTCGATGACACCGAGGAATCAAAATATGCAAGTTTCATGACAATGGAAGAAC
TGCTACAGACGGCAGAAAAGTACTGACCTTTTGCTGCTCGTTTTTTTCTTCTTTTGGAGCTTCCTTGGAAAGCATGGTATTGATCCACAATTTGACAGGCAAC
TCGAGGAGCCTGATGTTGATGATCTCAGCATTACTGATCTTGTCCATCTGGAAAACCAAGTTGAGACTGCTCTGACACAAACCAGATTTAGAAAGACACACT
TGTTGATTGAATCAATTAAGAATCTTCATGACAAGGAAAGCAGCTGATTGATGAAACAAAGTTCTGGAGGATGAGATAGGTACCATAAAGAACAGCGA
AGAAAATGAGATGGCAATGAATCTAAATAACATCGCACCAACCCATATGGATTGTGGACAACAGAGGGTGACCCTGAATTTTCTC
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Table S1: oligo's used in this study

FW SOC1	GCTTGAAACCTCATCCTTTAC
RV SOC1	GATGTGTATGTAAAAATGTCCATATG
FW pGEM-T (for SOC1 probe production)	CATGGCCGCGGGATT
RV pGEM-T (for SOC1 probe production)	GCGGCCGCACTAGTGATT
FW qPCR primer on MBP8-2exons	ATCCAGGTCGCAGAGACA
RV qPCR primer on MBP8-2exons	TGTCCTCCAGATGTTTCTTTTCC
FW qPCR primer on MBP8	ATCCAGGTCGCAGAGGTG
RV qPCR primer on MBP8	AGGTCAGTCAAAGTGAGACCA
FW qPCR primer on MBP25	AGAAGTCGATGACACAGAGGAAT
RV qPCR primer on MBP25	TCAGGCTCCTTGAGTTGCTT
FW qPCR primer on MBP25+intron	GACGGCAGAAAAGTACTGACC
RV qPCR primer on MBP25+intron	GCTCCTCGAGTTGCCTGTC

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Chapter 8

Discussion and conclusion

Chapter 8: Discussion and outlook

In this thesis, we investigated the role of alternative splicing in ambient temperature-dependent floral timing. The foundation of this research was an RNAseq experiment, which revealed that many splicing-related genes are differentially spliced upon a change in ambient temperature. The most interesting finding concerning floral timing was the differential splicing of the *A. thaliana* flowering time gene *FLM*. We uncovered by which mechanism alternative splice forms of *FLM* control flowering time, and investigated to what extent this is conserved in other plants. Moreover, we provided some first insights into the mechanism that causes differential splicing of *FLM* under different temperatures. In this final chapter, I will put the findings of this thesis into perspective, discuss several papers that commented on our findings, and give my advice for further research on this subject.

The alternative splicing response upon ambient temperature fluctuation

Differential splicing of splicing related genes might be a more general mechanism for sensing the environment

In chapter 3, we showed that many classes of genes related to the splicing machinery are target of alternative splicing when plants are subjected to changing ambient temperatures. We proposed a two-step model in which temperature influences directly the splicing of these splicing related genes, which alters the composition of the spliceosome, and consequently the splicing of downstream genes. One of the classes of splicing-related genes that we found differentially spliced concerns the SR genes, which have been receiving more and more attention recently. This was probably fuelled by the findings that in yeast, where alternative splicing is rare, most of the splicing reactions are carried out by a single SR protein (Kress et al., 2008), indicating that these proteins might be instrumental for alternative splicing. Several studies have shown that stresses, like salt, high-light irradiation, extreme temperatures, and hormones cause differential splicing in several SR genes (Duque, 2011, Filichkin et al., 2010, Iida et al., 2004, Palusa et al., 2007, Reddy and Shad Ali, 2011, Tanabe et al., 2007), as reviewed exhaustively by Staiger and Brown (Staiger and Brown, 2013). This prompted the authors of the above-mentioned studies to hypothesize that SR proteins play an important role in the stress response of the plant. In our study, we showed that upon ambient temperature alterations, not only SR genes, but many different classes of spliceosomal genes are differentially spliced. Thus, at least under ambient temperature-fluctuations, a large part of the spliceosome, rather than just the SR genes, are targeted for differential splicing. However, it is not unlikely that the splicing of many components of the splicing machinery is a general mechanism upon all kinds of environmental fluctuations. In fact, a study on insect herbivory in *Niccotiana attenuata* briefly mentioned that in root samples, the GO-terms "RNA splicing" and "mRNA metabolic process" are enriched (Ling et al., 2015). Another article focussing on the influence of environmental stress on intron

retention of circadian transcripts, reported that upon heat stress, genes harbouring differentially expressed introns are enriched in the GO-terms "RNA splicing" and "mRNA processing" (Filichkin et al., 2015). A study examining alternative splicing under salt stress also noticed enrichment of these GO-terms, but consequently focus on the splicing of SR genes (Ding et al., 2014). Thus, in retrospect, several studies suggested that splicing related genes are alternatively spliced when plants undergo environmental fluctuations, but it has never been fully assessed like in our study on ambient temperature (Chapter 3). Therefore, the two-step model we propose for ambient temperature directed alternative splicing might as well be applicable for environmental responses in general.

Conservation of the alternative splicing response

The RNAseq experiments in chapter 3 were executed on 2 accessions of the model plant *Arabidopsis thaliana*, and on one variety of the crop plant *Brassica oleracea var. botrytis* (cauliflower), which were all subjected to a change in ambient temperature. We showed that the genes encoding the spliceosome were the predominant targets of differential splicing in all three genotypes. Surprisingly, there was hardly any overlap between the differentially spliced genes, since the Gy-0 accession and cauliflower only showed 11% and 2% overlap with the Col-0 accession, respectively. This indicates that the alternative splicing response is highly divergent, and suggests that adaptation to the environment might- at least partly- be achieved by modifications in the splicing program. However, we should be cautious in drawing strong conclusions from these results. First of all, we only compared three different genotypes, and this small sample size might give a completely incorrect view of the true conservation. Moreover, there might also be a technical explanation that partly explains the low overlap of differentially spliced genes between the three genotypes we assessed. A study on detection of differential gene expression between closely related species showed that the ability of Illumina-based RNAseq to identify significant differentially expressed genes is positively associated with the extent of expression-fold change (Liu et al., 2011). Thus, the lower the expression-fold change, the higher the number of false-negatives. The same holds for differential splicing events, where we observed that the change in splicing isoform occurrence is generally low (approximately 85% of the cases <2 fold). Hence, we can expect a relatively high amount of false-negatives. As a consequence, the list of differentially spliced genes within one genetic background will not be completely exhaustive. This will probably not influence the outcome of our enrichment analysis a lot, but it might result in a reduced overlap of differential spliced genes in the three genotype datasets.

Nevertheless, the hypothesis that alternative splicing may have played an important role in adaptation to the environment during evolution, remains an intriguing concept. Hence, it is worthwhile to further investigate the conservation of the splicing response in more species and with other techniques, for example by PacBio Iso-Seq, that produces full-length transcript reads and bypasses the transcript-reconstruction process, resulting in a more

accurate measurement (Gonzalez-Garay, 2016). Alternatively, a digital array system
(Fluidigm) could be used. Researchers showed that using this system, they could accurately
quantify exon skipping levels in human material (Spitali et al., 2010).

FLM splicing, a complex system to regulate floral timing

Besides splicing related genes, we also found differential splicing of several flowering time
genes. _FLM_ appeared to be the most interesting one, since previous studies showed that this
gene is involved in the ambient temperature-directed flowering time pathway
(Balasubramanian et al., 2006, Werner et al., 2005). However, its mode of action had
remained enigmatic. The finding that this gene was alternatively spliced upon transition to
higher or lower ambient temperature led us to the hypothesis that regulation of flowering
time by ambient temperature might be controlled through alternative splicing of _FLM_. In the
Col-0 accession, the main splice forms _FLMβ_ and _FLMδ_ are differentially expressed when
ambient temperature changes, with β decreasing and δ increasing when the temperature
becomes higher (and vice versa). We demonstrated that FLMβ forms an obligate
heterodimer with SVP, forming a complex that suppresses the flowering process. With EMSA
experiments, we demonstrated that addition of FLMδ to a constant pool of FLMβ and SVP
resulted in a gradual decrease in FLMβ/SVP binding to the target DNA. This is a strong
indication that FLMδ functions as a dominant negative form, titrating out SVP and
preventing repressive SVP/FLMβ-complex formation. This is supported by the fact that
overexpression of FLMδ induced earlier flowering, which can be explained by a dominant-
negative effect of this splice form. Further research should focus on gathering _in planta_
proof for the model we present, and quantification of FLMβ and FLMδ isoforms at different
temperatures.

At the same time when we published our results (Posé et al., 2013), another study showed
that SVP becomes less stable as temperature rises, resulting in less SVP that can form
repressive complexes at higher ambient temperature (Lee et al., 2013). Most likely, these
two mechanisms act in parallel to efficiently reduce the amount of floral repressive
complexes and allow floral induction when temperatures rise. In fact, our model requires a
limiting amount of SVP to efficiently titrate out FLMβ by FLMδ, and reduced levels of SVP at
higher temperatures facilitate this.
A more recent study showed the possible involvement of other _FLM_ splice forms in the
regulation of flowering time (Sureshkumar et al., 2016). The authors of this study showed
that besides the known isoforms _β,δ,α_ and _γ_, _FLM_ produces more splice forms, of which two
were differentially produced between plants grown constantly at 23° and 27° C. They
showed that these novel splice forms are targets of nonsense-mediated decay (NMD), and
through producing more of these NMD forms at higher temperature, production of the
other isoforms, amongst which the repressive _FLMβ_ isoform, is reduced. These results show
that regulation of flowering time through alternative splicing of _FLM_ is even more

complicated than we previously assumed. However, caution should be taken by interpreting these results, since they are based on RNAseq experiments performed with 6-day-old seedlings grown under short day (SD) conditions. At this stage, the plantlets are most likely still in the juvenile phase, and have not yet acquired sensitivity to floral induction (see for example (Willmann and Poethig, 2011)). Under these conditions, at least in the hands of the authors, the *FLMβ/FLMδ* ratio was not significantly different in seedlings constantly grown under 23° C or 27° C, and it is very well possible that the observed splicing pattern of the NMD targeted isoforms is not completely representative for adult plants. Thus, further research is necessary to fully prove this hypothesis.

Altogether, it is clear that the mechanism by which FLM controls flowering time is less black and white as we first assumed. It is very conceivable that the different mechanisms mentioned above - the change in FLMβ/FLMδ ratio, the degradation of SVP at higher temperatures and the downregulation of *FLM* by production of NMD targets - work in a concerted manner as a powerful mechanism to facilitate the floral transition when temperature rises.

Family matters – a guide to identify *FLM*-like genes in plants lacking *FLM*

FLM is a key player in thermosensitive floral timing of *A. thaliana*, that originated relatively recently, and can only be found in the Camelineae clade of the Brassicaceae. Presence of *MAF2*, which has also been shown to act as a thermosensitive floral regulator in a less potent way, probably due to the lack of a dominant-negative isoform, is even restricted to *Arabidopsis* species. However, many flowering plant species from a wide variety of families are known to adapt their flowering time to ambient temperature, which raises the question how this is regulated. As reviewed in chapter 2, several mechanisms parallel to *FLM* and *MAF2* have been proposed to function in the thermosensitive floral pathway. Therefore, it is possible that in other plant species, *FLM or MAF*-like genes do not have a key position in this pathway, as is the case for *A. thaliana*. However, in Chapter 7 we showed that in tomato, a Solanaceae, at least two of the three *FLC*-like genes are alternatively spliced upon ambient temperature changes, suggesting that *FLC*-like genes may function in ambient temperature flowering time regulation through alternative splicing.

When searching for a gene functionally homologous to *FLM* or *MAF2* in a species of interest, there are a few conditions an *FLC*-like gene has to meet. First of all, the gene should show substantial expression in the period that the plant faces ambient temperature fluctuations. Second, the gene should produce several splice forms, that are differentially expressed under different ambient temperature conditions. Finally, the proteins encoded by these splice forms should include a flowering repressive form (one that binds both to the DNA and to an SVP-like protein), and isoforms that are either non-functional (like MAF2var2) or that function in a dominant-negative fashion (like FLMδ). Imaginably, non-functionality is easy to

acquire, for example by splicing in such a way that a premature stop codon is introduced. In that case, the transcript will either be targeted for nonsense-mediated decay, or the protein will be truncated. In Chapter 7, our preliminary EMSA data showed that a dominant-negative function is probably more widespread, since we found these kind of isoforms in both *A. thaliana MAF4* and *MAF5*. A dominant-negative function can be acquired when DNA-binding capacity is lost, while the ability to interact with SVP is still present, like in *FLM* and *MAF4/5*, but there are more possibilities. For example when higher order complex formation or transcriptional repression is lost, while dimerization and DNA binding activities are still intact.

Altogether, when searching for a gene functionally homologous to *FLM* or *MAF2* in a species of interest, the above-mentioned conditions are very relevant, and relatively easy to test before starting the often laborious (and in many plant species even not-established) procedure of targeted mutagenesis.

How plants feel temperature

Although our discoveries on alternative splicing in general, and more particularly of *FLM,* gave us a good idea how plants react to temperature and adapt flowering time at the molecular level, the question how plants perceive temperature remained largely enigmatic. In chapter 6, we gave a first insight into the possible role of RNA polII speed on alternative splicing. Previous experiments performed *in vitro*, as well as *in vivo* experiments in budding yeast have shown that ambient temperature influences transcriptional elongation rate by this molecule (Abbondanzieri et al., 2005, Miguel et al., 2013). Because polymerase II speed has been shown to affect alternative splicing, at least in human cells, we hypothesized that *FLM* is differentially spliced by changing temperatures through alteration of RNA polII-determined transcriptional elongation rate. To test this hypothesis, we had to investigate if an altered polII speed indeed influences *FLM* splicing. These kind of experiments are challenging in multicellular organisms, since it requires the possibility to inhibit (but not block) or increase polII speed, or the use of mutants expressing a crippled FRNA polII. In yeast, both drugs affecting RNA polII speed, as well as mutants with a slower RNA polII have been described. However, such protocols and mutants have not been developed in plants as yet. Therefore, we decided to use a different approach, in which we altered RNA polII speed by expressing *FLM* under a strong promoter. The preliminary results presented in chapter 6 are very promising, since we indeed observed an altered *FLMβ/FLMδ* splicing ratio and altered floral timing. When these data turn out to be reproducible, additional experiments should focus on proving that the observed altered splicing ratio is indeed caused by a higher RNA polII elongation rate, and not by other attributes of expression under the 35S CaMV promoter. Because it is very challenging to do this in plants, initial experiments could include introduction of the *FLM* gene in yeast mutants with altered RNA polII speed, to prove that transcriptional elongation rate can change the splicing ratios, as observed under the 35S

promoter in *A. thaliana*. Subsequently, a similar approach could be used in protoplasts, or, preferably, the experiments could be performed in plants with an altered RNA polII speed, if it turns out that such mutants can be generated.

When RNA polII speed is indeed responsible for differential splicing of *FLM*, the question remains why only a selected set of genes are differentially spliced when temperature changes. We may get some insights from recent advances regarding temperature dependent differential expression (Sidaway-Lee et al., 2014). In this study, the authors measured the mRNA synthesis and degradation rates of the *A. thaliana* transcriptome at low and high ambient temperature. They showed that transcription rates as well as decay rates of all genes increase as temperature rises. For most genes (98% of the transcriptome), the higher transcription rate is balanced out by the higher degradation rate, and net gene expression remains unaffected. However, for some genes, the transcription rate goes up more than in other genes, whereas the decay rates are roughly similar for the whole transcriptome, resulting in more mRNA accumulation of these genes at higher temperature. This means that certain genes are transcribed at a higher rate upon higher temperature perception than others. Although this study focusses on expression levels, the observed differences of transcription rate might affect splicing in a similar way as it affects expression. Regarding expression, the genes that show a lower-than-average transcription rate at low ambient temperatures, have enriched occupancy of the histone variant H2A.Z. As we reviewed in chapter 2, it was previously shown that this histone variant replaces the canonical histone H2A at lower temperatures, and is proposed to confer distinct DNA-wrapping properties on nucleosomes that inhibit transcription (Kumar and Wigge, 2010). Besides the high level of H2A.Z in genes with a low transcription rate, histone mark H3K27me3 was associated with genes showing an either higher or lower than average transcription rate. Thus, it appears that expression is regulated through the direct effect of temperature on transcription rate, but specificity is provided by the chromatin state of the genes. In chapter 3 we showed that only a subset of intron-containing genes are differentially spliced upon altered temperature, just like only a subset of genes is differentially expressed when temperature changes. Hence, it is a conceivable theory that a comparable mechanism is possible for alternative splicing. Further research has to demonstrate whether this is indeed the case, and which chromatin marks are involved.

Secondary RNA structure

Another way how temperature can directly affect splicing is trough secondary RNA structure. As reviewed in chapter 2, miRNAs seem to be involved in the ambient temperature flowering time pathway parallel to *FLM/SVP*. A recent article shows the involvement of the pri-miRNA structure in the temperature response of miR156. The study shows that miR156 accumulates differentially at different temperatures due to differential processing of pri-miR156a (Kim et al., 2016). The authors identified structural determinants for pri-miRNA processing and show that the second stem segment in the upper stem of pri-miR156a plays a role in ambient

temperature-responsive miR156a processing. Moreover, they demonstrated that overexpression (OX) of this structural variant resulted in an altered leaf number ratio compared to OX of the non-mutated pri-miR156a, suggesting that this specific region in the pri-miRNA is involved in thermosensitive floral timing. Although this theory is solely based on overexpression analyses, and mutant analysis should be performed to fully prove this model, the results are promising and show how RNA structure can directly affect flowering time.

Whether RNA structure is involved in other aspects of temperature-sensitive floral timing remains enigmatic. Interestingly, a study assessing conservation of secondary structures in the human genome, identified RNA-structure elements that associate with alternative splice site selection (Shepard and Hertel, 2008). Moreover, they found that these structural elements are enriched in splicing related genes. Because we also demonstrated that splicing related genes are enriched in differential splicing upon temperature changes, it would be worthwhile to investigate whether these structural elements are also enriched in the differentially spliced genes that we identified in chapter 3, including *FLM*.

Thermosensitive floral timing under natural conditions

The last few years, new knowledge about the mechanisms underlying ambient temperature directed floral timing has been generated, and our perception on this topic has advanced greatly. As described in chapter 2, besides our findings on FLM/SVP, thermosensitive floral timing can be controlled trough chromatin accessibility facilitated by histone-variant H2A.Z, miRNAs targeting flowering time accumulate at different amounts at different temperatures, and the basic-helix-loop-helix transcription factor PIF4 is proposed to activate flowering at higher temperatures under a non-inductive photoperiod, although more recently the role of this protein in the thermosensory flowering pathway is suggested to be minor (Galvão et al., 2015). For PIF4, it was shown that this protein is only involved under short days. Whether the other above-mentioned pathways are of equal importance, or act more specifically under particular conditions, remains to be determined. In general, all experiments have been executed under optimized laboratory conditions that are far from the conditions plants experience in a natural environment. An important aspect of this is that in most laboratories, experiments were conducted under constant temperatures, without a day/night temperature cycle. However, in nature, plants are exposed to considerable temperature fluctuations and temperature differences between days and nights. The importance of taking these temperature oscillations into account is illustrated by a recent study that shows that cool night-time temperatures induces *FT* expression, likely through SVP and FLMδ (Kinmonth-Schultz et al., 2016). The authors demonstrated that under a temperature regime of 22°/12° C (day/night, long day light conditions), SVP levels remained higher after dusk and into the night. Clearly, there is still a lot to discover about the interaction with other flowering pathways integrating environmental and internal cues.

Concluding words

How plants integrate ambient temperature to modulate flowering time was, until recently, largely unknown. Even the way how plants sense temperature had remained enigmatic, since temperature sensors like they exist in animals had never been discovered in plants. In the last few years, research on thermosensitive floral timing has made major advances. We and other researchers have demonstrated the presence of several –most likely parallel– pathways that regulate flowering time upon changing ambient temperatures. It became clear that a single thermosensor most likely does not exist in plants, and a model in which temperature is sensed through thermodynamic properties of DNA, RNA and proteins, is gaining support. However, first evidences on the true mechanisms are just emerging, and a lot of exciting discoveries are awaiting the researchers that will investigate this in the future.

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Summary

As a consequence of a sessile lifestyle, plants are constantly facing a fluctuating environment. In order to both profit maximally and protect themselves from these environmental cues, plants evolved ways to sense and respond to signals. Ambient temperature is one of the cues for which plants have acquired a strategy to enhance their chance of survival and reproduction. Small changes in ambient temperature can have major effects on plant architecture and development, such as the transition from the vegetative to the reproductive flowering phase. The moment of flowering is an important event in the life cycle of a plant, since reproductive success depends on it.

In **Chapter 1**, I introduced the concept of alternative splicing, a molecular mechanism with a pivotal role in ambient temperature regulation of flowering time. In the model plant *Arabidopsis thaliana*, approximately 60% of the intron-containing genes show alternative splicing. Gene splicing varies depending on developmental stage and tissue type, but also environmental changes trigger differential splicing. Splicing is conducted by a large cellular machinery called the spliceosome, which recognizes intron-defining sequences and other cis-regulatory elements acting as splicing enhancers or silencers. Moreover, factors like chromatin structure, histone marks, RNA polymerase II (polII) elongation speed and the secondary structure of the pre-mRNA all play a role in the splicing outcome. Due to alternative splicing, a single gene can yield various transcripts. However, this does not cause an equal expansion of the proteome. Part of the transcripts are targeted for nonsense-mediated decay, or will be translated into unstable proteins. This is a way of regulating gene expression at the post-transcriptional or –translational level. Other transcripts will be translated into functional proteins that may be structurally and functionally different. Hence, alternative splicing creates additional complexity in the transcriptome, providing plants with molecular tools to respond to their environment, including the translation of ambient temperature alterations into a flowering time response.

In **Chapter 2**, we reviewed the current knowledge on molecular mechanisms that control the ambient-temperature directed flowering time pathway in the plant model species *Arabidopsis thaliana*. Several different mechanisms have been proposed, like alternative splicing of *FLOWERING LOCUS M* (*FLM*) (described in Chapter 4) and protein degradation of *SHORT VEGETATIVE PHASE* (*SVP*), two mechanisms that probably work in a cooperative manner to release floral repression at higher ambient temperatures. Another mechanism that is involved at high ambient temperature is the replacement of the canonical histone H2A by the variant H2A.Z. As a consequence of this replacement, chromatin becomes less tightly wrapped around the nucleosomes, which allows transcription of flowering time activators, such as *PHYTOCHROME INTERACTING FACTOR 4 (PIF4)*. Lastly, we discuss microRNAs (miRNA) that can either repress or activate flowering (miR156 and miR172, respectively). These miRNAs have been proposed to be regulated by low and high ambient temperature. However, due to the lack of mutant analyses, more research is necessary to

show the true involvement of these factors. Altogether, there are several mechanisms acting partly in cooperation to regulate thermosensitive floral timing.

In **Chapter 3**, we analysed ambient temperature-directed alternative splicing events that occur after a temperature shift by RNAseq. We performed the experiment in two different accessions of *A. thaliana*, and in one variant of *B. oleracea* (cauliflower). We showed that flowering time genes are overrepresented amongst the ambient temperature induced alternatively spliced genes, but also genes encoding components of the splicing machinery itself, indicating that alternative splicing is one of the potential mechanisms by which plants are able to sense temperature and adapt floral timing. Analysis of a mutant for one of these alternatively-spliced splicing related factors, *ATU2AF65A*, showed a temperature-dependent flowering time phenotype, confirming its proposed role in the flowering time response upon temperature fluctuations. Based on these findings, we proposed a two-step model in which splicing related genes are targeted for differential splicing upon ambient temperature fluctuations, which results in changes in the composition of the spliceosome, causing differential splicing of downstream genes that affect the development and architecture of the plant, including flowering time.

In **Chapter 4**, we investigated the molecular mode-of-action of *FLM*, one of the differentially spliced flowering time regulating genes that we identified in Chapter 3. We showed that in *A. thaliana* Col-0, the main splice forms of *FLM* are *FLMβ* and *FLMδ*. FLMβ forms an obligate heterodimer with SVP, and this complex represses floral integrators like *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* by binding to the regulatory regions of these genes. FLMδ also dimerizes with SVP, but this complex is not able to bind to DNA. When temperature rises, more FLMδ is produced at the cost of FLMβ. Hence, less repressive complexes can be formed. However, the fact that FLMδ is still able to binds SVP makes it function as a dominant negative form, titrating out SVP and preventing repressive SVP/FLMβ-complex formation.

Chapter 5 is a short comment written to clarify the concept of thermoplasticity in flowering time control. Occasionally, this concept is confused with adaptation to different ambient temperature environments on the long term. Thermoplasticity is the ability to adapt flowering time to fluctuations in ambient temperature within one life cycle. Furthermore, some genes have been marked as players in the ambient temperature response, whereas these appear to be general flowering repressors or activators, affecting flowering time in a similar manner at low and high ambient temperature. In order to interpret novel findings on thermosensitive flowering time control, it is essential to distinguish between these various concepts.

In **chapter 6**, we unveiled the first indications that differential splicing of *FLM* can be caused by differences in polymerase II elongation rate. We mimicked a situation in which *FLM* is transcribed at a higher rate, by expressing the genomic *FLM* gene under a strong artificial promoter. Preliminary results showed that plants harbouring this construct have altered flowering time and temperature-responsiveness, which can be explained by the altered *FLMβ*/*FLMδ* ratio that we observed.

In chapter 7, we assessed the functional conservation between *FLM* and closely related genes at the intraspecific level in *A. thaliana*. *FLM* (also called *MAF1*) is a member of the *FLC*-clade, that consists of *FLC*, *FLM* and *MAF2-5*. *FLC* is widely known for its function in the vernalization pathway, whereas *MAF2* has been shown to regulate flowering time through alternative splicing in a way very similar to *FLM*. For the other *MAF* genes, not much is known. We showed that all of these genes produce splicing isoforms that function in a more or less similar way to *FLM* and *MAF2*. Despite the high functional conservation at the intraspecific level, *FLM* and *MAF* orthologues are not widely present. Through synteny analysis, we showed that *FLM* and *MAF2* are very recent genes, which are only present in a small group of Brassicaceae species. *MAF3-5* originated less recently, but are not present outside the Brassicaceae. For *FLC*, it was previously shown that it originated from an ancestor of the seed plants, and in many plant species belonging to other families, presence of more than one FLC-like gene has been reported. This raises the question what the function of these genes is. In tomato, we showed that the *FLC*-like gene *MBP8* becomes differentially spliced upon temperature changes, suggesting a function in the ambient temperature pathway. A binding assay showed high similarities of the different MBP8 isoforms to FLM and MAF isoforms, but suggests a slightly different functionality, since all three isoforms showed binding to the DNA. Further research is necessary to confirm the role of *MBP8* in thermosensitive flowering time control, and elucidate the functionality of the different splice forms.

In **Chapter 8**, I discussed the finding of this thesis in a broader perspective, and make suggestions for future research. Over the last few years, several mechanisms that act in the temperature-directed floral pathway have been revealed. In this thesis, we showed that alternative splicing plays an important role, and we demonstrated how temperature may affect the splicing outcome directly through the effect of temperature on transcription elongation rate. It is becoming clear that most likely a single thermosensor does not exist in plants, and a model in which temperature is sensed through thermodynamic properties of DNA, RNA and proteins, is gaining support. Future research is assigned to the exiting task to elucidate the exact mechanisms by which temperature-sensing is achieved in different plant species and to determine how conserved the currently identified molecular mechanisms are.

Acknowledgements

Several years ago I had booked a holiday to Madeira, the Portuguese island off the coast of Morocco. At that moment, I was an employee at Science Museum NEMO, where I was working on several projects that aimed to get children of all ages interested in science and technology. It was the first "real job" after finishing my study Biology in Wageningen, and I found it quite exciting to be working at a science museum. But as time went by, I started to feel that I was missing something. I was trying to convince young people to get involved in the exiting world of science, whereas I was not part of that world myself anymore.
I started to toy with the idea to go back to academia and start a PhD position, but as this felt like a major career change at that moment, I needed some time to think. With the holiday to Madeira coming up, I decided that this was a perfect opportunity to make this important decision. When I would get back on the plane to return to the Netherlands, I should have decided what I wanted to do with my life.

Flashing forward for five and a half years, here I am, writing this acknowledgement for my thesis. It is dark outside the Radix building and my thesis is ready for printing; except for this last part. I guess I don't have to explain what the outcome of that holiday was. Nevertheless, despite all the thinking I did that time, I couldn't have imagined what was lying ahead of me. If you think working at a science museum is exiting, try doing a PhD! Especially after having a "normal job" for two years, the dynamic life of a scientist was a big positive surprise to me.

And then the group I ended up in……..I quickly realised that I couldn't have ended up better. PDS, what an amazing group you are! Serious about science, serious about having a lot of fun together! I have so many people to thank, I don't know where to start. So let's start with the foundations of the lab; PDS is the most organized academic lab I have ever seen. We are never out of stock of something, if we change brands for a certain kit, it is thoroughly tested, problems are directly taken care of and good (or bad) suggestions are always taken serious. This all would not be possible without our great lab managers Michiel and Marco. Thanks guys! And who thinks that "organized" means boring, couldn't be more wrong. I probably have to apologize for turning the lab into a disco every now and then (Lab dancing just got a whole new definition), although most of the times it was not only me enjoying pipetting on some good music ;). Thanks Sam, my direct lab neighbour, we have enjoyed (and criticized) a lot of music together. Also Froukje, we had a lot of fun together in the lab, either making jokes or having good conversations. Martijn, thanks for all the ice in my neck, it was truly refreshing :P. Michiel, also you made my lab life unforgettable; sometimes the most unexplainable things would happen, when it seemed that nobody was around – and then later I would find out you were pranking me and were hiding behind the curtain of the dark room.

Outside of the lab, in the office space, there's also a lot of people to thank. First of all, the open workspace. We have enjoyed numerous dinners, movies and other outings together. For this, I want to thank Alice and Suraj, who I think are the main initiators of these events. Thanks for all the great evenings and weekends! And thanks everyone for sharing these nice moments together: Hilda, Suzanne, Anneke, Sam, Marian, Baojian, Mengfan, Rufang, Manjunath, Patricia, Vera, Han……and many people that have left the group: Cezary, Hui, and our many Brazilian guests who introduced me throughout the years into the Brazilian lifestyle and cuisine (and not to forget the caipirinhas): Eveline, Fabia, Nayara, Sinara, Haynna, Livia, Lilian, and a special thanks to "the three musketeers" Denis, Diego and José, and Greice. It was a very special time we had here together in Wageningen. Zé and Greicechje, I am happy we still talk every now and then, I miss you, and a visit to Brazil to see you guys is still on the planning! Throughout the years there have been many students visiting the lab, and I was lucky to supervise one of them: Gian Luca. I want to thank you for the work you did, without you Chapter 6 wouldn't have been the same. It was really nice to have you as my student, and we are still missing you on Fridays when we have too many fries ;).

Hilda and Suzanne, you have been very important to me. Doing a PhD is a very special job that challenges you to the max, not only because of the many hours, but also (and most importantly) on a personal level. And it is safe to say that it is not all roses. But luckily, we had each other (and each other's chocolate ;)) to get through the hardest parts. Thanks for everything girls, and good luck with the last part of this journey!

Than the people from the "real offices", the senior and junior scientists and the technicians. Thanks for all the fun and great scientific input. Ruud, Richard and Gerco, thanks for welcoming the group in your houses when we had something to celebrate or say goodbye to someone. I have great memories of these evenings we spent around the BBQ or a potluck dinner. Jan, thanks for statistical input and German Weizen.

Martijn and Tjitske (or Tjits-dinges, like Lejo called you), thanks for the amazing labuitjes. I really enjoyed our crazy outings, particularly when water was involved, and nobody was able to stay dry. And of course the years when we stayed overnight, accompanied with dance/karaoke evenings and many good Belgian beers. Last outing, when we were going wild on a self-declared dancefloor of an old farm house in the east of the country, a neighbouring group of people were surprised we could have so much fun with colleagues. I think this illustrates the atmosphere in our group very well.

Froukje and Michiel, my two "nimphjes", I am thankfull you will be at my side during my defence, you are very important to me!

Gerco, you are the mastermind behind the PDS group. You are a great scientist, a great manager and a great person. It is not often that a group leader possesses all these skills. Thank you for having me in your lab the last few years, the interesting scientific discussions, the personal support, and of course: the BBPA!

Richard, my direct supervisor, I couldn't have wished for a better mentor. You are an

amazing scientist, out-of-the box thinker and networker. You gave me space to develop myself as a scientist but were always there to brainstorm about cool scientific theories, even when you actually did not have time at all. I really enjoyed sparring with you about our ideas, especially because a half word is generally enough to understand each other. I really hope we will work together again in the future. Thanks for everything!

Outside the lab, there are also people I want to thank. My two dear friends Anja and Claudia, thank you for being there! Since we met 13 years ago, life has changed a bit. Claudia, you created a beautiful family, I wish you, Peter and the girls many good times, and I am looking forward to seeing your girls grow up. Anja, I am happy that you came back to the Netherlands before I am leaving, so we had some time to catch up after your Swiss adventures. We have many good memories together, and I am happy we are still making new ones. Edward called me to say that we might be turning grey (very slowly of course :P), but are definitely not losing our 'wild hairs'. Let's call it a win-win situation ;).

My housemates, Evert Jan, Kirsten, Koenraad and Joeri: I joined your house and you joined my life in the last 1,5 year of my PhD. It has really been great living with you guys, thanks for the good talks (I remember an evening where the philosophy books were gathered in the living room to support our discussion ;), the evenings watching television together, the nice apple cakes from the "garden apples" from Koenraad, and also for just being there when coming home.

And then last but not least. Besides my housemates, someone else has joined me in the last part of my PhD. Or as I like to say: I went to a congress and came home with a French guy :P Sam, we started our relationship on a distance, you in Lyon and me in Wageningen. Not necessarily easy, but it showed that together we are strong enough to overcome a thousand kilometres! Our apartment in Grenoble is ready for us, and I cannot wait to move in with you. Thank you for all your support during the last stretch of my PhD, you really helped me through the rough times. Je t'aime!

About the author

From the moment Leonie Verhage was born, on April 5th, 1984 in Zwijndrecht, The Netherlands, she was fascinated by nature, and especially by plants. This was fuelled by her grantfather, who took her on her wooden shoes into the garden for days and days during the summers of her youth. Together, they would sow, water, plant, graft and harvest. He tought her the names of all the different apple variaties in the yard by color-coded strings he would attach to the trees.

Hence, it was not a surprise that in 2003, Leonie decided to study Biology at Wageninen University. During her Bsc, she specialized in Plant Biology, and during her Msc she performed research in several molecular plant research labs, both in the Netherlands and in the USA.

After finishing her study, Leonie started a position at the educational department of Science Museum NEMO in Amsterdam, where she worked on projects to get children interested in science and technology. Soon however, she started to miss performing biology and science herself, and she decided to look for a PhD position in molecular plant science. In December 2011, she started as a PhD student in the lab of Gerco G.C. Angenent, under supervision of Richard G.H. Immink, where she performed research on the effect of ambient temperature on flowering time regulation, which resulted in the publication of this thesis. After her PhD, Leonie received an Agreenskills fellowship to perform structural and evolutionary research on floral regulator proteins at the group of François Parcy at the CEA in Grenoble, where she will continue her scientific carreer.

Publications

Leonie Verhage, Edouard I. Severing, Johan Bucher, Michiel Lammers, Jacqueline Busscher-Lange, Guusje Bonnema, Nicole Rodenburg, Marcel C.G. Proveniers, Gerco C. Angenent, Richard G.H. Immink *"Splicing-related genes are alternatively spliced upon changes in ambient temperatures in plants"* **Accepted with minor revisions in PLOS ONE**

A Pajoro, L Verhage, RGH Immink "*Plasticity versus Adaptation of Ambient Temperature Flowering Response"* **Trends in plant science** 21 (1), 6-8, 2016

L Verhage**,** GC Angenent, RGH Immink "*Research on floral timing by ambient temperature comes into blossom"* **Trends in plant science** 19 (9), 583-591, 2014

D Posé, L Verhage, F Ott, L Yant, J Mathieu, GC Angenent, RGH Immink "*Temperature-dependent regulation of flowering by antagonistic FLM variants"* **Nature** 503 (7476), 414-417, 2013

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to:	D.S.L. (Leonie) Verhage
Date:	17-01-2017
Group:	BU Bioscience and Laboratory of Molecular Biology
University:	Wageningen University & Research

1) Start-up phase	*date*
► **First presentation of your project** *Title:* The influence of ambient temperature on flowering time regulation	Feb 09, 2012
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day 2012 Amsterdam, NL	Nov 30, 2012
EPS PhD student Get2Gether 2016, Soest, NL	Jan 28-29 2016
► **EPS theme symposia**	
EPS theme 1 symposium "Developmental Biology of Plants" Wageningen, NL	Jan 19, 2012
EPS theme 1 symposium "Developmental Biology of Plants" Leiden, NL	Jan 17, 2013
EPS theme 1 symposium "Developmental Biology of Plants" Wageningen, NL	Jan 24, 2014
EPS theme 1 symposium "Developmental Biology of Plants" Leiden, NL	Jan 08, 2015
EPS theme 1 symposium "Developmental Biology of Plants" Wageningen, NL	Jan 21, 2016
► **Lunteren days and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren	Apr 03-04, 2012
Annual meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
Annual meeting 'Experimental Plant Sciences', Lunteren	Apr 14-15, 2014
Annual meeting 'Experimental Plant Sciences', Lunteren	Apr 13-14, 2015
Annual meeting 'Experimental Plant Sciences', Lunteren	Apr 11-12, 2016
► **Seminars (series), workshops and symposia**	
Thematic BU Bioscience seminars Aalt-Jan van Dijk "Predicting functional sites in protein sequences"	May 08, 2012
Thematic BU Bioscience seminars Sander Peters "150 tomato genomes project: exploring genetic variation"	Nov 27, 2012
Inv. Seminar Cristel Carles "Chromatin dynamics and cell fate in plants: From Genetics to Epigenetics"	Jan 16, 2013
EPS Flying Seminar Danny Tholen "Form and function of plant leaves"	Jan 22, 2013
EPS Flying Seminar Detlef Weigel "Arabidopsis thaliana as a model system for the study of evolutionary questions"	Feb 27, 2013
Thematic BU Bioscience seminars Jules Beekwilder "Synthetic biology of plant metabolites"	Jun 11, 2013
Thematic BU Bioscience seminars Elio Schijlen "Lessons learned and prospects in NGS"	Nov 26, 2013
Green Life Science Seminar Caroline Dean "Chromatin and antisense transcript dynamics regulating the switch to flowering"	Apr 14, 2014
Thematic BU Bioscience seminars Juan Carlos del Pozo Benito "Root development: Auxin, Cell cycle and the Ubiquitin pathway"	May 06, 2014
Inv. Seminar José María Seguí Simarro "New insights into the ultrastructural changes undergone by embryogenic microspores"	Sep 11, 2014
EPS Flying Seminar George Coupland "Seasonal flowering in annual and perennial plants"	Jan 19, 2015
Inv. Seminar Marcelo Dornelas "Using the non-model genus Passiflora to study the evolution of novelty in plant reproductive development"	Jan 27, 2015

EPS Flying Seminar Yves van de Peer "The evolutionary significance of gene and genome duplications"	Feb 03, 2015
EPS/PSG seminar series Martin Kater "Mining for Floral Meristem Regulatory Pathways in Arabidopsis and rice"	Mar 11, 2015
Inv. Seminar Francois Parcy "An integrated structural biology approach to flower development"	Oct 15, 2015
► **Seminar plus**	
► **International symposia and congresses**	
5th International PhD School in Plant Development, Siena, IT	Sep 25-28, 2012
Workshop on molecular mechanism controlling flower development, Presque'ile de Giens, FR	Jun 08-12, 2013
SPP1530 Workshop: Floral transition in Arabidopsis, Tübingen, DE	Sep 16-18, 2013
SPP1530 Workshop: Symposium genetic variation of flowering time genes and applications for cropt improvement, Bielefeld, DE	Mar 24-26, 2014
Workshop on molecular mechanism controlling flower development, Aiguablava, ES	Jun 07-11, 2015
► **Presentations**	
Talk: The effect of ambient temperature on flowering time regulation, Siena, IT	Sep 25, 2012
Poster: The role of alternative splicing in ambient temperature regulated flowering time control, Presque'ile de Giens, FR	Jun 08-12, 2013
Talk: The role of alternative splicing in ambient temperature regulated flowering time control, Tübingen, DE	Sep 16, 2013
Talk: Ambient temperature-directed alterternative splicing: conservation or divergence? Leiden, NL	Jan 08, 2015
Talk: Ambient temperature regulated alternative splicing, Aiguablava, ES	Jun 07, 2015
Poster: How do plants feel temperature? Lunteren, NL	Apr 11-12, 2016
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Sep 29, 2014
► **Excursions**	
Subtotal Scientific Exposure	*18.4 credits**

3) In-Depth Studies

	date
► **EPS courses or other PhD courses**	
EPS PhD course "Transcription Factors and Transcriptional Regulation", Wageningen, NL	Dec 17-19, 2013
EPS PhD course "the Power of RNA-seq", Wageningen, NL	Jun 05-07, 2013
Summer school "Integrated structural and cell biology", Les Houches, FR	Jul 07-Aug 01, 2014
► **Journal club**	
Jounal club of the PRI-PDS cluster	2012-2016
► **Individual research training**	
Subtotal In-Depth Studies	*10.9 credits**

4) Personal development

	date
► **Skill training courses**	
Mini-symposium "How to write a world-class paper"	Oct 17, 2013
Insight out (NWO conference for women in science)	May 24, 2016
KNAW discussion day ''PhD students on science 2.0''	May 01, 2015
► **Organisation of PhD students day, course or conference**	
Organisation of the 1st Wageningen PhD Symposium	Dec 10, 2013
Organisation of the WPC Impulse PhD lecture series	Feb 06-Apr 03, 2014
► **Membership of Board, Committee or PhD council**	
President of EPS PhD council	Mar 2013-Mar 2014
Subtotal Personal Development	*5.2 credits**
TOTAL NUMBER OF CREDIT POINTS*	**36.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits * A credit represents a normative study load of 28 hours of study.