TEMPRANILLO is a direct repressor of the microRNA miR172

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Running title: TEMs regulate miR172

Keywords: Arabidopsis, flowering, juvenile-to-adult transition, miR172, SPL, TEMPRANILLO, vegetative phase change.

Summary
In the age-dependent pathway, the microRNA 156 (miR156) is essential for the correct timing of developmental transitions. MiR156 negatively regulates several SPL genes, which promote the juvenile-to-adult and floral transitions in part through up-regulation of miR172. The transcriptional repressors TEMPRANILLO1 (TEM1) and TEM2 delay flowering in Arabidopsis thaliana at least through direct repression of FLOWERING LOCUS T (FT) and

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/tpj.14455
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gibberellins biosynthetic genes, and have also been reported to participate in the length of the juvenile phase. TEM mRNA and miR156 levels decrease gradually, allowing progression through developmental phases. Given these similarities, we hypothesized that TEMs and the miR156/SPL/miR172 module could act through a common genetic pathway.

We analyzed the effect of TEMs on miR156, SPL and miR172 levels, tested binding of TEMs to these genes using chromatin immunoprecipitation and analyzed the genetic interaction between TEMs and miR172. TEMs played a stronger role in the floral transition than in the juvenile-to-adult transition. TEM1 repressed MIR172A, MIR172B and MIR172C expressions and bound in vivo to at least MIR172C sequences. Genetic analyses indicated that TEMs affect the regulation of developmental timing through miR172.

INTRODUCTION

During their life cycle, plants go through several developmental transitions. The timing of these transitions is essential for proper development and adjustment of growth to environmental conditions. After germination, plants undergo a juvenile phase of vegetative growth, in which they are unable to flower, even under optimal environmental conditions. Following the juvenile period, there is a juvenile-to-adult transition, also termed vegetative phase change, leading to an adult phase in which plants become competent to flower (Huijser & Schmid, 2011). The juvenile-to-adult transition is associated with diverse morphological changes, and the appearance of trichomes on the abaxial side of rosette leaves is a conventional marker of this transition in Arabidopsis thaliana (Telfer et al., 1997; Huijser & Schmid, 2011). In response to environmental and endogenous signals, adult plants experience a vegetative-to-reproductive or floral transition (Amasino, 2010; Andrés & Coupland, 2012).

Flowering is an energy-consuming process and therefore plants need to accumulate enough reserves before inducing the floral transition. To ensure that flowering occurs under favorable conditions, the transition to the reproductive phase is regulated by a complex genetic network that responds to environmental and endogenous cues (Amasino, 2010; Andrés & Coupland, 2012). Arabidopsis flowers earlier under long day (LD) than short day (SD) conditions. We have previously shown that TEMPRANILLO 1 (TEM1) and TEM2 (also known as RAV2) inhibit flowering at early developmental stages under LDs and SDs (Castillejo & Pelaz, 2008; Osnato et al., 2012). TEMs belong to the RAV subfamily of transcription factors (Matías-
Hernández et al., 2014), which bind DNA at the consensus sequence C(A/C/G)ACA(N)_{2-8}(C/A/T)ACCTG (Kagaya et al., 1999). TEMs delay flowering through direct transcriptional repression of genes encoding the florigen component FLOWERING LOCUS T (FT), its paralog TWIN SISTER OF FT (TSF), and the gibberellin (GA) biosynthesis enzymes GIBBERELLIN 3-OXIDASE 1 (GA3OX1) and GA3OX2 (Castillejo & Pelaz, 2008; Osnato et al., 2012; Marín-González et al., 2015). Thus, in Arabidopsis, RAV proteins act as transcriptional repressors (Castillejo & Pelaz, 2008; Ikeda & Ohme-Takagi, 2009; Causier et al., 2012; Osnato et al., 2012; Feng et al., 2014; Marín-González et al., 2015). Recently, a role for TEMs in the control of juvenility in Arabidopsis has been reported (Sgamma et al., 2014). Therefore, TEMs play a role in two aspects of developmental timing, the juvenile-to-adult and the floral transitions.

Among the small RNAs involved in the regulation of plant developmental timing, the microRNAs (miRNAs) miR156 and miR172 play a very prominent role (Poethig, 2009; Rubio-Somoza & Weigel, 2011). In diverse plant species, miR156 delays vegetative phase change and flowering through downregulation of several SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes (Wang & Wang, 2015). In Arabidopsis, these miR156 target genes include SPL3, SPL4, SPL5, SPL9, SPL10 and SPL15 (Wu & Poethig, 2006; Gandikota et al., 2007; Schwarz et al., 2008; Wu et al., 2009; Hyun et al., 2016). Plants overexpressing miR156 (35S::miR156) show a delayed juvenile-to-adult transition, flower late and have reduced SPL mRNA levels (Schwab et al., 2005; Wu & Poethig, 2006; Gandikota et al., 2007; Wang et al., 2009; Wu et al., 2009). Conversely, plants in which miR156 function is reduced (35S::MIM156 plants) lack the juvenile phase, flower early and have increased SPL mRNA levels (Franco-Zorrilla et al., 2007; Wang et al., 2009; Wu et al., 2009).

Although single mutants of the miR156-targeted SPL genes show very weak or no obvious phenotypes under LDs, double spl9 spl15 mutants show delayed juvenile-to-adult and floral transitions, revealing functional redundancy within the SPL family (Wu & Poethig, 2006; Schwarz et al., 2008; Wang et al., 2008). The function of the miR156/SPL module has also been investigated using miR156-resistant versions of SPL genes (rSPL). Plants overexpressing rSPL3, rSPL4 or rSPL5 show early vegetative phase change and early flowering and plants expressing rSPL9 or rSPL15 under the control of their own promoters

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show even stronger phenotypes (Wu & Poethig, 2006; Gandikota et al., 2007; Wu et al., 2009; Hyun et al., 2016).

MiR156 and miR172 show opposite temporal expression patterns, such that miR156 decreases and miR172 increases with age (Aukerman & Sakai, 2003; Wu & Poethig, 2006; Jung et al., 2007; Wang et al., 2009; Wu et al., 2009). MiR156 represses miR172 expression via down-regulation of SPL9 and SPL15, which promote miR172 expression (Wu et al., 2009; Hyun et al., 2016). In turn, miR172 promotes the juvenile-to-adult and the floral transitions, given that miR172 overexpression accelerates these transitions and reduced miR172 function delays flowering (Aukerman & Sakai, 2003; Chen, 2004; Wu et al., 2009; Todesco et al., 2010; Jung et al., 2011). MiR172 down-regulates its target genes, which encode six APETALA2 family transcription factors (Aukerman & Sakai, 2003; Chen, 2004). All six redundantly repress flowering and at least three of them delay the juvenile-to-adult transition (Aukerman & Sakai, 2003; Chen, 2004; Jung et al., 2007; Mathieu et al., 2009; Wu et al., 2009; Yant et al., 2010; Jung et al., 2011). Therefore, miR156, SPLs, miR172 and the miR172 target genes constitute an age-dependent developmental pathway, in which complex feedback regulations have been reported (Schwab et al., 2005; Mathieu et al., 2009; Wu et al., 2009; Yant et al., 2010; Jung et al., 2011).

Similar to miR156, TEM1 and TEM2 mRNA levels decline with age (Castillejo & Pelaz, 2008) and have been reported to participate in juvenility length (Sgamma et al., 2014). Given the similar expression patterns and similar phenotypic effects of TEMs and miR156 on vegetative phase change and flowering, we hypothesized that TEMs and the miR156/SPL/miR172 module may act through a common genetic pathway. We show here that TEMs negatively regulate miR172 levels. TEM1 binds to MIR172C chromatin and directly regulates its expression, and also regulates that of MIR172A and MIR172B. In addition, miR172 partially mediates the effects of TEMs on vegetative phase change and flowering. Therefore, we conclude that TEMs regulate developmental timing in part through this miRNA involved in the age-dependent pathway.
RESULTS

Comparison of phenotypes caused by alterations in TEM1/TEM2 and the age-dependent pathway

In order to uncover the mechanism of TEM1/TEM2 action on phase change, we compared the phenotypes of plants with altered TEM levels with those of plants affected in the age-dependent pathway under SDs and LDs. It has been shown that miR156 maintains the juvenile traits by repressing SPLs, that in turn activate miR172 which will promote adult epidermal identity, such as the appearance of trichomes on the abaxial surface of rosette leaves (Poethig 2003, 2013, Huijser and Schmid, 2011, Yu et al., 2015; Wang et al., 2019). The timing of abaxial trichome formation is correlated with flowering time, consistent with the fact that juvenile-to-adult vegetative phase change contributes to the acquisition of the competence to flower. Thus, miR172 is involved in the timing of trichome formation, and its AP2 target genes, together with KANADI, have been shown to mediate the temporal and spatial integration for abaxial trichome formation (Wang et al., 2019). Interestingly, alterations of TARGET OF EAT 1 (TOE1) or miR172 levels affect the timing of abaxial, but not adaxial, trichome formation (Wang et al., 2019). Consequently, the length of juvenile or adult phases can be determined by counting the leaves without and with abaxial trichomes, respectively.

Under SDs, we confirmed previously reported results and used them as control for our tem mutant plants growing in parallel. Thus, 35S::miR156 plants showed a dramatically extended juvenile phase, consistent with the results of Wu et al. (2009), and a shortened adult phase (Figures 1a,b, S1a,b; Table S2a,b), resulting in late flowering compared with wild-type plants (Figures 2a,b, S2a,b; Table S3a,b). Plants in which miR156 activity was inhibited (35S::MIM156) and plants expressing a miR156-resistant form of SPL9 (pSLP9::rSPL9) lacked the juvenile phase, in agreement with a previous report (Wu et al., 2009), and had an adult phase similar to that of wild-type plants under SDs (Figures 1a,b, S1a-d; Table S2a,b). Therefore, 35S::MIM156 and pSLP9::rSPL9 plants flowered with fewer leaves than wild-type plants (Figures 2a, S2a,c; Table S3a) due to the absence of the juvenile phase. 35S::MIM172 plants showed longer juvenile and adult phases than wild-type plants (Table S2a,b), and flowered with statistically significant more leaves than wild-type plants (Figures 2a, S2c; Table S3a), consistent with previous reports (Todesco et al., 2010; Galvão et al., 2012).
Under SDs the length of the juvenile phase of *tem1* and *tem2* single mutants was not altered, whereas *tem1 tem2* double mutants displayed a slightly shorter juvenile phase than the wild type (Figures 1a,c, S1a,e; Table S2a,c), indicating that TEM1 and TEM2 delay the juvenile-to-adult transition in a redundant manner. *tem2* and *tem1 tem2* mutants had a significantly shorter adult phase than wild-type plants (Figures 1b,d, S1b,f; Table S2b,d). Consistent with a shortened adult phase and with previous reports (Castillejo & Pelaz, 2008; Osnato et al., 2012), early flowering was observed in *tem1*, *tem2* and, more markedly, in *tem1 tem2* (Figures 2a-d, S2a,b,c,f; Table S3a-d). Plants overexpressing *TEM1* (*35S::TEM1*) had a dramatically extended juvenile phase (>113 juvenile leaves) and neither transitioned to the adult phase nor flowered for at least 4 months under SDs. Overall, similar results were obtained under LDs (Figures 1e-j, 2e-j, S1g-l, S2g-l; Tables S2e-j, S3e-j). Under this condition, *35S::TEM1* plants also showed a substantially long juvenile phase, an unexpectedly short adult phase (Figures 1i,j, S1k,l; Table S2i,j) and flowered later than the wild-type (Figures 2i,j, S2k,l; Table S3i,j). Most *35S::TEM1* plants did not produce leaves with abaxial trichomes, probably due to the fact that these plants, in addition to having a late juvenile-to-adult transition, produce very few leaf trichomes (Matías-Hernández et al., 2016). Due to the similar results obtained with all genotypes under both photoperiods we therefore used LD conditions for all subsequent experiments.

Because of the lack of other *tem1* and *tem2* alleles, we analyzed the length of the juvenile and adult phases of *RNAi-TEM1/2* plants, in which both *TEM1* and *TEM2* are partially silenced (Castillejo & Pelaz, 2008) to confirm the effect of TEMs on the juvenile-to-adult transition. Consistent with the results obtained in *tem1 tem2* mutants, *RNAi-TEM1/2* plants exhibited shorter juvenile and adult phases than wild-type plants under LDs (Figures 3, S3; Table S4a,b). *RNAi-TEM1/2* also flowered earlier than the wild type under this condition (Figures 4, S4; Table S5a,b), in agreement with previous results (Castillejo & Pelaz, 2008).

We can conclude that TEMs and the miR156/SPL module affect the juvenile-to-adult and floral transitions to different extents, with the miR156/SPL module having a more dramatic effect on the juvenile phase and TEMs affecting more strongly the floral transition, with a much smaller effect on the regulation of vegetative phase change. Therefore, our results suggest that the miR156/SPL module and TEM1/TEM2 play partially overlapping but distinct roles in the regulation of phase transitions.
TEMs do not act through MiR156 or SPL genes

TEMs and miR156 affect the juvenile-to-adult and the floral transitions under both LDs and SDs (Figures 1-4, S1-S4; Schwab et al., 2005; Wu & Poethig, 2006; Franco-Zorrilla et al., 2007; Castillejo & Pelaz, 2008; Wu et al., 2009; Osnato et al., 2012; Sgamma et al., 2014). As miR156 and TEM1/TEM2 follow similar temporal expression patterns and have partially overlapping phenotypic effects, we tested whether miR156 regulates TEM1/TEM2 levels or vice versa. 35S::miR156 and 35S::MIM156 plants did not show significant alterations in TEM1 and TEM2 mRNA levels that could correlate with their flowering phenotypes under LD and SD conditions (Figure S5). For the analysis of the effect of TEMs on miR156, we collected samples at two time points under LD, one during the light period (ZT8), when TEM mRNA levels are low (Castillejo & Pelaz, 2008; Osnato et al., 2012) and the miR156-targeted SPL9 mRNA peaks (Figure S6), and one during the night (ZT18), when TEM mRNAs peak (Castillejo & Pelaz, 2008; Osnato et al., 2012). Although RNA blots with samples collected at one time point, ZT8 in 10-day-old plants, showed slight differences (Figure S7a,b), when we examined miR156 levels in tem1 tem2 mutants at different ages, we did not observe substantial differences relative to wild-type plants (Figure S7c). Even the miR156 level reduction happened at the same time, after day 12, in both genotypes and, therefore, we could not confirm that TEMs have an effect on miR156 (Figure S7c).

Then we tested the effect of TEMs on the expression of SPL genes under LDs. As previously, when we studied SPL3, SPL9 and SPL15 expressions at one time point we found that SPL3 transcript abundance was slightly increased in 10-day-old tem1 tem2 plants (Figure 5a-c) and that the expression of all three genes was reduced in 35S::TEM1 plants (Figure 5a-c), but an analysis of SPL mRNA levels at different ages confirmed that only SPL3 and SPL9 were down-regulated in 35S::TEM1 plants (Figure 5d-i) and none was affected in tem1 tem2 mutant plants (Figure 5d-i).

TEMs down-regulate MiR172 and bind to MiR172C chromatin

Since TEMs were reported to have a role in the length of the juvenile phase (Sgamma et al., 2014) and we have not observed expression changes of miR156 or SPL genes in tem1 tem2 mutant plants, we wondered if the effect on the phase transition could be through miR172 whose genes transcription is activated by SPLs (Wu et al., 2009). Consistent with this hypothesis, we found increased abundance of mature miR172 in tem1 tem2 and reduced abundance in 35S::TEM1 when we analyzed 10-day-old plants (Figure 6a,b). Similar results
were obtained by RNA blot (Figure 6a,b) and RT-qPCR (Figure S8). 35S::miR156 and pSPL9::rSPL9 plants used as controls showed slightly reduced and increased miR172 levels, respectively (Figure 6a), as expected (Wu et al., 2009). Then we examined mature miR172 levels in tem1 tem2 mutants at different ages. Again, we found that tem1 tem2 mutants show increased miR172 levels up to day 12 (Figure 6c), indicating that TEMs down-regulate miR172 at early developmental stages, consistent with their effect on the juvenile-to-adult and floral transitions.

We found putative RAV binding sites in four MIR172 genes (Table S6). We chose MIR172C gene because it has several putative RAV binding sites and it was possible to design specific oligos to test binding of TEM1 and TEM2 by ChIP. TEM1 clearly bound to a fragment containing two putative RAV binding sites and TEM2 also bound to some extent (Figure 6d). This suggests that TEMs repress miR172 expression through direct binding to, at least, MIR172C chromatin. We used transient expression assays in Arabidopsis protoplasts to test whether this is the case. Indeed, we found that TEM1 represses expression of a reporter construct that carries a fragment of the MIR172C gene containing the two RAV binding sites bound in ChIP experiments (Figure 6e). In addition, MIR172C transcript levels are increased in tem1 tem2 and RNAi-TEM1/2 plants, and are decreased in 35S::TEM1 plants (Figure 6f). Similarly, we found that MIR172A and MIR172B transcript levels are increased when TEM genes are down-regulated and that MIR172A levels are decreased when TEM1 is overexpressed (Figure 6f). Upregulation of the three MIR172A, MIR172B and MIR172C genes resulted in the high mature miR172 levels observed in tem1 tem2 mutant plants (Figure 6a,c and S8). TEMs, therefore, down-regulate miR172 levels by binding to at least the MIR172C gene and repressing its transcription.

TEMs act partially through miR172

If the early vegetative phase change and early flowering of tem1 tem2 and RNAi-TEM1/2 plants is due to increased miR172 abundance, silencing of miR172 should suppress the vegetative phase change and flowering phenotypes of tem1 tem2 and RNAi-TEM1/2. Unfortunately, we found that several transgenes were silenced when introduced in tem1 and 35S:TEM1 mutant backgrounds (Figure S9a,b,c). Silencing of transgenes by T-DNA insertion mutations has already been reported (e.g. Daxinger et al., 2008; Wu et al., 2009). Therefore, to analyze the genetic interaction between TEMs and miR172 we crossed 35S::MIM172 with RNAi-TEM1/2 and checked that the 35S::MIM172 transgene was not
silenced (Figure S10). If miR172 acts downstream of TEM1 to control vegetative phase change and flowering, we would expect that inactivation of miR172 would suppress the phenotypes of RNAi-TEM1/2 plants. Our results showed that 35S::MIM172 plants had longer juvenile and adult phases and flowered later and after producing more leaves than WT plants (Figures 7, 8, S11, S12; Tables S7a,b, S8a,b). RNAi-TEM1/2 35S::MIM172 plants had intermediate phenotypes between 35S::MIM172 and RNAi-TEM1/2: they had longer juvenile and adult phases than RNAi-TEM1/2 plants, but had shorter phases than 35S::MIM172 plants (Figures 7, S11; Table S7a,b), and they flowered with more leaves than RNAi-TEM1/2, but with fewer leaves than 35S::MIM172 plants (Figures 8, S12; Table S8a). These results indicate that 35S::MIM172 suppresses partially the early juvenile-to-adult transition and the early flowering of RNAi-TEM1/2. Taking together these results and the effect of TEMs on miR172 levels, we can conclude that TEMs delay flowering, and to a lesser extent the vegetative phase change, partially through miR172.

**DISCUSSION**

**TEMs regulate miR172 levels**

Several flowering-time regulators, such as FCA, GIGANTEA, SHORT VEGETATIVE PHASE, SUPPRESSOR OF OVEREXPRESSION OF CO 1, SPL9, and SPL15, affect miR172 levels. Several of these genes regulate transcription of MIR172 genes, whereas others affect processing of miR172 primary transcripts (Jung et al., 2007; Wu et al., 2009; Lee et al., 2010; Cho et al., 2012; Jung et al., 2012b; Tao et al., 2012; Hyun et al., 2016). In addition, proteins of the POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) and PRC2, which establish and maintain transcriptional repression through histone modifications, repress MIR172B expression (Picó et al., 2015). Our work establishes that TEMs are new miR172 regulators (Figure 6). Whereas all the previously reported miR172 regulators have been shown to affect expression or processing of MIR172A and/or MIR172B, we show here that TEMs regulate MIR172A, MIR172B and MIR172C expressions. Therefore, multiple flowering-time regulators fine-tune mature miR172 levels by regulating several MIR172 genes. Further work will be required to understand whether there are interactions among all these factors and how they contribute to establish the temporal and spatial pattern of miR172 expression.
TEM1 and TEM2 had been described as transcriptional repressors that directly down-regulate FT, TSF, GA3ox1 and GA3ox2 (Castillejo & Pelaz, 2008; Osnato et al., 2012; Marín-González et al., 2015). We show here that TEMs down-regulate miR172 levels through direct binding to at least one MIR172 gene, MIR172C, and transcriptional repression of MIR172A, MIR172B and MIR172C (Figure 6). Although the molecular mechanism of this repression has yet to be elucidated, TEM1 and TEM2 interact with the transcriptional corepressor TOPLESS (Causier et al., 2012), which forms a complex with and requires the activity of histone deacetylases (Long et al., 2006; Krogan et al., 2012; Wang et al., 2013). It is possible, then, that TEMs repress transcription by recruiting histone modification factors through their interaction with TOPLESS, or that these interactions contribute to maintenance of transcriptional repression.

Our work and that of other groups suggests that TEMs can repress SPL expression (Figure 5) and function through several mechanisms. Given that FT and GAs are positive regulators of several SPL genes (Yamaguchi et al., 2009; Galvão et al., 2012; Jung et al., 2012a; Park et al., 2013), the repression of FT expression and GA biosynthesis by TEMs (Castillejo & Pelaz, 2008; Osnato et al., 2012) may lead to indirect transcriptional down-regulation of SPLs. TEMs can also exert post-translational regulation of SPLs through the repression of GA biosynthesis (Osnato et al., 2012), as DELLA proteins interfere with SPL transcriptional activity through direct interaction, and GA releases this interaction (Yu et al., 2012). We hypothesize that TEMs can also indirectly repress SPL expression through feedback loops mediated by miR172 target genes. There is feedback regulation of SPL genes by miR172 target genes, such that miR172 target genes negatively regulate several SPLs or positively regulate miR156 (Yant et al., 2010; Jung et al., 2011). This indirect feedback on miR156 might result in the observed differential effect on SPL expressions, as SPL3 transcript has recently been shown to be more sensitive to miR156 cleavage than SPL9 or SPL15 (He et al., 2018). It is possible, therefore, that the down-regulation of SPL3 and SPL9 observed in 35S::TEM1 plants (Figure 5a-i) is an indirect consequence of the downregulation of miR172 (Figure 6b) – and the expected up-regulation of miR172 targets - in these plants. All this would contribute to ensure that SPLs do not promote precocious juvenile-to-adult transition and flowering.
The regulation of flowering by TEMs is partially mediated by miR172

The flowering phenotypes of tem1 tem2 double mutants and RNAi-TEM1/2 plants are stronger than those of tem1 and tem2 single mutants (Figures 2, 4; Castillejo & Pelaz, 2008; Osnato et al., 2012), indicating that both TEM1 and TEM2 are required for delaying flowering. Our work shows that the early flowering phenotype of RNAi-TEM1/2 plants is partially suppressed by the inactivation of miR172 function in RNAi-TEM1/2 35S::MIM172 plants (Figure 8). Together with the binding of TEMs to MIR172C chromatin, the effect of TEMs on MIR172A, MIR172B and MIR172C RNAs and mature miR172 levels (Figure 6), indicates that TEMs regulate flowering partly through miR172, by repressing the expression of MIR172A, MIR172B and directly that of MIR172C. Taking into account previous reports, we can conclude that the regulation of flowering time by TEMs is mainly mediated by FT, TSF and gibberellin (Castillejo & Pelaz, 2008; Osnato et al., 2012; Marín-González et al., 2015), but also partially by miR172.

TEMs play a more modest role in the juvenile-to-adult transition

We had previously shown that TEM1 and TEM2 play a role in the timing of the floral transition in Arabidopsis (Castillejo & Pelaz, 2008; Osnato et al., 2012). We show here that they also somewhat affect the timing of vegetative phase change. In a previous work, it was showed a more dramatic effect of TEMs on the juvenile phase (Sgamma et al., 2014). However, they determined the length of the juvenile phase by the response of flowering to inductive photoperiods and measured number of days from germination, whereas we measured the number of leaves without abaxial trichomes (Telfer et al., 1997; Huijser & Schmid, 2011; Wang et al., 2019). In addition, we cannot rule out that the difference can be due to the use of different growth conditions. Taking into account that determining the juvenile phase length by measuring the competence to flower after LD exposure may not be the same as determining it by counting the number juvenile leaves, our results (Figure 1 and 3), although with a much weaker effect, seem consistent with those of Sgamma et al. (2014) as we both observed that TEM1 and TEM2 redundantly delay vegetative phase change.

Unexpectedly, when TEM1 is overexpressed, instead of an extension of the adult phase, we observed a dramatic lengthening of the juvenile phase under both LDs and SDs, together with an extreme shortening or even suppression of the adult phase under LDs (Figure 1i,j). However, 35S::TEM1 plants show a dramatically reduced trichome density (Matías-Hernández et al., 2016). Therefore, it is possible that the alteration of the number of leaves

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without and with abaxial trichomes in these plants is a combination of the effects on the juvenile-to-adult transition and on trichome density.

The different genetic pathways acting in the flowering time network share several genes (Bouché et al., 2016). By revealing the role of TEMs in the age-dependent flowering pathway, our work suggests that TEMs link this pathway to the photoperiod, GA and ambient temperature pathways, in which TEMs are also involved (Castillejo & Pelaz, 2008; Osnato et al., 2012; Marín-González et al., 2015), pointing to additional genetic pathway inter-communication within the flowering network.

MATERIALS AND METHODS

Plant material and growth conditions
Arabidopsis thaliana (L.) Heynh. Columbia-0 (Col-0) was used as the wild type for all the experiments. tem1-1, tem2-2, tem1-1 tem2-2, RNAi-TEM1/2, 35S::TEM1, 35S::TEM2, pTEM1::GUS, 35S::miR156, 35S::MIM156, 35S::MIM172 and pSPL9::rSPL9 plants have been previously described (Schwab et al., 2005; Franco-Zorrilla et al., 2007; Castillejo & Pelaz, 2008; Wang et al., 2008; Todesco et al., 2010; Osnato et al., 2012). Seeds were stratified on wet filter paper in the dark at 4°C for 3-4 d and then sown on soil and grown in controlled environment chambers at 22°C under LDs (16 h light : 8 h dark) or SDs (8 h light : 16 h dark) at a light intensity of 80-90 µmol m⁻² s⁻¹.

Phenotypic analyses
To determine the juvenile-to-adult transition we counted the rosette leaves without and with abaxial trichomes (juvenile and adult leaves, respectively). For flowering time experiments, the shoot apex was carefully checked for visible signs of flowering every two days. Flowering time was measured as the number of days from sowing to the appearance of the floral bud and as the total number of rosette and cauline leaves produced on the main stem. We used at least 8 plants per genotype in each experiment, although in most experiments we used 15-20 plants per genotype.
Analyses of miRNA and transcript levels

For miRNA analyses, total RNA was extracted from pools of at least 10 plants using the Real ARNzol Spin kit (+PVP; Durviz) or using Trizol (Ambion) following manufacturer’s instructions. RNAs were treated with DNase I using the DNA-free kit (Ambion) and were precipitated with sodium acetate. Stem-loop reverse transcription followed by quantitative real time PCR (stem-loop RT-qPCR) and RNA blots were performed as previously described (Martin et al., 2009). Primer and probe sequences are shown in Table S1.

For analyses of transcript levels by RT-qPCR, total RNA was extracted from pools of at least 10 plants with the Real ARNzol Spin kit (+PVP; Durviz), the PureLink RNA Mini kit (Ambion) or Trizol (Ambion), and was treated with DNase I using the DNA-free kit (Ambion). Reverse transcription was performed with 1-4 µg of RNA using Superscript III reverse transcriptase (Invitrogen), following manufacturer’s instructions. qPCR was performed on a LightCycler® 480 Real-Time PCR System (Roche Diagnostics Ltd) with gene-specific primer pairs. The reactions, performed in triplicate in a volume of 14 µl, contained 0.2 µl of cDNA (except for MIR172C, see below), 1X Light Cycler 480 SYBR Green I Master mix (Roche), 0.3 µM forward primer and 0.3 µM reverse primer, and were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of PCR was checked with dissociation curves and quantification was standardized to UBIQUITIN10 (UBQ10) mRNA levels. Data from RT-qPCR were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Primer sequences are listed in Table S1.

The analysis of MIR172C RNA levels was performed using TaqMan assays provided by Applied Biosystems (assay IDs: ARKA3K9 for MIR172C and At02163341_gH for IPP2). The qPCR reactions, performed in triplicate in a volume of 14 µl, contained 2 µl of cDNA, 1X TaqMan Multiplex Master mix (Applied Biosystems) and 1X TaqMan assay, and were incubated as indicated above. Quantification of MIR172C RNA was standardized to IPP2 mRNA levels and data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

GUS staining

Histochemical analyses of GUS expression were performed as previously described (Blázquez et al., 1997).
Identification of putative RAV binding sites

To find putative RAV binding sites, sequences of interest were searched for the pattern C[ACG][ACG]CAN(2,9)[ACT]NNCTG using Fuzznuc (http://emboss.bioinformatics.nl/cgi-bin/emboss/fuzznuc) or DNA Pattern Find (http://www.bioinformatics.org/sms2/dna_pattern.html).

Chromatin immunoprecipitation analyses

Chromatin immunoprecipitation (ChIP) experiments were performed using a modified version of a previously reported protocol (Matias-Hernandez et al., 2010). Direct binding of TEM1 and TEM2 to the regulatory regions of putative targets was assayed using the 35S:TEM1-HA and 35S:TEM2-HA lines previously described (Castillejo & Pelaz, 2008). Wild-type plants were used as negative controls. The crosslinked DNA was immunoprecipitated with an anti-HA antibody (Sigma) and purified using Protein A-Agarose resin (Millipore). Enrichment of the target regions was determined by qPCR using different primer sets specific for putative direct targets, as listed in Table S1. The qPCR assay was conducted in triplicate using a SYBR Green Assay (SYBR Green Supermix, Roche) and was performed in a Roche LightCycler® 480 System. For the binding of TEM1 and TEM2 to the selected genomic regions, the affinity of the purified sample obtained in the 35S:TEM1-HA and 35S:TEM2-HA lines was compared with the affinity-purified sample obtained in the wild-type background, which was used as negative control. Fold enrichment, relative to wild-type input DNA immunoprecipitated with no antibody, was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Transient expression assays

To generate the set of reporter vectors, different regions of MIR172C were cloned as SalI-PstI fragments in a modified pGreenII 0800-LUC vector carrying p35S::LUC (as reporter) and p35S::REN (as internal control to estimate the proportion of transformed protoplasts). Arabidopsis protoplasts were isolated from leaf-derived calli by digesting cell walls with Macerozyme R-10 and Cellulase (Yakult Pharmaceuticals). Protoplasts were transfected with different combinations of effector p35S::TEM1 (Castillejo & Pelaz, 2008) and reporter constructs using PEG. After an overnight incubation in darkness at 24°C, transformed protoplasts were pelleted and resuspended in homogenization buffer for RNA extraction and DNase treatment with the Maxwell RSC Plant RNA kit (Promega), according to
manufacturer’s protocol. Transcriptional repression activity of TEM1, based on the relative ratio of *Luciferase* (*LUC*) and *Renilla* (*REN*) mRNA abundance, was assessed by RT-qPCR. Primers used to generate the reporter vectors and for qPCR are listed in Table S1.

**Genetic crosses**

*RNAi-TEM1/2* and *35S::MIM172* plants were crossed and the F₁ generation was allowed to self-pollinate. F₂ plants were selected based on resistance to kanamycin and Basta and were genotyped by checking resistance of the F₃ generation to kanamycin and Basta. F₃ homozygous plants were used for all the experiments.

**Statistical analyses**

Statistical analyses were performed with GraphPad Prism 6 or 7 software (GraphPad Software, Inc). Shapiro-Wilk normality tests were performed. For comparisons we used Student’s t test and ANOVA analyses, multiple comparisons were then corrected with Dunnett’s or Tukey’s test.

**Data statement**

Materials and data are available upon request to the corresponding authors.

**Acknowledgements**

We thank Detlef Weigel and Scott Poethig for seeds; Rossana Henriques for primers, discussion and critical reading of the manuscript; Mauricio Soto for advice with miRNA blots; Martí Bernardo for help with statistical analyses and data visualization; Laura Ossorio, Manel Giménez, Cristina Valdivieso and CRAG greenhouse staff for technical assistance; and Ángel Sánchez for pictures. This work was supported by the Spanish Ministry of Economy and Competitiveness/European Regional Development Fund (grants BFU2012-33746, BFU2015-64409-P and CSD2007-00036), the Catalonian Government (CERCA Programme, Consolidated Research Group no. 2014 SGR 1406 and Investigator Training Program PhD fellowship to A.E.A.-J.), and the Spanish Government (FPI fellowship to E.M.-G.). We acknowledge financial support from the Spanish Ministry of Economy and Competitiveness, through the “Severo Ochoa Programme for Centres of Excellence in R&D” 2016-2019 (SEV 2015 0533). A.E.A.-J. performed this work within the frame of a Ph.D. Program of the Universitat Autònoma de Barcelona.

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Author contributions

P.S.-L., S.P. and E.M.-G. conceived and designed the experiments. A.E.A.-J., E.M.-G., L.M.-H., M.O. and P.S.-L. performed the experiments. A.E.A.-J., E.M.-G., L.M.-H., M.O., S.P. and P.S.-L. analyzed the data. P.S.-L. and S.P. wrote the manuscript, with input and comments from all other authors.

The authors declare that there is NO conflict of interest.

Supporting Information

Figure S1 Effect of TEMs, miR156, SPL9 and miR172 on the juvenile-to-adult transition.

Figure S2 Effect of TEMs, miR156, SPL9 and miR172 on flowering time.

Figure S3 Juvenile-to-adult transition of RNAi-TEM1/2 plants.

Figure S4 Flowering time of RNAi-TEM1/2 plants.

Figure S5 TEM1 and TEM2 mRNA levels in 35S::miR156 and 35S::MIM156 plants.

Figure S6 Diurnal oscillation of SPL9 mRNA levels.

Figure S7 Mature miR156 levels in tem1 tem2 and 35S::TEM1 plants.

Figure S8 Mature miR172 levels in tem1 tem2 and 35S::TEM1 plants.

Figure S9 Silencing of transgenes by tem1 and 35S::TEM1.

Figure S10 MIM172 RNA levels in RNAi-TEM1/2 35S::MIM172 plants.

Figure S11 Juvenile-to-adult transition of RNAi-TEM1/2 35S::MIM172 plants.

Figure S12 Flowering time of RNAi-TEM1/2 35S::MIM172 plants.
Table S1 Primer sequences

Tables S2a-S2j Statistical analyses of data from Figure 1

Tables S3a-S3j Statistical analyses of data from Figure 2

Tables S4a-S4b Statistical analyses of data from Figure 3

Tables S5a-S5b Statistical analyses of data from Figure 4

Table S6 Putative RAV binding sites in MIR172 genes

Tables S7a-S7b Statistical analyses of data from Figure 7

Tables S8a-S8b Statistical analyses of data from Figure 8

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**Figure 2.** Effect of miR156, SPL9, miR172 and TEMs on flowering time in *Arabidopsis thaliana*. Boxplots of the number of total leaves (a,c,e,g,i) and days to flowering (b,d,f,h,j) of the indicated genotypes grown under short day (SD, a-d) and long day (LD, e-j) conditions. The lines within the boxes represent the median and the whiskers represent the minimum and maximum values. Data represent the combination of two (a-d,f), three (e,i,j) or four (g,h) independent experiments, n=15-68. Asterisks indicate statistically significant differences relative to the WT (*, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$, ****, $P \leq 0.0001$; ns, not significant), using one-way ANOVA followed by Dunnett’s multiple comparison test (a-h) or using the Student’s t test (i,j). Histograms and statistical analyses of these data are shown in Figure S2 and Tables S3a-S3j, respectively.

**Figure 3.** Juvenile-to-adult transition of *Arabidopsis thaliana* RNAi-TEM1/2 plants. (a,b) Boxplots of the number of juvenile (a) and adult (b) leaves of WT and RNAi-TEM1/2 plants grown under long day conditions. The number of juvenile and adult leaves was determined by counting the rosette leaves without and with abaxial trichomes, respectively. The lines within the boxes represent the median and the whiskers represent the minimum and maximum values. Data represent the combination of two independent experiments, n=52-59. Asterisks indicate statistically significant differences relative to the WT (***, $P \leq 0.001$), using the Student’s t test. Histograms and statistical analyses of these data are shown in Figure S3 and Tables S4a-S4b, respectively.

**Figure 4.** Flowering time of *Arabidopsis thaliana* RNAi-TEM1/2 plants. (a,b) Boxplots of the number of total leaves (a) and days to flowering (b) of WT and RNAi-TEM1/2 plants grown under long day conditions. The lines within the boxes represent the median and the whiskers represent the minimum and maximum values. Data represent the combination of two independent experiments, n=51-60. Asterisks indicate statistically significant differences relative to the WT (*, $P \leq 0.05$, ***, $P \leq 0.001$), using the Student’s t test. Histograms and statistical analyses of these data are shown in Figure S4 and Tables S5a-S5b, respectively. (c) WT and RNAi-TEM1/2 plants grown under LD conditions for 21 days. Bars, 1 cm.

**Figure 5.** TEM1 and TEM2 affect SPL mRNA levels in *Arabidopsis thaliana*. (a-i) SPL3 (a,d,g), SPL9 (b,e,h) and SPL15 (c,f,i) mRNA levels were determined by RT-qPCR in rosettes of 10-day-old tem1 tem2 and 35S::TEM1 plants (a-c) or of plants of different ages (d-i) grown under long days (LDs), collected at ZT8 (a-f) and ZT18 (a-c,g-i). UBQ10 was used for normalization. Histograms and statistical analyses of these data are shown in Figure S6 and Table S6a-S6b, respectively.
as normalization control and normalized levels in the wild type (a-c) or in the wild type at day 6 (d-i) were set to 1. Each symbol represents the mean of the combination of 2-5 independent experiments, n=2-5, and lines represent the standard error of the mean (SEM).

**Figure 6.** TEM1 and TEM2 regulate miR172 levels in *Arabidopsis thaliana*. (a-c) Mature miR172 levels in *tem1 tem2* (a,c) and *35S::TEM1* (b) plants were determined by RNA blot in rosettes of 10-day-old plants (a-b) or of plants of different ages (c) grown under long days (LDs), collected at ZT8 and ZT18. Hybridization to U6 small nuclear RNA was used as a loading control. The numbers below each lane indicate the fold change relative to the miR172 level in the wild type at the corresponding ZT. (d) Binding of TEM1 to MIR172C chromatin by ChIP-qPCR. Chromatin from 11-day-old *35S::TEM1-HA* and *35S::TEM2-HA* plants grown under LDs was immunoprecipitated with an anti-HA antibody. Precipitated chromatin was used as template for qPCR using specific primers for a MIR172C fragment containing two putative RAV binding sites 109 and 186 nt downstream of the transcription start site. Data are presented as fold enrichment relative to WT input DNA immunoprecipitated without antibody. Error bars represent the standard deviation of three technical replicates. Three independent experiments gave similar results and one was chosen as representative. A schematic diagram of the MIR172C gene is shown above the graph. Black boxes indicate exons, an arrow indicates the transcription start site, inverted triangles indicate putative RAV binding sites and arrowheads indicate the fragment amplified by qPCR. (e) Repression of MIR172C expression by TEM1 in transient assays in Arabidopsis protoplasts. RT-qPCR was used to determine the relative ratio of Luciferase (LUC) and Renilla (REN) mRNA levels in protoplasts transformed with reporter constructs, with or without a p35S::TEM1 effector construct. The reporter constructs carried the LUC gene under the control of a 35S promoter (p35S::LUC) or this promoter fused to fragments of the MIR172C gene containing or not containing putative RAV binding sites [MIR172C(+)-p35S::LUC and MIR172C(-)-p35S::LUC, respectively]. Data represent the combination of three independent experiments, n=3. (f) MIR172C (left), MIR172A (middle) and MIR172B (right) RNA levels in *tem1 tem2, RNAi-TEM1/2* and *35S::TEM1* plants were determined by RT-qPCR in rosettes of 19-day-old plants grown under LDs, collected at ZT8 and ZT18. IPP2 (left) and UBQ10 (middle and right) were used as normalization control and normalized levels in the wild type were set to 1. The symbols represent the mean of the combination of 3 independent experiments, n=3, and lines represent the SEM. Spaces in a) denote the removal of additional lanes bearing unrelated samples.

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**Figure 7.** Juvenile-to-adult transition of *Arabidopsis thaliana* RNAi-TEM1/2 35S::MIM172 plants. (a,b) Boxplots of the number of juvenile (a) and adult (b) leaves of WT, RNAi-TEM1/2, 35S::MIM172 and RNAi-TEM1/2 35S::MIM172 plants grown under long day conditions. The number of juvenile and adult leaves was determined by counting the rosette leaves without and with abaxial trichomes, respectively. The lines within the boxes represent the median and the whiskers represent the minimum and maximum values. Data represent the combination of 3 independent experiments, n=59-60. Asterisks indicate statistically significant differences (**, $P \leq 0.01$, ****, $P \leq 0.0001$; ns, not significant), using one-way ANOVA followed by Tukey’s multiple comparison test. Although the WT is shown as reference, only the relevant statistical analyses for the transgenic lines are shown. Histograms and statistical analyses of these data are shown in Figure S11 and Tables S7a-S7b, respectively.

**Figure 8.** Flowering time of *Arabidopsis thaliana* RNAi-TEM1/2 35S::MIM172 plants. (a,b) Boxplots of the number of total leaves (a) and days to flowering (b) of WT, RNAi-TEM1/2, 35S::MIM172 and RNAi-TEM1/2 35S::MIM172 plants grown under long day (LD) conditions. The lines within the boxes represent the median and the whiskers represent the minimum and maximum values. Data represent the combination of 3 independent experiments, n=60. Asterisks indicate statistically significant differences (***, $P \leq 0.001$; ns, not significant), using one-way ANOVA followed by Tukey’s multiple comparison test. Although the WT is shown as reference, only the relevant statistical analyses for the transgenic lines are shown. Histograms and statistical analyses of these data are shown in Figure S12 and Tables S8a-S8b, respectively. (c) WT, RNAi-TEM1/2, 35S::MIM172 and RNAi-TEM1/2 35S::MIM172 plants grown under LD conditions for 23 days. Bars, 1 cm.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

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Figure 8