Functional analysis of splice variant expression of MADS AFFECTING FLOWERING 2 of Arabidopsis thaliana

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Abstract The MADS-AFFECTING FLOWERING 2 (MAF2) gene of Arabidopsis thaliana has been characterized as a repressor of flowering. The molecular basis of MAF2 gene function and role of alternative MAF2 transcripts in flowering time modulation is not understood. MAF2 splice variant expression was quantified in cold-acclimated plants by quantitative RT-PCR. Cold influenced the abundance of splice variants and prompted a functional study of splice forms. Individual variants were overexpressed in the Col background and were assayed for their ability to delay flowering. Overexpression of MAF2 variants 2 and 4 had limited effect on flowering time. Overexpression of MAF2 splice variant 1 resulted in early flowering and affected the expression of the endogenous MAF2 gene and its paralogues, confounding functional assessment. In the L1-2 Arabidopsis accession, a MAF2, MAF3, MAF4 and FLC null line, MAF2 var1 was consistent in its effect on reproductive delay under ambient and reduced temperatures, indicating that it acts as a repressor of flowering.

Keywords Arabidopsis thaliana · Flowering time · Alternative splicing · Response to cold · MAF2

Introduction

The number of genes discovered in early genome sequencing projects was lower than anticipated considering the apparent complexity of eukaryotic organisms (Rubin et al. 2000; International Human Genome Sequencing Consortium 2001). However, a small gene set may result in complex phenotypes if genes produce multiple products with diverse functions (Pesole 2008). Alternative splicing is one of the mechanisms that can expand the functional diversity of genes (Modrek and Lee 2002; Reddy 2007). Alternative splicing operates through selection of variable donor and acceptor sites during pre-mRNA processing and can result in a suite of divergent mRNAs produced from a single gene (Sharp 1994). Alternative splicing can instigate a spectrum of functional outcomes that may considerably complicate the assignment of gene function.

In plants, alternative splicing is wide-spread. In Arabidopsis and rice, it affects 32.5 and 23.5 % of genes, respectively (Campbell et al. 2006). Many alternative splicing events are shared between these species, suggesting a functional role for splice product variation (Wang et al. 2008). Alternative splicing is particularly apparent during environmental stress responses (Iida et al. 2004; Robinson and Parkin 2008). Splice site decisions are sensitive to salt, temperature, wounding, metal contamination...
of the soil, light levels and stress hormones (Simpson et al. 2008; Bove et al. 2008; Palusa et al. 2007, Iida et al. 2004; Marrs and Walbot 1997; Robinson and Parkin 2008). Thus, alternative splicing may be an additional mechanism of sensing or responding to environmental cues. Despite the importance of alternative splicing, most reports only describe alternative gene products but do not elucidate the function of alternative transcripts (Reddy 2007).

The initiation of flowering is crucial for plant reproductive success. Plants integrate both intrinsic developmental and environmental cues such as day-length and temperature during the transition from the vegetative to reproductive development and some of these decisions are influenced by components of the spliceosome or are splice variant specific (Xing et al. 2008; Quesada et al. 2003). Fifty-nine out of the approximately 80 genes in Arabidopsis that affect timing of flowering display transcripts with splice or poly-adenylation site variation and 24 of these genes are known to be involved in pre-mRNA processing (Terzi and Simpson 2008; Herr et al. 2006; Lopato et al. 1999; Wang and Brendel 2006; Wang et al. 2007; Noh et al. 2004). Though flowering time control is influenced at multiple levels, alternative splicing may provide an additional responsive sensor of environmental changes. MADS-AFFECTING FLOWERING 2 (MAF2) is a member of a tandem quadruplication of MADS-box transcription factor genes MADS-AFFECTING FLOWERING 2–5 (MAF2, MAF3, MAF4, MAF5) in Arabidopsis. These genes are homologous to FLOWERING LOCUS M (FLM/MAF1) and FLOWERING LOCUS C (FLC). Genes from the FLC/MAF clade are major determinants of natural flowering time variation, as Quantitative Trait Loci (QTLs) at FLC, MAF1, MAF2–5 account for 19, 15 and 15 % of natural variation in flowering time in the studied populations, respectively (Salome et al. 2011). All six genes display between 2 and 5 unique splice variants and expression of all genes is responsive to temperature (Sung et al. 2006; Caicedo et al. 2004; Ratcliffe et al. 2003; Micheals and Amasino 1999). The effect of temperature on gene expression, downstream pathways and strength of the phenotypic effect has diverged between the best known clade members FLC, FLM/MAF1 and MAF2 (reviewed in Alexandre and Hennig 2008). Alteration of the splice variant profile by temperature (at 27 °C) has been reported for FLM/MAF1 and MAF2, though the effect has not been studied quantitatively nor is the qualitative function of alternative splice variants known (Balasubramanian et al. 2006). Neither FLM/MAF1 nor MAF2 major alternative variants are predicted to be targeted by the Nonsense Mediated Decay pathway and therefore may be translated (Severing et al. 2012). FLM/MAF1 variants display mutually exclusive exon and intron retentions and the MAF2 alternative variants display an alternative acceptor site and premature transcript termination (Severing et al. 2012, Ratcliffe et al. 2003). Unlike FLM/MAF1, the splice variant profile of MAF2 is conserved in Brassica napus and Brassica rapa orthologues, suggesting a possible functional relevance (Severing et al. 2012). Also, unlike FLM/MAF1, the two major MAF2 splice variants, MAF2 variant (var) 1 and 2, are expressed distinctly and strongly, increasing the likelihood of functional relevance and rendering them more tractable to study (Balasubramanian et al. 2006). Therefore, MAF2 is a good candidate to understand the role of alternative splicing in the determination of flowering time, though the conclusions made for this gene may not be transferable to other clade members.

Though the effect of temperature on the MAF2 splice variant profile has been reported at moderately high temperatures, the effect of MAF2 on plant phenotype is best understood at low, 4 °C, and optimal growth temperatures, 21 °C. Study of MAF2 function showed that maf2 plants that are lacking MAF2 expression displayed a weak acceleration of flowering when grown at 21 °C (Ratcliffe et al. 2003). MAF2 had a stronger capability to delay flowering after a short, 16–21 day, cold treatment, suggesting that MAF2 prevents flowering in the event of autuminal temperature fluctuations that might be misinterpreted as the arrival of spring (Ratcliffe et al. 2003). Unlike plants lacking FLC function, maf2 plants lacking MAF2 function exposed to a long, 85-day cold treatment flowered similarly to controls (Ratcliffe et al. 2003). Notwithstanding, it is unclear if a complete knock-out of transcription at an alternatively spliced gene can adequately explain gene function.

In this study, we show that expression levels of the MAF2 splice variants are temperature-dependent. During the course of the cold treatment the accumulation of MAF2 var1, the transcript form predicted to generate the full-length protein, is maintained, and the abundance of MAF2 var2 transcript, predicted to encode the truncated MAF2 protein decreases. Phenotypic analysis of gain-of-function transgenic plants revealed that the MAF2 var1 has the most consistent effect on repression of flowering, indicating that it acts as a repressor of flowering.

Materials and methods

Plant material

The seed of Col and L1-2 Arabidopsis accessions were acquired from the Arabidopsis Biological Resource Center (ABRC). The seed of the Ler accession was acquired from X. Chen (Chen and Meyerowitz 1999) and flc-3 from R. Amasino (Micheals and Amasino 1999). Col and Ler have wild-type MAF2 alleles, however, FLC and MAF2 expression is not detectable in L1-2 (Lempe et al. 2005;
Rosloski et al. 2010). Plants were grown at 22 °C under a long day (LD), 16 h light/8 h dark, or a short day (SD) photoperiod, 8 h light/16 h dark, under 100–150 μmol/m²/s cool-white fluorescent lights. All seedlings were given at least a 3-day stratification period prior to germination. Cold treatment was administered by growing seedlings on half-strength MS media in petri plates in a temperature-controlled 4 °C incubator under low light conditions 16 days before shifting to LD or SD conditions. T₁ transgenic lines overexpressing individual MAF2 transcripts in Col and flc-3 backgrounds were initially grown at 4 °C for 10 days and were then transferred to LD conditions at 16 °C to enhance potential differences in flowering time. Tissue was collected and bulked from 5 T₁ transgenic plants and assayed in triplicate to assess, on average, if plants were expressing the transgene using qRT-PCR and primers 25 and 26 (Supplemental Table 1). A spectrum of flowering phenotypes from T₁ transgenic lines in the Col background was used to derive T₄ transgenic plants. T₄ transgenic lines with a single copy of transgenes were selected.

Generation of gain-of-function transgenic plants

cDNA clones corresponding to splice variants AY231441 (var1, full-length), AY231442 (var2), AY231444 (var4) and the full-length genomic construct of MAF2 (MAF2g) were cloned into the SmaI site of the pCHF3 binary vector, and were placed under control of the strong constitutive 35S promoter (Fig. 2a). The Col, flc-3 and Li-2 accessions were transformed using the floral dip method (Clough and Bent 1998). Transformants were selected on the half-strength MS media supplemented with 50 mg/L kanamycin.

Analysis of the effect of cold on gene expression

For the comparative analysis of temperature on MAF2 expression, sterile seeds were stratified in the dark for 3 days at 4 °C on half-strength MS media in sealed Petri plates. As Col plants grown in LD conditions are committed to reproductive development at around 7 days after germination (dag) and as we are interested in the role of temperature-sensitive MAF2 splice variant expression in the determination of flowering time, we cold-treated plants 3 dag under a non-inductive SD photoperiod (Bradley et al. 1997). Seeds were transferred to a 21 °C chamber with a SD photoperiod for 3 days. At this time, seedlings had emerged from the seed coat without extensive expansion of the cotyledons. These seedlings were sampled and are referred to as day 0. The petri plates were then either transferred to a 4 °C, SD growth chamber or remained at 21 °C SD for 3, 6, 12 and 18 days. Seedlings in the 4 °C treatment remained arrested at the cotyledon stage and did not display expansion of true leaves. Seedlings in the 21 °C treatment developed true leaves as the experiment progressed and displayed ~4 small leaves by day 12. Tissue for cold-treated seedlings was collected at 4 °C and all tissues were frozen immediately in liquid nitrogen prior to RNA extraction.

Analysis of the effect of cold on flowering time

T₂ seeds were collected from plants overexpressing MAF2 genomic or var1 constructs in Li-2, as analysed in Fig. 5. Cold-treated seeds were sterilized and plated on half-strength MS media supplemented with 50 mg/L kanamycin and placed in a 4 °C chamber for 16 days. On the 13th day, another set of seeds was sterilized, plated on half-strength MS media supplemented with 50 mg/L kanamycin and stratified for 3 days at 4 °C. Both 16 day cold-treated and 3 day stratified seedlings were planted simultaneously in at least 3 replicates of 4 plants per pot and grown under SD conditions, as previously described. Over half of Li-2 T₂ plants expressing both MAF2g and MAF2 var1 transgenes displayed severe leaf deformities and failed to maintain the apical meristem after germination. Only plants that were phenotypically normal and attained reproductive development were analysed. Time to flowering was determined by counting rosettes leaves (RLN) produced prior to bolting.

Analysis of gene expression

RNA was extracted using the RNeasy Plant RNA Extraction Kit (Qiagen) as per manufacturer’s instructions. For semi-quantitative RT-PCR (sqRT-PCR), the number of PCR cycles was determined to reflect the log phase of product accumulation for various primer combinations (see Supplemental Table 1). Quantitative RT-PCR (qRT-PCR) primer combinations were optimized for melting temperature and MgCl₂ concentration. Real-time qRT-PCR amplification of MAF2 var1 and MAF2 var2 using primers 15 and 16, or 17 and 18, respectively, and reaction conditions are found in Supplemental Table 1. qRT-PCR was conducted on the Corbett Rotor Gene 3000 using two biological replicates and at least three technical replicates. Relative target amplification was expressed using the 2T (-Delta Delta C) method (Livak and Schmittgen 2001). Expression of the TUBULIN2 (TUB) was monitored as a loading control in all semi-quantitative and quantitative RT-PCR experiments using primers and reaction conditions specified in Supplemental Table 1.
Statistical analysis

Statistical analysis was conducted by ANOVA in the program PAST version 1.81 (http://folk.uio.no/ohammer/past/index.html).

Results

MAF2 gene expression is affected by the ambient temperature

To understand the regulation of MAF2 splice variation by temperature, Col and Ler seeds were germinated for 3 days at 21 °C and seedlings were then transferred to either 4 or 21 °C. Seedlings were collected for RNA extraction from both temperature treatments at 3, 6, 12 and 18 days after the transfer. Seedlings were kept under a short-day (SD) photoperiod to insure that all were developing vegetatively throughout the duration of the experiment.

Temperature treatments altered the relative expression levels of MAF2 splice variants compared to control seedlings. Using qRT-PCR, the same pattern is obtained in both Col and Ler, characterized by two bands corresponding to the lower band MAF2 var1 (Genbank accession AY231441, Ratcliffe et al. 2003), and the upper band corresponding to MAF2 var2 (Genbank accession AY231442, Ratcliffe et al. 2003), (Fig. 1a, b; Supplemental Table 1). Another reported MAF2 splice variant, MAF2 var4 (Genbank accession AY231444, Ratcliffe et al. 2003), consisting of only the short, MADS-Box DNA-binding domain, cannot be specifically amplified because of the highly conserved nature of this region preventing us from assessing the effect of temperature on this particular transcript. MAF2 var1 is predicted to encode a full-length, MADS-box transcription factor, while MAF2 var2 encodes a truncated protein that is predicted to have a MADS-box DNA binding domain but lacks the K-box protein interaction domain (Supplemental Fig. 1). The two MAF2 variants remained at an equivalent relative staining intensity for the entire time series in seedlings grown at 21 °C (MAF2, to 21 °C, Fig. 1b). In contrast to controls, the splice variant profiles of seedlings transferred to 4 °C changed. The expression of MAF2 var1 increased and the expression of MAF2 var2 decreased (MAF2, to 4 °C, Fig. 1b). To obtain a quantitative measure of these changes, we performed qRT-PCR (Fig. 1c). This analysis showed that cold significantly decreases the abundance of the MAF2 var2, predicted to encode a truncated MAF2 protein. In contrast, the expression of MAF2 var1, predicted to encode the full-length MAF2 protein, has been maintained at a high level. Change in the abundance of these two transcripts during the short period of cold prompted us to address their roles in the regulation of flowering time.

Overexpression of MAF2 splice variants in the Col background

To address whether alternative splice variants produced by MAF2 differ in their ability to modulate flowering time, we generated transgenic plants expressing the MAF2 genomic clone (MAF2g), MAF2 var1, MAF2 var2 and MAF2 var4 under the transcriptional control of the constitutive 35S promoter in Col and flc-3 backgrounds (Fig. 2). Overexpression of all constructs in T1 plants was verified and resulted in a similar flowering time distribution pattern for Col and flc-3 transgenic populations (Fig. 2b, c; Supplemental Table 2). Overexpression of MAF2g delayed flowering, consistent with the proposed role for MAF2 as a floral repressor. However, no individual splice variant was capable of conferring a late-flowering phenotype. Instead, MAF2 var1 overexpression resulted in pronounced early flowering in both Col and flc-3 backgrounds. MAF2 var2 produced slightly early flowering plants in Col and had no effect on flowering time in flc-3 in Col, while MAF2 var4 had no effect on flowering time in either Col or flc-3. As such, results of overexpression of MAF2 splice variants in Col do not conform to our expectation that any variant individually encodes a floral repressor but that they act either as a powerful accelerator of flowering (MAF2 var1) or have little or no effect on flowering (MAF2 var2 and MAF2 var4).

To substantiate these unexpected results, we analyzed the flowering time and transgene expression of T4 homozygous, single-insert progeny that represent the spectrum of flowering times within each T1 MAF2 variant class (Fig. 3). The early flowering 35S:MAF2 var1 T4 lines were the only lines with high expression of the transgene (transgene expression could not be uniquely determined, but can be inferred from the total MAF2 expression (MAF2-T, which reflects the expression from both the endogenous MAF2 gene and the transgene) and endogenous MAF2 expression (MAF2-E)), Fig. 3c. These plants flowered earlier than the maf2 null allele (Fig. 2b, 35S:MAF2 var1, n = 12, flowered after forming 5.2 ± 0.40 and 6.3 ± 0.45 leaves; maf2, n = 12, flowered after forming 10.3 ± 0.65 leaves). Consistent with this pronounced early flowering phenotype we found reduced expression of the endogenous MAF2 var1 and var2 splice forms (Fig. 3c). These lines also showed reduced expression of the FLC gene. Clear resolution of the MAF3 expression profile revealed that it is changed in these early flowering lines as well. A single MAF3 band was absent from the early flowering MAF2 var1 plants (arrow in Fig. 3c; Supplemental Fig. 2). Cloning and sequencing of bands in the 610–728 bp region (Supplemental Fig. 2) showed that a band at 706 bp, corresponding to MAF3 var1.
is absent in the early flowering, MAF2 var1 transgenic lines. Therefore, pronounced early flowering in 35S:MAF2 var1 in T4 plants is associated with high transgene expression and suppressed expression of the endogenous MAF2 gene and some of its paralogues. In summary, the analysis of T4 lines linked the early flowering phenotype of MAF2 var1 transgenics with non-target effects on endogenous MAF genes. These non-target effects, as well as the failure of remainder of T4 single insert lines to maintain strong overexpression of the transgene across generations prevented examination of the effect of splice variant overexpression on plant phenotypes in Col background.

MAF2 var1 partially phenocopies the effect of the MAF2 genomic fragment in the accession LI-2.

Given the confounding effects of strong MAF2 var1 overexpression on MAF2, MAF3 and FLC gene expression leading to the recovery of mostly early flowering transgenic plants, we sought to determine the effect of overexpression of the MAF2 var1 in plants that do not express MAF2 and its paralogues. The LI-2 accession is known to be null for FLC (Lempe et al. 2005). In addition, MAF2 expression was not detectable using primers in the 5′ and 3′-UTR (Fig. 4a) and within the coding region (Rosloski Fig. 1 Temperature influences the splice variant profile of MAF2. a Graphic representation of two MAF2-Col/Ler splice variants and position of primers used (arrows). Grey boxes, UTR; black boxes, exons; black lines, introns. b Representative semi-quantitative RT-PCR of MAF2 var1 and var2 transcripts amplified by using primers 3 and 4. The upper band corresponds to MAF2 var2 and the lower band to MAF2 var1. 0d (days) corresponds to 3-day-old seedlings grown at 21 °C. Subsequently, control seedlings remained at 21 °C, and cold-treated seedlings were transferred to 4 °C for 3, 6, 12 and 18 days. TUB amplification was used as a loading control. c Comparison between the expression of MAF2 var1 and MAF2 var2 in Col and Ler seedlings grown at 21 °C (black bars) and 4 °C (grey bars) at 6 and 18 days after the transfer of seedlings to 4 °C. Primers 15 and 16, and 17 and 18 were used for quantitative RT-PCR analysis of MAF2 var1 and MAF2 var2 expression, respectively. TUB amplification was used as a loading control. Statistical analysis was conducted on two biological replicates and three technical replicates by ANOVA. *p < 0.05; **p < 0.01; ***p < 0.0001
et al. 2010), showing that L1-2 is also null for MAF2 expression. While checking the expression of the remaining paralogues, we discovered that L1-2 also fails to properly express MAF3 and MAF4 despite presence of the RT-PCR primer sites at these loci (Fig. 4a). Although genomic DNA sequences of MAF2, MAF3 and MAF4 in L1-2 does not give indication as to why expression of these genes is not detectable, the failure of primer sets to detect expression at these loci lend credence to the proposition that L1-2 is null for wild-type expression of MAF2 and most of the other clade members. Thus, the L1-2 accession provides a valuable genetic background in which one could assess the phenotypic effect of overexpression of MAF2 splice variants without confounding, non-target effects on the endogenous MAF2 gene and its paralogues.

L1-2 is an extremely early flowering accession that flowers after producing ~4 rosette leaves under a long-day (LD) photoperiod (Fig. 4b). It was transformed with the same constructs as previously used for Col transformation, and the effect of the transgenes on timing to flowering was determined by counting rosettes leaves (RLN) produced prior to bolting. RLN was determined in T1 plants under LD and SD photoperiods at 22 ºC (Fig. 4b). Similar to the Col background, overexpression of the MAF2g construct in L1-2 resulted in delayed flowering in both LD and SD day photoperiods. Overexpression of MAF2 var1 did not affect flowering time of L1-2 plants grown under LD conditions compared to controls, with the exception of a single, extremely late flowering plant (57 leaves). However, under SD conditions, 35S:MAF2 var1 plants displayed a similar range of flowering times as seen for the MAF2g construct, (Fig. 4b, c) MAF2 var2 and MAF2 var4 plants did not flower differently from the controls in SD conditions, though one and two late-flowering plants were observed in these populations, respectively. As MAF2 var2 and var4 populations were largely undifferentiated from the control we conducted further analysis only on MAF2g and MAF2 var1 populations.

To understand the effect of MAF2 transgenes on flowering time in L1-2, we determined the transgene expression in some early and late flowering MAF2g and MAF2 var1 T1 plants (Fig. 4d). Transgene expression was determined by two sets of RT-PCR primers: primers 5 and 20, placed at the 5' -end of the transgene, showed that both MAF2g and var1 transgenes are overexpressed in transgenic plants; primers 5 and 6 (MAF2g) or 15 and 16 (MAF2 var1), at the 3'-end of the transgene, showed that only a subset of transgenic plants have normal transgene expression by expressing both parts of the transgene. While the mechanism is not known, the frequency of proper expression of MAF2g and MAF2 var1 transgenes associates with the phenotypic distribution of T1 lines. Importantly, plants that flower late in both populations of transgenic plants express transgenes properly, suggesting that the late-flowering phenotype under SD conditions is induced by overexpression of MAF2g and MAF2 var1 transgenes.

L1-2 plants overexpressing MAF2 genomic and var1 transgene are weakly responsive to cold treatment

MAF2 has been proposed to function as a repressor of flowering during short periods of cold (Ratcliffe et al. 2003). Consistent with this role, the maf2 T-DNA line is more responsive to cold-induced acceleration of flowering than Col (Fig. 5). Since properly expressed MAF2g and var1 transgenes in the L1-2 background were able to delay flowering under ambient temperature, we tested the response of representative T2 transgenic lines to a short period of cold. We have analysed two and three T2 transgenic lines expressing MAF2g and MAF2 var1 transgenes, respectively, whose T1 progenitor plants flowered in the mid-range of the corresponding T1 populations. Under a SD photoperiod, L1-2 plants flower after producing 9.8 ± 1.8 leaves and are insensitive to vernalization. Like T1 progenitor plants, all T2 transgenic lines significantly delayed flowering without a cold treatment compared to L1-2, confirming that both MAF2g and MAF2 var1 transgenes function as repressors of flowering. The ability to delay flowering after a short period of vernalization was variable among the T2 lines of both constructs and was weak. Therefore, both MAF2g and MAF2 var1 transgenes are capable of repressing flowering and neither of them have capability to strongly prevent the acceleration of flowering after a moderate period of cold.
Discussion

Ratcliffe et al. (2003) postulated that MAF2 functions to prevent flowering after a moderate cold treatment of 16–21 days. This conclusion was based on phenotypic characterization of a T-DNA insertion line that has no detectable MAF2 transcription and plants overexpressing the full-length cDNA form, MAF2 var1. Given that the MAF2 locus generates multiple transcripts, it is unclear if a transcriptionally inactive locus provides insight into the function of an alternatively spliced gene. 35S:MAF2 var1 T1 overexpression plants described in that study were mostly early flowering, although several late flowering plants were also observed (Ratcliffe et al. 2003). The
Fig. 4 Overexpression of MAF2 genomic fragment and splice variants in Ll-2 accession. a Expression of the FLC/MAF gene family in Col and the Ll-2 accessions. b Flowering time distribution in T1 plants overexpressing MAF2 genomic fragment (MAF2g), MAF2 var1 (var 1), MAF2 var2 (var 2) and MAF2 var4 (var 4). The number of transgenic plants analyzed, the average rosette leaf number (RLN) ± standard deviation and results of an ANOVA test indicating significant differences from the control at * <0.05, ** <0.01, *** <0.001 are shown in the right hand corner of each graph. VLF, a late-flowering plant that did not flower during the course of the experiment. c Representative transgenic plants from populations shown in b. d Analysis of transgene expression in a sample of early and late flowering 35S:MAF2g and 35S:MAF2 var1 T1 plants. The 5' end of cDNA was amplified with primers 5 and 20 and the 3' end of cDNA was amplified with primers 5 and 6 in 35S:MAF2g plants and primers 15 and 16 in 35S:MAF2 var1 plants (Supplemental Table 1).
authors concluded that \textit{MAF2} acts as a repressor of flowering based on the heritability of late flowering in advanced transgenic lines. Our study expands the understanding of \textit{MAF2} gene function and the role of the individual splice variants. We show that cold favors expression of \textit{MAF2} var1, the transcript predicted to encode the full-length MADS-box protein, but that it significantly affects the expression of \textit{MAF2} var2, a splice form predicted to encode a truncated \textit{MAF2} protein. Our data further reveal that \textit{MAF2} var1 has the greatest ability to delay flowering compared to other observed splice variant forms.

Cold affects the expression of \textit{MAF2} splice variants

We previously reported that higher ambient temperature affects the expression patterns of \textit{MAF2} (Balasubramanian et al. 2006). Like elevated temperatures, cold treatment affects the abundance of the \textit{MAF2} splice variants, indicating that temperatures at both ends of the spectrum can affect the expression of alternatively spliced genes. However, high and low temperatures affect \textit{MAF2} splicing in different ways: at 25 °C the expression of \textit{MAF2} var2 is favored, while at 4 °C its expression is downregulated (Balasubramanian et al. 2006; Fig. 1c). The retention of \textit{MAF2} var1 expression in the cold renders it as a candidate repressor of flowering during a moderate cold treatment. The inability of the \textit{MAF2} var2 form to delay flowering when overexpressed portrays this splice variant as a non-functional product of transcription (Figs. 2, 3). The protein encoded by the \textit{MAF2} var2 is predicted to have a MADS-box DNA binding domain, but to lack the protein-interaction K-box domain. Given this putative structure, the protein could interfere with the function of the \textit{MAF2} var1 gene product. However, the high potency of the overexpressed \textit{MAF2} genomic clone in flowering repression in Col, \textit{flc}−3 and LI-2 backgrounds, giving rise to both \textit{MAF2} var1 and var2 transcripts, suggests that \textit{MAF2} var2 does not impede the functionality of the \textit{MAF2} locus.

Of interest to this study is the series of natural \textit{MAF2} alleles resulting from structural rearrangements that are frequent across the eastern European range of \textit{Arabidopsis thaliana} (Rosloski et al. 2010). These \textit{MAF2} alleles are characterized by large insertions of \textit{MAF3} gene into the \textit{MAF2} coding region. Despite the diversity of rearranged \textit{MAF2} alleles, these alleles are predicted to be incapable of producing \textit{MAF2} var1 proteins but instead produce truncated proteins identical to those predicted from \textit{MAF2} var2, the major wild-type \textit{MAF2} alternative variant, via a premature stop codon. Knock-down of several \textit{MAF3} insertion alleles had no effect on flowering time, thereby corroborating our assertion that \textit{MAF2} var2 protein does not contribute strongly to the repression of flowering (Rosloski et al. 2010).

Overexpression of \textit{MAF2} var1 in Col affects the expression of the endogenous \textit{MAF2}, \textit{MAF3}, \textit{MAF4} and \textit{FLC} genes

If \textit{MAF2} var1 is the functional repressor of flowering, then this transcript should be able to phenocopy the effect of the overexpression of the \textit{MAF2} genomic fragment. However, similar to Ratcliffe et al. (2003), we found that most T$_1$ 35S: \textit{MAF2} var1 in Col plants were very early flowering, failing to support the hypothesis that \textit{MAF2} var1 acts as a

![Fig. 5](image_url)
floral repressor. However, our analysis of T₄, single insert transgenic lines in Col showed that the extreme early flowering of the MAF2 var1 overexpression lines associates with the suppressed expression of the endogenous MAF2, MAF3 and FLC genes (Fig. 3b, c). The strongest effect on paralogous MAFs was seen only in plants that robustly expressed MAF2 var1. Given the high homology of clade members, it is possible that the effect of the MAF2 var1 transgene on paralogues occurs via a cosuppression-like mechanism, as has been observed during the gene silencing experiments within other gene families (Miki et al. 2005; Travella et al. 2006). It is also possible that artifacts of transgene expression such as truncated transcripts observed in Ll-2, also complicated the analysis of flowering time in Col. Given the high homology of the 5′ MAF2 gene to paralogues in Col, it is difficult to study the formation of truncated transcripts during overexpression in this accession. However, as extreme early flowering and wide phenotypic distributions have been noted in populations of transgenic plants overexpressing other MAF clade genes (Sheldon et al. 1999; Ratcliffe et al. 2001), suppression of MAF/FLC paralogue expression in transgenic plants or the formation of truncated transcripts may be significant impediments to the functional analysis of single MAF clade genes.

Overexpression of MAF2 var1 in Ll-2 represses flowering

To avoid non-target effects of transgene overexpression on endogenous copies of MAF/FLC gene family members, we also expressed MAF2 var1 in the Ll-2 accession that is lacking the expression of the MAF2, MAF3, MAF4 and FLC genes. Under SD conditions, repression of flowering was observed in the population over-expressing both the MAF2g and MAF2 var1 transgenes. Late flowering transgenic T₁ plants of both constructs produced late-flowering T₂ progeny, showing that the effects of these transgenes are stable across generations (Fig. 5).

Early flowering in Ll-2 was associated with the expression of truncated transcripts representing only the 5′-end of each construct. The number of plants expressing truncated transcripts was substantial, representing 17–40 % of T₁ Ll-2 plants. Plants expressing truncated transcripts were invariably on the early end of the flowering spectrum, showing that transcript truncation generally disrupts the repression of flowering by MAF2. MAF2 var4 is a truncated transcript, being composed only of the MADS-box region of MAF2, and, accordingly, 35S:MAF2 var4 plants rarely displayed late flowering in any population. Because truncated transcripts are predicted to give rise to proteins that retain the DNA binding capability of the MADS-box, they may function to alter the flowering phenotype through non-specific pathways. Of interest, plants expressing truncated transcripts were less frequent in the 35S:MAF2g plants, suggesting that the presence of introns may aid either proper transgene insertion or its expression. The presence of truncated transcripts in Ll-2 and the non-target effects in Col recommends analysis of transgenic plant populations for these artifacts when assessing the function of MAF-expressing transgenic plants.

There was a distinct effect of day-length on the ability of MAF2 var1 to delay flowering. In Ll-2, 35S:MAF2g consistently repressed flowering under both LD and SD photoperiod, while late flowering was generally not observed in 35S:MAF2 var1 plants under LD. The MAF2g construct contains both exonic and intronic sequences and may be more strongly or stably expressed through a phenomenon termed Intron-Mediated Enhancement (Rose 2002, 2004). The extremely rapid flowering of Ll-2 under LD conditions may necessitate high levels of repressor expression to prevent the initiation of reproduction.

Temperature-modulated alternative splicing at MAF2 may be an adaptation of plants to temperature fluctuation

The exposure of plants to cold favors the expression of the MAF2 var1 that is fully spliced and that is expected to generate the full-length protein. However, the formation of fully spliced transcripts in the cold may not be the norm in Arabidopsis. Increased complexity of the splice variant profiles and increased retention of introns is regularly observed during the response of the Arabidopsis transcriptome to cold (Ner-Gaon et al. 2004; Robinson and Parkin 2008). The evolution of splice-site temperature optima, differential stability of alternative transcripts in the cold or altered protein–protein interactions at low temperatures might be different ways of altering the splicing efficiency and producing optimal transcripts from genes that function at low temperatures. Similar to MAF2 var1, expression of the full-length transcript in Black Spruce β-hydroxyacyl ACP dehydratase, a gene product that is likely important for membrane acclimation to low temperatures, is produced only at low temperatures through intron exclusion (Tai et al. 2007). Our data show that MAF2 var1 has the capability to repress flowering when overexpressed in transgenic plants under ambient temperatures or after a moderate period of cold. The ability of both the MAF2g and MAF2 var1 construct to delay flowering after a moderate period of cold was variable among T₂ lines and was weak. The weak effect on the vernalization response suggests that MAF2 may be a locus of minor effect in the vernalization response in comparison to the major effect locus FLC (Sánchez-Bermejo et al. 2012; Strange et al. 2011).
It is possible that MAF2 has functions with more prominent phenotypic outcomes compared to the weak cold response. Expression of the non-functional MAF2 splice variant, MAF2 var2, is increased at high temperatures (Balasubramanian et al. 2006). Low temperature decreases expression of the non-functional MAF2 var2 but does not significantly increase expression of MAF2 var1, the repressor of flowering (Fig. 1c). Although the phenotypic and gene expression response of maf2 plants to high temperatures has not been tested quantitatively, alleviation of flowering repression at high temperatures via increased production of a non-functional splice variant may be a more salient response of MAF2 expression to temperature fluctuation compared to the cold response.

Gene expression divergence in the FLC/MAF clade

FLC and MAF2 are paralogous repressors of flowering. The parallel effects of overexpression of MAF2 splice variants in Col and flc-3 (Fig. 2) indicate that these paralogues affect flowering time independently. In addition, FLC and MAF2 have distinct gene expression patterns in response to cold. FLC expression is slowly reduced over a lengthy cold period via epigenetic modulation (He et al. 2004; March-Díaz et al. 2007; Oh et al. 2008), until a low level of FLC expression permits the initiation of flowering (Bastow et al. 2004; Sung and Amasino, 2004). Conversely, MAF2 expression is modulated in two separate ways. Ratcliffe et al. (2003) found that total MAF2 expression, sampled when plants were returned to 21 °C, was not affected by 3–21 days of cold treatment, but was reduced after 76–85 days of cold. Of possible relevance, Sheldon et al. (2009) demonstrated that repression of MAF2 expression by a long period of cold was affected by the VIN3, VRN1 and VRN2 genes that regulate FLC epigenetically. Here, we show the second dimension of MAF2 gene regulation: the temperature-responsive, short to moderate-term alteration of the splice variant profile at 4 °C. This dimension of MAF2 regulation is distinct from that of FLC. Considering the divergence in MAF2 and FLC expression, it will be interesting to discover how the molecular mechanisms of MAF2 regulation have diverged from those known to control FLC, and to determine crucial regulatory elements that allow for the temperature-sensitive alteration of MAF2 gene expression.

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References

Alexandre CM, Hennig L (2008) FLC or not FLC: the other side of vernalization. J Exp Bot 59:1127–1135
Balasubramanian S, Sureshkumar S, Lempe J, Weigel D (2006) Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. PLoS Genet 2:e106
Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C (2004) Vernalization requires epigenetic silencing of FLC by histone methylation. Nature 427:164–167
Bove J, Kim C, Gibson C, Assmann S (2008) Characterization of wound-responsive RNA-binding proteins and their splice variants in Arabidopsis. Plant Mol Biol 67:71–88
Bradley D, Ratcliffe O, Vincent C, Carpenter R, Coen E (1997) Inflorescence commitment and architecture in Arabidopsis. Science 275:80–83
Caicedo AL, Stinchcombe JR, Olsen KM, Schmitt J, Purugganan MD (2004) Epistatic interaction between Arabidopsis FRI and FLC flowering time genes generates a latitudinal cline in a life history trait. Proc Natl Acad Sci USA 101:15670–15675
Campbell M, Haas B, Hamilton J, Mount S, Buell CR (2006) Comprehensive analysis of alternative splicing in rice and comparative analyses with Arabidopsis. BMC Genomics 7:327
Chen X, Meyerowitz EM (1999) HUA1 and HUA2 are two members of the floral homeotic AGAMOUS pathway. Mol Cell 3:349–360
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743
He Y, Doyle MR, Amasino RM (2004) PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev 18:2774–2784
Herr AJ, Molnar A, Jones A, Baulcombe DC (2006) Defective RNA processing enhances RNA silencing and influences flowering of Arabidopsis. Proc Natl Acad Sci USA 103:14994–15001
Iida K, Seki M, Sakurai T, Satou M, Akiyama K, Toyoda T, Konagaya A, Shinozaki K (2004) Genome-wide analysis of alternative pre-mRNA splicing in Arabidopsis thaliana based on full-length cDNA sequences. Nucl Acids Res 32:5096–5103
International Human Genome Sequencing Consortium (2001) Initial sequencing and analysis of the human genome. Nature 409:860–921
Lempe J, Balasubramanian S, Sureshkumar S, Singh A, Schmid M, Weigel D (2005) Diversity of flowering responses in wild Arabidopsis thaliana strains. PLoS Genet 1:e6
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408
Lopato S, Kalyana M, Dorner S, Kobayashi R, Krainer AR, Barta A (1999) atSRp30, one of two SF2/ASF-like proteins from Arabidopsis thaliana, regulates splicing of specific plant genes. Genes Dev 13:987–1001
March-Díaz R, Garcia-Domínguez M, Chen X, Meyerowitz EM (1999) atSRp30, one of two SF2/ASF-like proteins from Arabidopsis thaliana, regulates splicing of specific plant genes. Genes Dev 13:987–1001
March-Díaz R, Garcia-Domínguez M, Chen X, Meyerowitz EM (1999) atSRp30, one of two SF2/ASF-like proteins from Arabidopsis thaliana, regulates splicing of specific plant genes. Genes Dev 13:987–1001
March-Díaz R, Garcia-Domínguez M, Chen X, Meyerowitz EM (1999) atSRp30, one of two SF2/ASF-like proteins from Arabidopsis thaliana, regulates splicing of specific plant genes. Genes Dev 13:987–1001

Springer
Micheals SD, Amasino RM (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11:949–956

Miki D, Itoh R, Shimamoto K (2005) RNA silencing of single and multiple members in a gene family of rice. Plant Physiol 138: 1903–1913

Modrek B, Lee C (2002) A genomic view of alternative splicing. Nat Genet 30:13–19

Ner-Gaon H, Halachmi R, Savaldi-Goldstein S, Rubin E, Ophir R, Fluhr R (2004) Intron retention is a major phenomenon in alternative splicing in Arabidopsis. Plant J 39:877–885

Noh Y, Bizzell CM, Noh B, Schomburg FM, Amasino RM (2004) EARLY FLOWERING 5 acts as a floral repressor in Arabidopsis. Plant J 38:664–672

Oh S, Park S, van Nocker S (2008) Generic and global functions for Paf1C in chromatin modification and gene expression in Arabidopsis. PLoS Genet 4:e1000077

Palusa SG, Ali GS, Reddy ASN (2007) Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses. Plant J 49:1091–1107

Pesole G (2008) What is a gene? An updated operational definition. Gene 417:1–4

Quesada V, Macknight R, Dean C, Simpson GG (2003) Autoregulation of FCA pre-mRNA processing controls Arabidopsis flowering time. EMBO J 22:3142–3152

Ratcliffe OJ, Nadzam GC, Reuber TL, Riechmann JL (2001) Regulation of flowering in Arabidopsis by an FLC Homologue. Plant Phys 126:122–132

Ratcliffe OJ, Kumimoto RW, Wong BJ, Riechmann JL (2003) Analysis of the Arabidopsis MADS AFFECTING FLOWERING gene family: MAF2 prevents vernalization by short periods of cold. Plant Cell 15:1159–1169

Reddy ASN (2007) Alternative splicing of pre-messenger RNAs in plants in the genomic era. Annu Rev Plant Biol 58:267–294

Robinson S, Parkin I (2008) Differential SAGE analysis in Arabidopsis uncovers increased transcriptome complexity in response to low temperature. BMC Genomics 9:434

Rose AB (2002) Requirements of intron-mediated enhancement of gene expression in Arabidopsis. RNA 8:1444–1453

Rose AB (2004) The effect of intron location on intron-mediated enhancement of gene expression in Arabidopsis. Plant J 40:744–751

Rosloski SM, Jali SS, Balasubramanian S, Weigel D, Gricic V (2010) Natural diversity in flowering responses caused by variation in MADS-AFFECTING FLOWERING 2. Genetics 186:263–276

Rubin GM, Yandell MD, Wortman JR, Miklos GLG, Nelson CR, Haritaran IK, Fortini ME, Li PW, Apweiler R, Fleischmann W et al (2000) Comparative genomics of the eukaryotes. Science 287:2204–2215

Salome PA, Bomblies K, Laitinen RAE, Yant L, Mott R, Weigel D (2011) Genetic architecture of flowering-time variation in Arabidopsis thaliana. Genetics 188:421–433

Sánchez-Bermúdez E, Méndez-Vigo B, Pico FX, Martínez-Zapater JM, Alonso-Blanco C (2012) Novel natural alleles at FLC and LVR loci account for enhanced vernalization responses in Arabidopsis thaliana. Plant Cell Environ. doi:10.1111/j.1365-3040.2012.02518.x

Severing EI, van Dijk ADJ, Morabito G, Busscher-Lange J, Immink RGH, van Ham RCHJ (2012) Predicting the impact of alternative splicing on plant MADS domain protein function. PLoS ONE 7:e30524

Sharp PA (1994) Split genes and RNA splicing. Cell 77:805–815

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The FLF MADS Box Gene: A Repressor of Flowering in Arabidopsis Regulated by Vernalization and Methylation. Plant Cell 11:445–458

Sheldon CC, Finnegan EJ, Peacock WJ, Dennis ES (2009) Mechanisms of gene repression by vernalization in Arabidopsis. Plant J 59:488–498

Simpson CG, Monika Maronova JF, Kalyana M, Davidson D, McNicol J, Bart A, Brown JWS (2008) Monitoring changes in alternative precursor messenger RNA splicing in multiple gene transcripts. Plant J 53:1035–1048

Strange A, Li P, Lister C, Anderson J, Warthmann N, Shindo C, Irwin J, Nordborg M, Dean C (2011) Major-effect alleles at relatively few loci underlie distinct vernalization and flowering variation in Arabidopsis accessions. PLoS ONE 6(5):e19949. doi:10.1371/journal.pone.0019949

Sung S, Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427:159–164

Sung S, Schmitz RJ, Amasino RM (2006) A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes Dev 20:3244–3248

Tai H, Williams M, Iyengar A, Yeates J, Beardmore T (2007) Regulation of the β-hydroxyacyl ACP dehydratase gene of Picea mariana by alternative splicing. Plant Cell Rep 26:105–113

Terzi LC, Simpson GG (2008) Regulation of flowering time by RNA processing. In: Reddy ASN, Golovkin M (eds) Nuclear pre-mRNA Processing in plants: current topics in microbiology and immunology, vol. 326. Springer, Berlin, pp 201–218

Travella S, Klimm TE, Kelle B (2006) RNA interference-based gene silencing as an efficient tool for functional genomics in hexaploid bread wheat. Plant Physiol 142:6–20

Wang B, Brendel V (2006) Molecular characterization and phylogeny of U2A2F35 homologs in Plants. Plant Physiol 140:624–636

Wang C, Tian Q, Hou Z, Mucha M, Aukerman M, Olsen O (2007) The Arabidopsis thaliana AT PRP39-1 gene, encoding a tetratricopeptide repeat protein with similarity to the yeast pre-mRNA processing protein PRP39, affects flowering time. Plant Cell Rep 26:1357–1366

Wang B, O’Toole M, Brendel V, Young N (2008) Cross-species EST alignments reveal novel and conserved alternative splicing events in legumes. BMC Plant Biol 8:17

Xing D, Ruqiang X, Li QQ (2008) Arabidopsis PCFS4, a homologue of yeast polyadenylation factor Pcf11p, regulates FCA alternative processing and promotes flowering time. Plant J 54:899–910