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FLOWERING NEWSLETTER REVIEW

The role of microRNAs in the control of flowering time

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Abstract

The onset of flowering in plants is regulated by complex gene networks that integrate multiple environmental and endogenous cues to ensure that flowering occurs at the appropriate time. This is achieved by precise control of the expression of key flowering genes at both the transcriptional and post-transcriptional level. In recent years, a class of small non-coding RNAs, called microRNAs (miRNAs), has been shown to regulate gene expression in a number of plant developmental processes and stress responses. MiRNA-based biotechnology, which harnesses the regulatory functions of such endogenous or artificial miRNAs, therefore represents a highly promising area of research. In this review, the process of plant miRNA biogenesis, their mode of action, and multiple regulatory functions are summarized. The roles of the *miR156*, *miR172*, *miR159/319*, *miR390*, and *miR399* families in the flowering time regulatory network in *Arabidopsis thaliana* are discussed in depth.

Key words: *Arabidopsis thaliana*, flowering time, microRNA (miRNA), *miR156*, *miR159/miR319*, *miR172*, *miR390*, *miR399*, plant development, SPL.

Introduction

Plants progress through several developmental phases in their lifetimes; these are characterized by the expression of distinct morphological traits and/or the development of new organs (Huijser and Schmid, 2011; Jin *et al.*, 2013). In angiosperms, one such developmental transition is from vegetative growth to the reproductive growth phase during which flowers are produced. The correct timing of this vegetative to reproductive phase transition is crucial for the reproductive success of a species as its timing must coincide with suitable conditions for fertilization and seed dispersal (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). This is of particular importance in non-self-fertilizing species, as these require their flowering to be synchronized with others in the population, and also to coincide with the activity of pollinators (Huijser and Schmid, 2011; Srikanth and Schmid, 2011). A complex gene network consisting of multiple overlapping, cross-regulating pathways has evolved to coordinate this developmental switch. Environmental and endogenous cues are integrated by the network in order to control the expression of a set of key flowering genes in the shoot apical meristem (SAM) (Srikanth and Schmid, 2011; Yamaguchi and Abe, 2012). When expression of these genes exceeds a threshold level, the SAM switches from a vegetative meristem to a floral meristem. As the timing of flowering significantly impacts both plant fitness and crop yield, a detailed understanding of the regulatory mechanisms governing flowering time is essential for continued improvements in agricultural practice (Huijser and Schmid, 2011; Srikanth and Schmid, 2011).

In recent years, microRNAs (miRNAs), a class of small non-coding RNA molecules ranging from 18 to 24 nucleotides in length, have been identified as central regulators of gene expression in both plants and animals (Yamaguchi and Abe, 2012). These mediate direct, or indirect, transcriptional and post-transcriptional gene silencing (TGS and PTGS) to modulate the activity of the networks underlying various developmental programmes and plant stress adaptations (Rubio-Somoza and Weigel, 2011; Khraiwesh *et al.*, 2012; Jin *et al.*, 2013). Several miRNA families have been shown to play important roles in a number of the pathways controlling flowering, serving either to inhibit or to promote the reproductive

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phase transition. The main players are the *miR156* and *miR172* families, the activities of which control both the juvenile to adult vegetative phase transition and reproductive phase transition (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). In addition, the *miR159*, *miR319*, *miR390*, and *miR399* families have also been shown to play a role in the control of flowering time (Jones-Rhoades *et al.*, 2006; Kim *et al.*, 2011; Rubio-Somoza and Weigel, 2011; Jin *et al.*, 2013).

Plant miRNAs are the subject of intense research, as these gene regulators have potential applications for the control of almost every aspect of plant development. The manipulation of plant miRNA expression levels, as well as the use of target-specific artificial miRNAs, allows for the control of target gene expression and thus entire gene programmes (Schwab *et al.*, 2006; Zhou and Wang, 2013). Developmental processes such as plant growth and stature, flowering, seed set, and yield could potentially be regulated and optimized. Furthermore, expressing miRNAs specifically targeting the RNA genomes of major plant viruses in transgenic plants may be a mechanism to engineer viral disease resistance (Qu *et al.*, 2012).

This review will provide a summary of the biogenesis and mechanism of action of plant miRNAs, and a detailed analysis of those miRNA families that are involved in the control of flowering time.

Biogenesis, processing, and stability of plant microRNAs

Plants possess a large repertoire of evolutionarily conserved, and more recently evolved species-specific miRNAs that regulate various aspects of plant physiology and development (reviewed in Voinnet, 2009; Luo *et al.*, 2013). The process of miRNA biogenesis is evolutionarily conserved within plants, and there is considerable homology with the process of miRNA biogenesis in animals (Liu *et al.*, 2012; Naqvi *et al.*, 2012).

Plant miRNAs are encoded by *MIR* genes, which are found mostly in the intergenic regions of the *Arabidopsis* genome (Voinnet, 2009; Naqvi *et al.*, 2012). These genes are highly variable in length and regulated by a conserved TATA-box (Xie *et al.*, 2005). Like the promoters of protein-coding genes, *MIR* promoters contain a number of regulatory elements for transcription factor binding (Voinnet, 2009). The promoters of evolutionarily conserved miRNAs, such as *miR156*, contain a number of biotic and abiotic stress response elements, indicating that miRNA expression levels can be modulated by stress-induced transcription factors (Megraw *et al.*, 2006; Naqvi *et al.*, 2012). Furthermore, the specific temporal and spatial expression pattern of miRNAs suggests that their promoters contain tissue- and cell type-specific *cis*-acting regulatory elements (Naqvi *et al.*, 2012). For instance, many *Arabidopsis MIR* promoters contain binding sites for the auxin response factors (ARFs), LEAFY (LFY) and MYC2, transcription factors which are in turn regulated by plant hormones involved in flowering time regulation (Megraw *et al.*, 2006; Voinnet, 2009). A complex regulatory network therefore exists which controls the expression of plant miRNAs at specific developmental time points and under certain environmental conditions.

The process of miRNA biogenesis commences in the nucleus with the transcription of a *MIR* gene by RNA polymerase II (Guleria *et al.*, 2011; Naqvi *et al.*, 2012). A primary miRNA transcript (pri-miRNA) containing a 5′ cap and 3′ polyadenylated tail is produced and spliced to remove introns (Xie *et al.*, 2005; Liu *et al.*, 2012). Pri-miRNA stability is dependent on their interaction with the DAWDLE (DDL) protein, an RNA-binding protein capable of recruiting additional processing factors (Yu *et al.*, 2008; Voinnet, 2009; Liu *et al.*, 2012). The characteristic feature of pri-miRNAs, an imperfect double-stranded fold-back structure, is recognized and processed to produce the precursor miRNA (pre-miRNA) by a protein complex located in perinuclear D/SmD3bodies (Vaucheret, 2006; Liu *et al.*, 2012). The RNase III family protein DICER-LIKE 1 (DCL1), a component of this complex, mediates the endonucleolytic cleavage of the primary transcript to liberate this stem–loop region (Papp *et al.*, 2003). The interaction is aided by the accessory proteins within the complex, which include the double-stranded RNA (dsRNA)-binding protein HYPONASTIC LEAVES 1 (HYL1), the C2H2-zinc finger protein SERRATE (SE), and the cap-binding complex (CBC) proteins (CBP20 and CBP80) (Fang and Spector, 2007; Laubinger *et al.*, 2008; Voinnet, 2009; Khraiwesh *et al.*, 2012) (Fig. 1). Incorrect pri-miRNA processing in plants with mutations in the *DCL1*, *HYL1*, and *SE* genes results in reduced levels of mature miRNAs. At the level of the whole organism, this results in an embryonic lethal, or severely developmentally compromised, phenotype (Han *et al.*, 2004; Yang *et al.*, 2006; Liu *et al.*, 2012).

The pre-miRNA is further processed by DCL1 and other accessory proteins to generate a miRNA/miRNA* duplex consisting of the guide strand miRNA and the passenger strand (miRNA*). This occurs predominantly by a stem to loop processing mechanism, though loop to base processing is required for the maturation of a subset of pre-miRNAs (reviewed in Naqvi *et al.*, 2012). The strands of the miRNA/miRNA* duplex are left with two nucleotide overhangs on their 3′ ends (Voinnet, 2009; Naqvi *et al.*, 2012). These nucleotides are subsequently methylated by the *S*-adenosylmethionine-dependent methyltransferase HUA ENHANCER 1 (HEN1) on their 2′ hydroxyl groups (Yu *et al.*, 2005; Yang *et al.*, 2006; Guleria *et al.*, 2011). This, as well as the addition of a poly(U) tail to the miRNAs, prevents the degradation of the miRNAs by SMALL RNA DEGRADING NUCLEASE-1 (SDN1) (Ramachandran and Chen, 2008; Liu *et al.*, 2012). The methyl group may furthermore act as an export signal for the miRNA/miRNA* duplex to the cytoplasm via the nuclear shuttle protein HASTY 1, though HASTY-independent cytoplasmic transport also occurs (Fig. 1) (Park *et al.*, 2005; Vaucheret, 2006; Naqvi *et al.*, 2012).

In the cytoplasm, the miRNA duplex dissociates into the guide strand and passenger strand through the action of unknown helicases (Guleria *et al.*, 2011). The dsRNA-binding protein DRB1, which interacts with DCL1, is responsible for guide strand selection (Eamens *et al.*, 2009). Subsequently,

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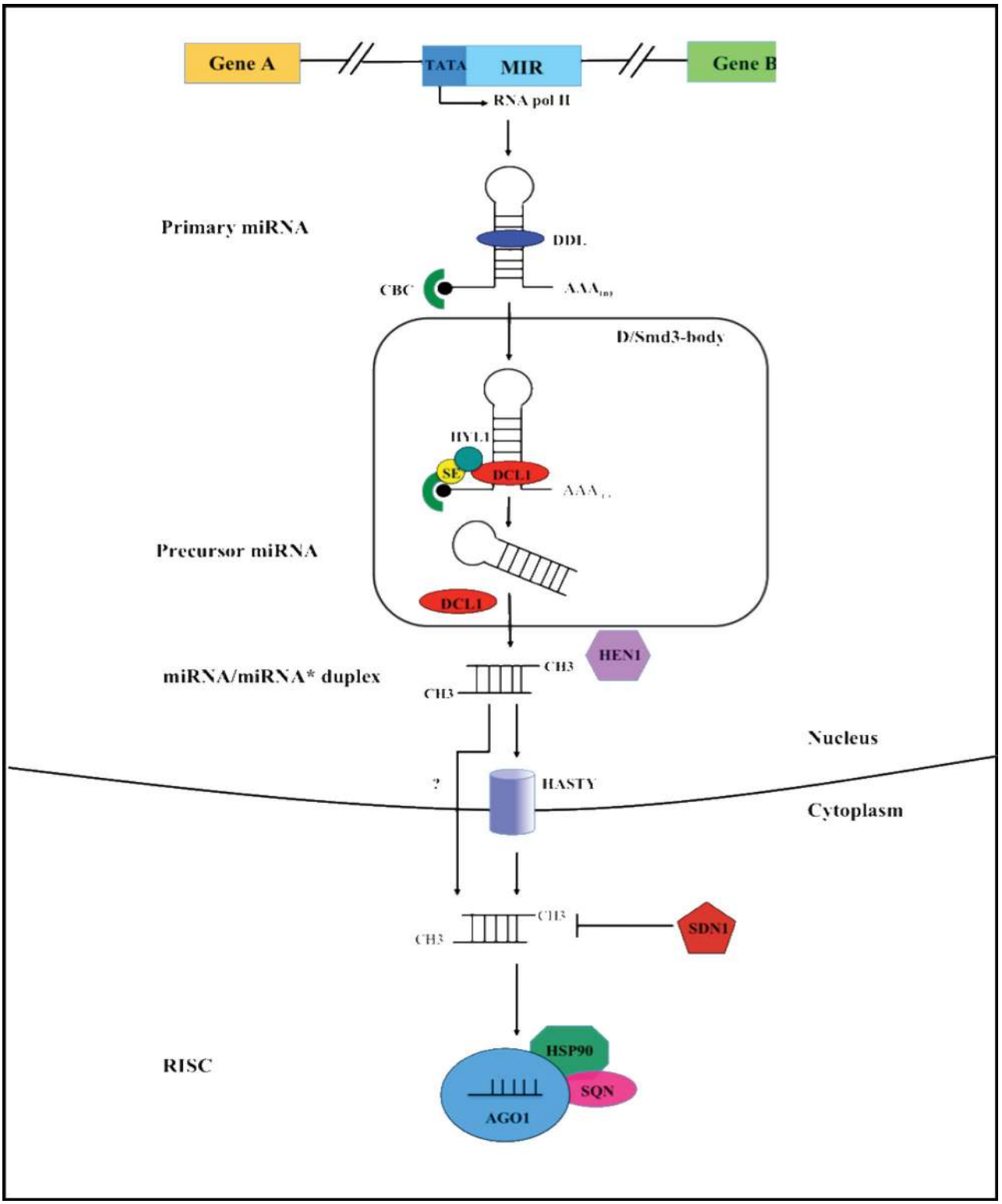

Fig. 1. The process of miRNA biogenesis, processing, and assembly into the RNA-induced silencing complex (RISC). After transcription by RNA polymerase II, the primary miRNA transcript is processed by DCL1 within perinuclear D/SmD3 bodies. This generates the precursor miRNA, which is further spliced to generate the miRNA/miRNA* duplex. The duplex is subsequently shuttled to the cytoplasm by either a HASTY-dependent or -independent mechanism. Once in the cytoplasm, the duplex dissociates into the miRNA and miRNA* strand. The former associates with AGO1 (or AGO10), as well as other accessory proteins to form the RISC. In most cases, the miRNA* strand is degraded. Abbreviations: AGO1, ARGONAUTE 1; CBC, CAP-BINDING COMPLEX; Cyc 40, CYCLOPHILIN 40; DCL1, DICER_LIKE PROTEIN 1; DDL, DAWDLE; HEN1, HUA ENHANCER 1; HSP90, HEAT SHOCK PROTEIN 90; HYL1, HYPONASTIC LEAVES 1; MIR, microRNA gene; SDN1, SMALL RNA DEGRADING NUCLEASE 1; SE, SERRATE; SQN, SQUINT. Adapted from *Cell*, 136, Voinnet O. Origin, biogenesis, and activity of plant microRNAs, 669–687, Copyright (2009), with permission from Elsevier.

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ARGONAUTE 1 (AGO1) or, in some cases, its paralogue AGO10 (two of the 10 AGO proteins found in *Arabidopsis*) binds to the guide strand (Lanet *et al.*, 2009; Guleria *et al.*, 2011; Naqvi *et al.*, 2012). AGO1 binding and activity is aided by the cyclophilin 40-homologue SQUINT (SQN) and the HEAT SHOCK PROTEIN 90 (HSP90) (Khraiwesh *et al.*, 2012; Yamaguchi and Abe, 2012). The guide miRNA strand is retained within the AGO1 complex due to its weaker pairing 5′ nucleotide and lower thermodynamic stability. Together these form the miRNA-induced silencing complex (RISC) (Brodersen and Voinnet, 2009; Naqvi *et al.*, 2012; Shao *et al.*, 2013). The miRNA* in turn is degraded, though recent reports have also identified a regulatory role for those miRNAs* which evade degradation (Shao *et al.*, 2013).

MicroRNAs and gene silencing

MiRNAs act as master regulators of gene expression by inducing both the transcriptional and post-transcriptional silencing of specific genes. PTGS occurs post-RISC assembly once the complex binds to a target transcript by virtue of the associated miRNA (Ding *et al.*, 2012; Khraiwesh *et al.*, 2012). Plant miRNAs display perfect or near perfect sequence complementarity to target sites in mRNA open reading frames (ORFs), which therefore limits the number of cognate mRNAs a miRNA can regulate (Rhoades *et al.*, 2002; Wang *et al.*, 2004; Voinnet, 2009). A given miRNA family and its targets therefore form a regulatory unit termed a miRNA–target node or module (Rubio-Somoza and Weigel, 2011). Binding of the RISC to a target mRNA predominantly results in the AGO1-dependent slicing of the transcript (Llave *et al.*, 2002; Voinnet, 2009; Naqvi *et al.*, 2012). The resulting mRNA cleavage products can be detected by the rapid amplification of 5′ cDNA ends (5′ RACE) technique and northern blot analysis for the identification of stable 3′-cleavage fragments (Voinnet, 2009).

An inconsistency between the levels of mRNA transcripts and loss of protein production was observed in a number of studies, however, which indicated that miRNAs also silence gene expression by translational inhibition (Bari *et al.*, 2006; Fang and Spector, 2007; Lanet *et al.*, 2009; Khraiwesh *et al.*, 2010; Naqvi *et al.*, 2012). Brodersen and colleagues showed that AGO1 slicing activity could be uncoupled from its translational repression activity in *ago1-27* mutants, presumably by preventing its interaction with various accessory proteins (Brodersen *et al.*, 2008). AGO10 was also shown to mediate translational repression of a subset of mRNAs in specific tissues or developmental phases (Brodersen *et al.*, 2008; Voinnet, 2009).

MiRNA-dependent TGS involves epigenetic changes that alter DNA structure to inhibit the production of target gene mRNAs (Wu *et al.*, 2010; Yaish *et al.*, 2011). In *Arabidopsis* it has been postulated that extensive methylation of the genes encoding the transcription factors PHABULOSA (PHB) and PHAVULOTA (PHV) is due to the interaction of the *miR165/166* family with the nascent *PHB* transcript and a chromatin-modifying complex (Bao *et al.*, 2004; Wu *et al.*, 2010). The expression of these two transcription factors, which play key roles in leaf and root development, is thus strictly controlled by this negative feedback loop.

Plant miRNAs therefore act by multiple mechanisms to silence the expression of specific target genes. These different mechanisms may serve organ-, tissue-, or even cell type-specific functions. Irreversible gene silencing is required, for instance, for cell differentiation, and may be mediated by TGS. Reversible gene silencing, on the other hand, is required for transient plant stress responses, and would be mediated by transcript cleavage or translational inhibition (Voinnet, 2009).

The diverse roles of plant microRNAs

In 2009, merely 7 years after the discovery of plant miRNAs, the plant microRNA database (PMRD) listed nearly 8500 mature miRNAs from 121 different plant species that had been discovered by using computational and experimental approaches (Zhang *et al.*, 2010). Of these known miRNAs, the majority were identified in a small subset of plant species including rice (*Oryza sativa*) and *Arabidopsis* (Sun, 2012). For instance, 1427 mature miRNAs have been identified in *Arabidopsis* alone (Zhang *et al.*, 2010). This review mainly focuses on the roles of the miRNAs involved in flowering time control in *A. thaliana*. The roles of miRNAs in other aspects of plant development, in biotic and abiotic plant stress responses, are referred to only briefly as several in-depth reviews have been published recently which describe the roles of miRNAs in these responses (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012; Sun, 2012; Jin *et al.*, 2013).

The plant life cycle begins with embryogenesis, which is followed by seed germination, the vegetative phase (which is further divided into the juvenile and adult vegetative phases), the reproductive phase, seed set, and finally senescence (Huijser and Schmid, 2011; Jin *et al.*, 2013). MiRNAs directly regulate both the timing of these transitions and the expression of certain morphological traits by targeting the expression of key transcription factors. Furthermore, miRNAs indirectly affect the expression of these genes by modulating the expression of phytohormones, *trans*-acting small interfering RNAs (tasiRNAs), and miRNAs themselves (Jin *et al.*, 2013).

The recognition of bacterial, viral, fungal, and nematode pathogen-associated molecular patterns (PAMPs) triggers a defence response in plants in which miRNAs play a key role (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012). Their activity underlies certain dramatic changes in gene expression, and in phytohormone and nutrient levels that are required for the induction of plant resistance to invading pathogens (Sunkar *et al.*, 2012). For instance, several miRNAs, including *miR160*, *miR167*, and *miR393*, are up-regulated in *Arabidopsis* leaves upon their infection with a virulent strain of the bacterium *Pseudomonas syringae* (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012). These miRNAs limit pathogen growth by inhibiting various aspects of auxin signalling. *MiR393* is induced by the bacterial PAMP flagellin-22 and down-regulates the expression of the auxin receptors TRANSPORT INHIBITOR

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RESPONSE 1 (TIR1) and AUXIN SIGNALING F-BOX (AFB1-3) (Kruszka *et al.*, 2012; Sunkar *et al.*, 2012). *MiR160* and *miR167*, on the other hand, target *ARF* transcripts for degradation (Sun, 2012). However, it is important to note that while miRNA expression patterns may change in response to different stresses, this does not necessarily imply that these are involved in plant stress adaptation (Khraiwesh *et al.*, 2012).

Drought, extreme temperatures, salinity, nutrient starvation, radiation, and/or oxidative stress all challenge plant survival. In order to survive such abiotic stressors, plants have developed complex gene networks that facilitate the rapid adaptation to adverse environmental conditions, in which miRNAs mediate transient gene silencing (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012). The concerted activity of these networks re-establishes cellular homeostasis, often at the price of plant development and growth rate (Sunkar *et al.*, 2012). In *Arabidopsis*, for instance, miRNA expression is either up- or down-regulated depending on the stress, their targets being inhibitors of stress responses or components of stress-inhibited processes (Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012). Stress can often cause plants to flower early, and recent findings by Xu *et al.* (2014) suggest that the *miR169* family is involved in stress-induced flowering. The up-regulation of *miR169* family members by abiotic stress reduces levels of the AtNF-YA transcription factor which in turn results in de-repression of genes involved in the promotion of flowering. While some miRNA families have conserved functions in many plant species, other stress-responsive miRNA families may exhibit distinct expression profiles in different plant species, or even in related genotypes of the same species that have distinct stress sensitivities (Sunkar *et al.*, 2012; Jin *et al.*, 2013).

MicroRNAs and the control of flowering time

In *Arabidopsis*, the main pathways regulating flowering in response to environmental cues are the photoperiod, ambient temperature, and vernalization pathways, which respond to daylength, surrounding temperature, and prolonged cold exposure, respectively (Jackson, 2009; Fornara *et al.*, 2010). The autonomous, gibberellic acid (GA), nutrient-responsive, and ageing pathways in turn are controlled by endogenous factors, such as phytohormones and carbohydrate status (Srikanth and Schmid, 2011; Kim *et al.*, 2012; Matsoukas *et al.*, 2012; Yamaguchi and Abe, 2012). These pathways form a complex gene regulatory network that converges on a set of floral pathway integrators, namely *FLOWERING LOCUS T* (*FT*) and its paralogue *TWIN SISTER OF FT* (*TSF*), as well as *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *AGAMOUS-LIKE 24* (*AGL24*) (Jang *et al.*, 2009; Fornara *et al.*, 2010; Srikanth and Schmid, 2011; Matsoukas *et al.*, 2012).

FT, which is induced in leaves by *CONSTANS* (*CO*) in response to an inductive photoperiod [in *Arabidopsis* this is long days (LDs)], acts as a long-distance signal to the SAM where it interacts with the locally transcribed FLOWERING LOCUS D (FD) transcription factor to activate other floral integrators (An *et al.*, 2004; Abe *et al.*, 2005; Amasino, 2010; Matsoukas *et al.*, 2012). GIGANTEA (GI), a component of both the circadian clock and photoperiod pathway, acts together with FLAVIN BINDING, KELCH REPEAT F-BOX 1 (FKF1) to regulate *CO* expression in different photoperiods (Sawa *et al.*, 2007; Jackson, 2009). The floral pathway integrators in turn activate the floral meristem identity genes, which include *LEAFY* (*LFY*), *APETALA 1* (*AP1*), and *FRUITFUL* (*FUL*) (Zhou and Wang, 2013). *LFY* is also directly up-regulated by the activity of the GA-dependent flowering pathway. The expression of the floral repressors *FLOWERING LOCUS C* (*FLC*), *SHORT VEGETATIVE PHASE* (*SVP*), and *MADS AFFECTING FLOWERING* (*MAF*), which suppress these regulatory hubs, is down-regulated by the autonomous and vernalization pathways (Helliwell *et al.*, 2006; Lee *et al.*, 2007; Terzi and Simpson, 2008; Zhou and Wang, 2013). Once the expression of the floral meristem identity genes reaches a threshold level, the floral organ genes are expressed and flower production is initiated (Huijser and Schmid, 2011). Within this complex gene network, various miRNA families play a number of key regulatory roles.

The *miR156* and *miR172* miRNA families have the greatest influence on flowering time. These are major constituents of the ageing pathway and act sequentially to regulate the onset of reproductive competency (Wang *et al.*, 2009; Wu *et al.*, 2009; Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). Whilst *miR156* is highly expressed in the embryo and early seedling stage and declines with increasing plant age, *miR172* accumulates in the leaves and floral buds over time (Fahlgren *et al.*, 2006; Wu *et al.*, 2009; Nodine and Bartel, 2010; Zhu and Helliwell, 2010). These temporally opposite expression patterns form the basis for the control of both the juvenile to adult vegetative phase change and the subsequent reproductive phase transition (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). The activity of the ageing pathway ultimately results in the expression of the floral pathway integrators *FT*, *SOC1*, and *AP1*, as well as the direct activation of several floral meristem identity genes (Yamaguchi and Abe, 2012). Leaf morphology (juvenile versus adult) and trichome distribution (adaxial/abaxial) are also affected by the activity of these miRNAs, generating the distinct morphological traits of the juvenile and adult vegetative phases (reviewed in Huijser and Schmid, 2011).

The role of the miR156 *family in flowering time regulation*

In *Arabidopsis*, the *miR156* family is encoded by the loci *MIR156a–j* (Yamaguchi and Abe, 2012). It targets 11 of the 17 SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors, down-regulating their expression by transcript cleavage (Park *et al.*, 2005; Franco-Zorrilla *et al.*, 2007; Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). The age-dependent decrease in *miR156* levels is therefore accompanied by a concomitant increase in *SPL* expression (Wu and Poethig, 2006; Yamaguchi and Abe, 2012). The

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functions of the various components of the *miR156–SPL* module were elucidated by a series of loss- and gain-of-function studies. The role of *miR156* in the control of flowering time, for instance, was first identified by studying a transgenic line engineered to overexpress *miR156* constitutively from the *Cauliflower moisiac virus* (CaMV) *35S* promoter (*35S::miR156*). These plants exhibited a delayed-flowering phenotype and a prolonged juvenile phase, as was evidenced by the increased production of juvenile leaves and lack of abaxial trichomes (an adult trait) (Wu and Poethig, 2006; Huijser and Schmid, 2011). As in *Arabidopsis*, overexpression of *miR156* causes delayed flowering in rice, tomato, and maize, suggesting an evolutionarily conserved role for *miR156* in flowering (Xie *et al.*, 2006; Chuck *et al.*, 2007; Zhang *et al.*, 2011). Conversely, it was shown that the down-regulation of *miR156* activity by use of a *miR156* target mimic (*MIM156*), which sequesters the available *miR156*, produced an early-flowering mutant with adult features (Franco-Zorrilla *et al.*, 2007). High *miR156* levels early in plant development therefore suppress flowering and are necessary and sufficient for the expression of the juvenile phase (Huijser and Schmid, 2011).

The loss of a single SPL protein often had no effect on plant phenotype, indicating a high level of functional redundancy amongst the SPL proteins (Yamaguchi and Abe, 2012). The SPL targets of *miR156* are therefore grouped into four separate clades according to phylogeny and paralogous relationships, two of which greatly influence the transition to flowering (Guo *et al.*, 2008; Wu *et al.*, 2009). One of these (clade VI) consists of the small *SPL3*, *SPL4*, and *SPL5* genes (Huijser and Schmid, 2011). The expression of *miR156*-resistant *SPL3* (*rSPL3*) (or *rSPL4*/*rSPL5*), which lacks the 3′-untranslated region (UTR) miRNA recognition element to which *miR156* binds, resulted in an early-flowering phenotype (Guo *et al.*, 2008; Wu *et al.*, 2009; Huijser and Schmid, 2011). This was a consequence of SPL3-dependent induction of *LFY*, *FUL*, and *AP1* expression, a result of its direct interaction with the promoter elements of these floral meristem identity genes. Furthermore, the *miR156–SPL3* node was shown to modulate ambient temperature-responsive flowering and induce the expression of *FT* (Yamaguchi *et al.*, 2009; Kim *et al.*, 2012).

SPL9 and *SPL15* comprise another clade of *SPL* genes (clade VIII) that are involved in the control of flowering. These act redundantly, and double loss-of-function mutants showed a distinct phenotype similar to that of mutants overexpressing *miR156* (Guo *et al.*, 2008; Schwarz *et al.*, 2008). Conversely, transgenic lines expressing *rSPL9* or *rSPL15* flowered extremely early and produced adult leaves (Wu and Poethig, 2006; Wu *et al.*, 2009; Huijser and Schmid, 2011). It was revealed that this phenotype resulted from the induction of *miR172* expression by SPL9 (Wu *et al.*, 2009; Zhu and Helliwell, 2010). There is redundancy in *miR172* regulation as its expression is induced by SPL10 and SPL11 (Zhu and Helliwell, 2010). In addition to *miR172*, SPL9 can also directly induce the expression of *FUL*, *AP1*, *SOC1*, and *AGL24* by binding their respective promoters (Wang *et al.*, 2009; Huijser and Schmid, 2011) (Fig. 2).

Until recently, the upstream effectors mediating the age-dependent decline in *miR156* levels were largely unknown. Loss- and gain-of-function studies of genes that are involved in the vernalization-, photoperiod-, and GA-dependent flowering pathways revealed little to no effect on *miR156* levels. Ambient temperature was shown to affect *miR156* expression mildly, with higher levels of this miRNA being detected at lower ambient temperatures (16 °C versus 23 °C) (Lee *et al.*, 2010; Zhu and Helliwell, 2010). While *miR156* levels are largely unaffected by the activity of these pathways, studies revealed that the GA and photoperiod pathways regulate SPL levels in a *miR156*-independent manner; for instance, the photoperiod pathway components PENNYWISE (PNY) and POUND-FOOLISH (PNF) up-regulate *SPL3*, *SPL4*, and *SPL5* levels (Huijser and Schmid, 2011; Zhou and Wang, 2013). In recent studies, however, a correlation between plant nutritional status and *miR156* levels was identified where increasing nutrient abundance acts as a proxy signal for plant age (Wahl *et al.*, 2013; Yang *et al.*, 2013; Yu *et al.*, 2013).

Two independent research groups determined that the accumulation of metabolically active sugars, such as sucrose and glucose, selectively regulates the expression of the *MIR156A* and *MIR156C* genes, which play a dominant role in the vegetative phase transition (Yang *et al.*, 2013; Yu *et al.*, 2013). These studies were based on previous findings, which had revealed the importance of a leaf-derived signal in mediating the age-dependent decline of *miR156* levels (Yang *et al.*, 2011). Sugar may act as a proxy signal for plant age, accumulating as the plant increases its photosynthetic capacity throughout the juvenile and adult vegetative phases. While sugar accumulation was shown to reduce *miR156* expression, sugar deprivation resulted in an increase in *miR156* expression and a consequent decrease in SPL levels. One study revealed that the effects of both sugar deprivation and sugar accumulation were in part mediated by the glucose-sensing enzyme and signalling protein hexokinase 1 (HXK1) (Yang *et al.*, 2013).

HXK1 participates in a nuclear complex that positively regulates the expression of *MIR156A* and *MIR156C* under low sugar conditions, possibly by recruiting DNA-binding transcription factors (Yang *et al.*, 2013). In this model, increasing glucose levels bind to and inhibit HXK1 activity, thereby reducing *miR156* expression. Sugar may also act post-transcriptionally, either by activating sugar-specific *cis*-acting regulatory elements or by directly destabilizing the pri-miRNAs (Yang *et al.*, 2013). Regardless of the role of HXK1, it is not the only regulator of *miR156* expression, as an age-dependent decrease in *miR156* levels was still observed in *gin2-1* (HXK1-null) mutants. Photosynthesis-defective mutants with a defective chlorophyll *a* oxygenase (*ch1-4*) also showed an age-dependent decrease in *miR156* levels, albeit at a much slower rate, indicating that sugar alone does not regulate *MIR156* expression (Yang *et al.*, 2013; Yu *et al.*, 2013).

While the focus of these studies was the juvenile to adult vegetative phase transition, this mechanism of *miR156* regulation is directly relevant to the reproductive phase transition. Whether or not sugar accumulates in the SAM during the vegetative phase change remains to be determined. Further

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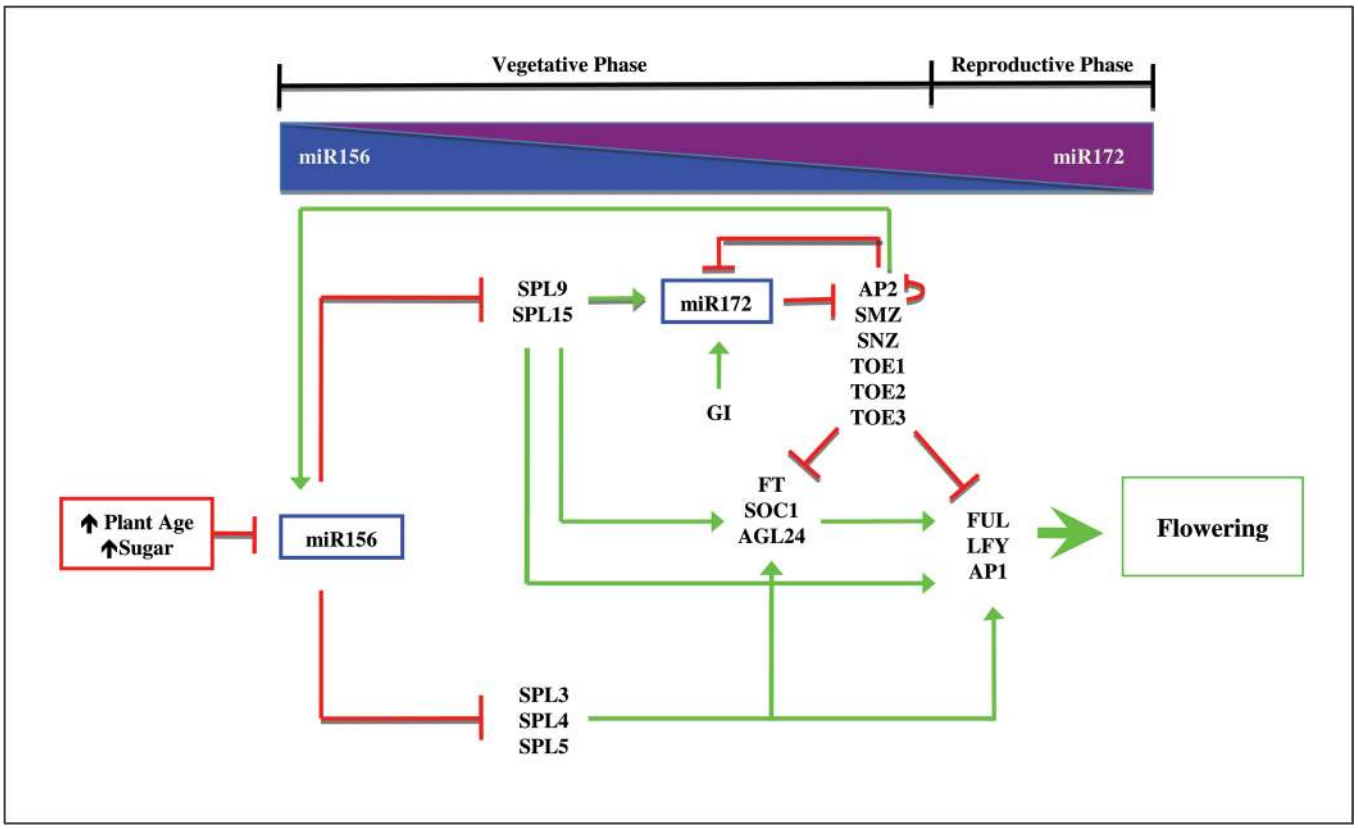

Fig. 2. The *miR156–SPL* and *miR172–AP2* modules are the main components of the ageing pathway. As the plant ages, *miR156* levels decline, resulting in a concomitant increase in *SPL* and therefore *miR172* expression. In addition, GI mediates a *miR156*-independent increase in *miR172* levels by promoting *miR172* transcript processing. *MiR172* in turn down-regulates the *AP2* floral repressors, which inhibit the floral pathway integrators (*FT*, *SOC1*, and *AGL24*) and floral meristem identity genes (*FUL*, *LFY*, and *AP1*) necessary for flower induction. Abbreviations: AGL24, AGAMOUS-LIKE 24; AP1/2, APETALA1/2; FT, FLOWERING LOCUS T; FUL, FRUTIFUL; GI, GIGANTEA; LFY, LEAFY; SOC1, SUPPRESSOR OF CONSTANS1; SPL, SQUAMOSA PROMOTER BINDING-LIKE protein; SMZ, SCHLAFMÜTZE; SNZ, SCHNARCHZAPFEN; TOE1-3, TARGET OF EAT1-3 (Vaucheret, 2006; Jung *et al.*, 2007; Zhu and Helliwell, 2010; Yaish *et al.*, 2011).

studies examining loss- and gain-of-function mutations in genes involved in sugar transport from the leaves to the SAM are required to determine the role of sugar in the age pathway (Yang *et al.*, 2013; Yu *et al.*, 2013).

Another recent study established a link between plant carbohydrate status and *miR156* expression. The enzyme trehalose-6-phosphate synthase 1 (TPS1) produces trehalose-6-phosphate (T6P) which serves as a signal for carbohydrate availability in the plant (Wahl *et al.*, 2013). TPS1 regulates the expression of *SPL* genes in the SAM via the T6P pathway and by a *miR156*-dependent mechanism; this function is distinct from its role in the photoperiodic pathway in the leaves (Wahl *et al.*, 2013). Microarray analysis was used to compare gene expression between 21-day-old wild-type plants and *tps1-2*, *GVG:TPS1* plants, in which *TPS1* expression could be induced by dexamethasone application. The results revealed that *SPL3*, *SPL4*, and *SPL5* levels were significantly reduced in transgenic plants. Subsequently it was shown that mature *miR156* levels were significantly elevated in *tps1-2*, *GVG:TPS1* plants 10 d post-germination when compared with wild-type plants, with an associated decrease in expression of target *SPL* genes (Wahl *et al.*, 2013). However, *miR156* decline is still partially independent of the T6P pathway, as *miR156* levels still declined with age in the *tps1-2* mutant (Wahl *et al.*, 2013). Therefore, while these nutrient-dependent signals have shed light on the mechanism of *miR156* regulation, the age-dependent decline in the levels of this miRNA is still not fully understood.

The role of the miR172 *family in flowering time regulation*

The *miR172* family encoded by the *MIR172a–e* loci acts downstream of *miR156* and has the opposite effect on plant flowering time (Wu *et al.*, 2009; Zhu and Helliwell, 2010). As previously mentioned, the *miR156* targets SPL9 and SPL10 are direct transcriptional activators of *miR172b* expression. This was revealed by chromatin immunoprecipitation and the use of transgenic lines in which overexpression of *SPL9* resulted in elevated *miR172* levels. The temporally opposite expression pattern of *miR172* and *miR156* is therefore a direct consequence of *miR156* decline (Wu *et al.*, 2009). The targets of *miR172* in *Arabidopsis* are the six *APETALA-2* (*AP2*) type genes: *AP2*, *TARGET OF EAT 1* (*TOE1*), *TOE2*, *TOE3*,

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SCHLAFMÜTZE (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) (Aukerman and Sakai, 2003; Chen, 2004; Yamaguchi and Abe, 2012). These act as floral repressors and are silenced by *miR172* primarily by translational inhibition, although transcript cleavage has also been observed (Aukerman and Sakai, 2003; Schwab *et al.*, 2006; Fang and Spector, 2007). AP2-type protein levels are high in the early seedling and decline as *miR172* levels rise with increasing plant age, thus relieving the repression of flowering as the plant matures (Jung *et al.*, 2007; Zhu and Helliwell, 2010).

MiR172 (*35S::miR172b*) overexpression in the *Arabidopsis* activation-tagged line *early activation tagged*, *dominant* (*eat-D*) resulted in an extremely early-flowering phenotype in both inductive LD and non-inductive short-day (SD) conditions, the first indication of the role of *miR172* in the control of plant flowering (Aukerman and Sakai, 2003; Zhu and Helliwell, 2010). *MiR172* overexpression resulted in the up-regulation of *FT* and the floral meristem identity genes *LFY* and *AP1* (Zhu and Helliwell, 2010). Whilst AP2-type protein levels were down-regulated by *miR172* overexpression, the gene transcript levels for *TOE1*, *TOE2*, and *AP2* were not reduced in these mutants, indicating that gene silencing is a result of translational inhibition (Aukerman and Sakai, 2003). Only hextuple mutants for all *AP2* genes flowered as early as the *eat-D* mutants, highlighting the extensive functional redundancy of these *miR172* targets (Wu *et al.*, 2009; Yant *et al.*, 2010; Yamaguchi and Abe, 2012). Conversely, overexpression of the *AP2*-type genes such as *SMZ* and *SNZ* results in a late-flowering phenotype (Schmid *et al.*, 2003; Mathieu *et al.*, 2009; Yamaguchi and Abe, 2012). A direct interaction of AP2 with sites upstream of the transcription initiation site for the *AP1*, *FUL*, and *SOC1* floral meristem identity genes was demonstrated. In addition, *SMZ* and *TOE1* were shown to regulate *FT* expression negatively (Mathieu *et al.*, 2009; Yant *et al.*, 2010; Zhu and Helliwell, 2010). Interestingly, *miR156* and *miR172* are up-regulated and down-regulated, respectively, by AP2 activity in a feedback loop that helps to fine-tune the flowering response (Yant *et al.*, 2010; Huijser and Schmid, 2011). Further complexity arises from AP2-type proteins binding to and regulating the expression of other *AP2*-type genes, thereby generating a complex negative feedback loop to fine-tune the transition to flowering (Zhu and Helliwell, 2010; Zhou and Wang, 2013) (Fig. 2).

MiR172 is not only regulated by the age-dependent increase in *SPL* gene expression, but also by the photoperiod and ambient temperature flowering pathways (Jung *et al.*, 2007; Lee *et al.*, 2010; Yamaguchi and Abe, 2012). It thus represents a hub for the integration of these flowering pathways. The photoperiod/circadian clock component GI mediates a CO-independent increase in *miR172* expression (Yamaguchi and Abe, 2012; Zhou and Wang, 2013). In the *gi* mutant, *miR172* levels are reduced; however, levels of the primary *MIR172* transcript (*pri-MIR172*) are in fact increased, indicating that GI affects processing of *miR172* rather than its transcription (Jung *et al.*, 2007). SVP, a floral repressor, and the RNA-binding protein FCA of the ambient temperature pathway inhibit *miR172* expression (Zhu and Helliwell, 2010; Kim *et al.*, 2012). The up-regulation of these components under low ambient temperature therefore results in decreased *miR172* expression, with the temperature-dependent increase in *miR156* expression contributing to this phenomenon. Loss of *SVP* consequently resulted in ambient temperature-insensitive flowering (Kim *et al.*, 2012; Yamaguchi and Abe, 2012).

Flowering in perennial plants: the role of the age and vernalization pathways

Recent studies in the perennial plants *Cardamine flexuosa* and *Arabis alpina* showed that the activities of the age and vernalization pathways are coordinated in these species. This ensures that competence to flower occurs at an age when these plants have sufficient resources to sustain repeated annual cycles of flowering (Bergonzi *et al.*, 2013; Zhou *et al.*, 2013).

In *C. flexuosa*, flowering can only occur following the down-regulation of the floral repressors *CfFLC* and *CfTOE1*, homologues of the *Arabidopsis FLC* (*AtFLC*) and *TOE1* (*AtTOE1*) floral repressors, respectively. Both repressors modulate the activity of the floral integrator *CfSOC1*, the expression of which promotes flowering in *C. flexuosa*. Whilst *CfFLC* expression is down-regulated upon exposure to cold, the plant does not become competent to flower until *CfTOE1* levels decline as well. Just as in *A. thaliana*, *CfTOE1* expression levels decline with increasing plant age as a consequence of declining *miR156* levels and a concomitant increase in *SPL9* and *miR172* expression. Only then does *CfSOC1* expression rise, resulting in flower induction (Zhou *et al.*, 2013).

A similar process takes place in *A. alpina*, a perennial relative of *Arabidopsis*. However, a key distinction between these two species is that in *A. alpina* the decline in *miR156* levels is not coupled to an increase in *miR172* expression. The *miR156–SPL* module determines the age at which a plant becomes competent to flower in response to vernalization. *MiR156* levels are highest in *A. alpina* seedlings and reach a trough at ~5 weeks of age, with a concomitant increase in the expression of *A.alpina SPL* homologues (*AaSPL*). These AaSPL transcription factors are essential for the induction of flowering following vernalization. Bergonzi *et al.* (2013) established that exposure of seedlings to prolonged periods of cold increased the age at which they could respond to vernalization, and that this phenomenon was a result of a delay in the decline of *miR156* levels, and determined that the transcription of *MIR156* genes is regulated by cold exposure.

The *miR172–AP2* module in turn confers the vernalization requirement in *A. alpina*. Bergonzi and colleagues identified PEP2 as an orthologue of the AP2 transcription factor of *Arabidopsis*, and which is also regulated by *miR172*. PEP2 was shown to be an upstream positive regulator of the *A. alpina* floral repressor *PEP1*, an orthologue of *A.thaliana FLC*. PEP1 acts by down-regulating the *A. alpina SOC1* orthologue (*AaSOC1*), a promoter of flowering. During the vegetative phase, or in young plants that are <5 weeks of age and exposed to winter cold, *miR172* levels remain constant. Therefore, *PEP2* and *PEP1* expression remains high and inhibits flowering even with rising SPL levels. However, once older plants are exposed to winter cold, *miR172* levels increase and flowering occurs as a result of declining *PEP1*

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levels and the age-dependent increase in *SPL* expression (Bergonzi *et al.*, 2013; Zhou *et al.*, 2013).

The role of the miR159/miR319 *superfamily in flowering time regulation*

The closely related *miR159* and *miR319* target the MYB and TCP transcription factors, respectively. These two miRNA–target nodes display a degree of functional redundancy, as both regulate the *miR167–ARF6/ARF8* node (Fig. 3) (Rubio-Somoza and Weigel, 2013). This, and the direct interaction of the MYB and TCP transcription factors may explain their overlapping roles in flowering onset and floral development (Jones-Rhoades *et al.*, 2006; Rubio-Somoza and Weigel, 2013). Despite extensive sequence similarity, these miRNAs do not cross-regulate *TCP* and *MYB* transcripts. Whilst *miR319* is capable of binding *MYB* transcripts, it exhibits a limited spatial and temporal expression pattern in comparison with the abundant *miR159*. *MiR159*, on the other hand, cannot bind *TCP* transcripts. For these reasons, *miR159* and *miR319* can also play distinct regulatory roles in plant development (Jones-Rhoades *et al.*, 2006; Palatnik *et al.*, 2007).

The miR159–MYB *module*

The role of the *miR159* family in flowering time control is not as clear-cut as that of *miR156* and *miR172* due to conflicting evidence (Achard *et al.*, 2004; Amasino, 2010). In *Arabidopsis*, *miR159* is encoded by three loci (*MIR159a–c*), and regulates the expression of the transcription factors MYB33, MYB65, and MYB101, homologues of the GAMYB transcription factors found in rice and barley (Rhoades *et al.*, 2002; Achard *et al.*, 2004; Allen *et al.*, 2007). The *miR159–MYB* node has been implicated in playing a role in the GA pathway, which promotes flowering under non-inductive SD conditions in *Arabidopsis* (Terzi and Simpson, 2008; Yamaguchi and Abe, 2012).

GA induces flowering by binding and activating the three GIBBERELLIC INSENSITIVE DWARF (GID1–GID3) receptors. These mediate the 26S proteasome-dependent degradation of the DELLA proteins, the negative regulators of the GA response (Achard *et al.*, 2004; Griffiths *et al.*, 2006; Hartweck, 2008; Yamaguchi and Abe, 2012). GA treatment and DELLA degradation result in an increase in *miR159* levels, as well as of its targets the GAMYB transcription factors which bind to GA-response elements (GAREs) located in the *LFY* promoter to induce its transcription (Achard *et al.*, 2004; Jin *et al.*, 2013). Paradoxically, MYB33 may feed-back to regulate *miR159* expression positively (Fig. 3), as putative GARE-like sites have been identified in the *miR159* promoter (Achard *et al.*, 2004). Therefore, *miR159* may act as a putative homeostatic regulator of GA-induced *MYB33*, *MYB65*, and *MYB101* expression (Achard *et al.*, 2004; Jin *et al.*, 2013).

Achard and colleagues demonstrated that the overexpression of *miR159a* delayed the onset of flowering in SD conditions and was accompanied by a decrease in *MYB33* and *LFY* transcript levels. Overexpression of a *miR159*-resistant *MYB33* (*mMYB33*) did not, however, have a significant impact on flowering time, possibly due to the redundant action of the *miR319–TCP* node (Achard *et al.*, 2004). These mutants produced curled leaves with shortened petioles and were short in stature (Achard *et al.*, 2004; Jones-Rhoades *et al.*, 2006).

A study by Alonso-Peral and colleagues (2010), however, found that GA did not alleviate *miR159*-dependent repression of *MYB33* and *MYB65* in *Arabidopsis*. It was concluded that these two GAMYB-type transcription factors play no role in the onset of flowering, as *miR159* is constitutively expressed in vegetative tissues and therefore continually represses these transcription factors (Alonso-Peral *et al.*, 2010). The study proposed that the principal role of the *miR159-MYB* nodes is the regulation of seed development and flower maturation, primarily through the regulation of the *miR167–ARF6/8* node (Alonso-Peral *et al.*, 2010; Rubio-Somoza and Weigel, 2013). Thus, whilst the *miR159-MYB* node has been shown to play a clear role in *Sinningia speciosa* flowering time control, further studies are required to clarify its role in *Arabidopsis* (Li *et al.*, 2013).

The miR319–TCP *module*

The targets of the *miR319* family, which is encoded by the *MIR319a–c* loci, are five *TCP* mRNAs (*TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*) of the TCP subclass II (Schommer *et al.*, 2012; Rubio-Somoza and Weigel, 2013). The *miR319–TCP* transcript interaction is unusual in that as many as six base mismatches can occur within this duplex (Palatnik *et al.*, 2003; Schommer *et al.*, 2012). Most plant miRNA–mRNA interactions display no more than three mismatches, given that perfect sequence complementarity is required for miRNA function (Schommer *et al.*, 2012). *TCP* regulation by *miR319* is nonetheless considered to be feasible, as the Gibbs free energy value of the interaction is negative (Schommer *et al.*, 2012).

The TCP transcription factors are involved in multiple aspects of plant growth, including flower production, and leaf and gametophyte development (Schommer *et al.*, 2012). Furthermore, these transcription factors act as the central regulators of the circadian clock by activating and interacting with its core components (Schommer *et al.*, 2012). The role of these TCPs and their regulation by *miR319* was first identified from microarray experiments in *jaw-D* mutants (Jones-Rhoades *et al.*, 2006; Schommer *et al.*, 2012). These *Arabidopsis* mutants overexpressed *miR319* and displayed a late-flowering phenotype in LD conditions (Palatnik *et al.*, 2003; Jones-Rhoades *et al.*, 2006; Terzi and Simpson, 2008). Loss of function of the *miR319* target *TCP4* also generated a late-flowering phenotype (Sarvepalli and Nath, 2011; Schommer *et al.*, 2012). As the factors regulating *miR319* expression are yet to be identified, further research is required to clarify the role of this miRNA in the regulation of flowering time (Schommer *et al.*, 2012).

The role of the miR390 *family in flowering time regulation*

The *miR390* family plays a role in multiple developmental processes, including leaf morphogenesis, lateral root

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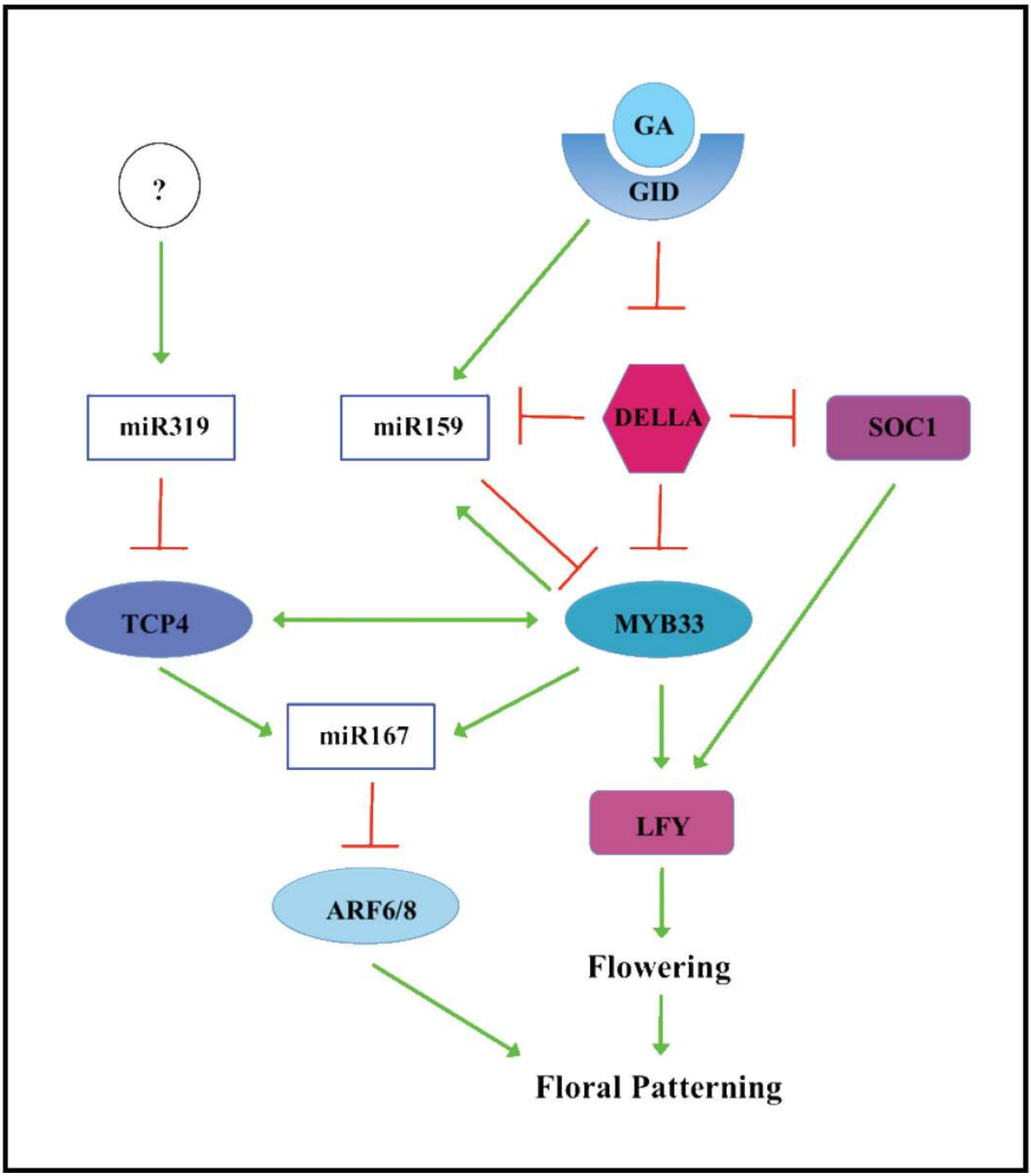

Fig. 3. The closely related *miR159* and *miR319* families target the MYB and TCP transcription factors, respectively. Evidence indicates that the *miR159–MYB* node plays a role in the GA flowering pathway; increased GA levels relieve the inhibition of *miR159* expression by DELLA proteins, *miR159* then inhibits MYB activity, which has increased due to the lack of repression by the DELLA proteins, forming a homeostatic regulatory loop. The *miR159–MYB* node, as well as the *miR319–TCP* node, positively regulates *miR167* expression. This results in the expression of genes required for floral patterning. (Achard *et al.*, 2004; Rubio-Somoza and Weigel, 2013). Abbreviations: ARF6/8, AUXIN RESPONSE FACTOR 6/8; GA, gibberellic acid; GID, GIBBERELLIC ACID INSENSITIVE DWARF; LFY, LEAFY; SOC1, SUPPRESSOR OF CONSTANS 1.

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development, and, indirectly, flowering time control (Rubio-Somoza and Weigel, 2011). *MiR390*, which is unique in that it associates with AGO7 during RISC assembly, indirectly represses the ARF3 and ARF4 transcription factors by promoting the production of another type of small RNA involved in PTGS, the tasiRNAs, from the *TAS3* locus (Garcia, 2008; Montgomery *et al.*, 2008; Rubio-Somoza and Weigel, 2011; Endo *et al.*, 2013). As ARF3/4 activity promotes the juvenile to adult vegetative phase transition, *miR390* activity delays flowering onset by prolonging the juvenile phase (Fahlgren *et al.*, 2006; Rubio-Somoza and Weigel, 2011). In *Arabidopsis*, loss-of-function mutants of RNA-dependent RNA polymerase 6 (*rdr6*), DCL4 (*dcl4*), or AGO7 (*ago7*), key components of the tasiRNA biogenesis machinery, result in an accelerated juvenile to adult vegetative phase transition as evidenced by premature production of adult leaves and abaxial trichomes (Fahlgren *et al.*, 2006; Garcia, 2008; Rubio-Somoza and Weigel, 2011). Plants expressing tasiRNA-insensitive *ARF3* (*ARF3:ARF3mut*) displayed the same phenotype (Fahlgren *et al.*, 2006; Garcia, 2008). Increased ARF3 (and ARF4) activity may produce this phenotype by inducing *SPL3* and/or *SPL4* expression in a *miR156*-independent manner (Rubio-Somoza and Weigel, 2011). Furthermore, the changes in leaf patterning in these mutants may be a consequence of increased *SPL9* and *SPL15* expression due to heightened ARF3 signalling. This interaction with the *miR156–SPL* node, as well as AP2-mediated silencing of *ARF3*, is evidence for the involvement of *mi390–TAS3–ARF3/4* in certain aspects of the ageing pathway (Rubio-Somoza and Weigel, 2011).

The role of the miR399 *family in flowering time regulation*

The *miR399–PHO2–IPS1* module is an example of miRNA involvement in both abiotic stress responses and flowering time control. Phosphorus, primarily in the form of phosphate, is essential for maintaining multiple cellular processes such as kinase cascades. Its depletion from the environment can therefore have serious deleterious effects on plant growth (Kruszka *et al.*, 2012). *MiR399* was first identified as a key player in phosphate homeostasis due to its regulation of transcript levels of the E2 ubiquitin-conjugating enzyme PHOSPHATE 2 (PHO2) (Fuji *et al.*, 2005; Bari *et al.*, 2006; Kim *et al.*, 2011; Kruszka *et al.*, 2012). This phloem-mobile miRNA acts in a complex regulatory network with sucrose to generate a systemic signal for phosphate deficiency, culminating in the induction of various phosphate-scavenging mechanisms (reviewed by Liu and Vance, 2010). In *Arabidopsis*, *miR399* is encoded by the *MIR399a–f* loci and is expressed primarily in the shoot from where it is transported to the roots in order to reduce *PHO2* expression by transcript cleavage (Liu and Vance, 2010; Kim *et al.*, 2011; Kruszka *et al.*, 2012). PHO2 is part of a complex that mediates protein turnover via the ubiquitin–proteasome pathway in the roots. Its targets include key proteins involved in phosphate uptake in the roots (Liu and Vance, 2010; Kim *et al.*, 2011).

MiR399 activity is therefore up-regulated under conditions of phosphate starvation to increase phosphate availability, and down-regulated under high phosphate conditions to avoid phosphate toxicity (Chiou *et al.*, 2006; Liu and Vance, 2010; Matsoukas *et al.*, 2012). Interestingly, *miR399*-dependent regulation of *PHO2* is attenuated by the phosphate starvation-induced expression of the short non-coding RNA molecule *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*) (Chiou *et al.*, 2006). *IPS1* is a target mimic for *miR399* that serves to sequester its activity, as there is a mismatch in the cleavage region that prevents AGO1-mediated slicing (Franco-Zorrilla *et al.*, 2007; Khraiwesh *et al.*, 2012; Kruszka *et al.*, 2012). The activity of *miR399* is therefore tightly controlled to prevent excessive phosphate accumulation and tissue necrosis (Kruszka *et al.*, 2012).

More recently, a potential role for *miR399* as an ambient temperature-responsive flowering time regulator was identified. Ambient temperature had previously been shown to regulate *miR399* accumulation, as it was found to be more abundant in plants grown at 23 °C than in those grown at 16 °C (Lee *et al.*, 2010; Kim *et al.*, 2011). Kim and colleagues (2011) determined that *miR399* overexpressors (*p35S::miR399b*) or *PHO2* loss-of-function mutants grown at a normal ambient temperature (23 °C) under LD conditions flowered early. No change in flowering time was seen in these mutants when grown at a low ambient temperature (16 °C), indicating that the *miR399–PHO2* module is involved in the ambient temperature-dependent flowering pathway (Kim *et al.*, 2011). Increased expression of the floral pathway integrator *TSF* at 23 °C may account for the early flowering phenotype of these *Arabidopsis* mutants. However, the authors highlighted that this early flowering phenotype could also be an indirect consequence of phosphate toxicity given that high levels of phosphate accumulated in these *miR399*-overexpressing and *PHO2*-deficient shoots (Kim *et al.*, 2011).

Optimizing flowering time: applications of miRNA technology

The manipulation of plant miRNAs has numerous potential applications to improve agricultural and horticultural output. By altering the levels of endogenous miRNAs, or by using target-specific artificial miRNAs, almost every aspect of plant development may be manipulated and therefore optimized. As previously mentioned, Franco-Zorrilla and colleagues revealed that the activity of specific miRNAs may be sequestered by the expression of target mimics (Franco-Zorrilla *et al.*, 2007). Conversely, miRNA activity may be enhanced by overexpressing the *MIR* genes using the CaMV *35S* promoter (Li *et al.*, 2013). Both of these methods were employed in a recent study by Li and colleagues to control the onset of flower production in gloxinia (*Sinningia speciosa*) plants (Li *et al.*, 2013). Transgenic lines were generated which either over- or underexpressed *miR159*, a negative regulator of flowering onset in gloxinia. Transgenic lines overexpressing this regulator exhibited a delay in the onset of

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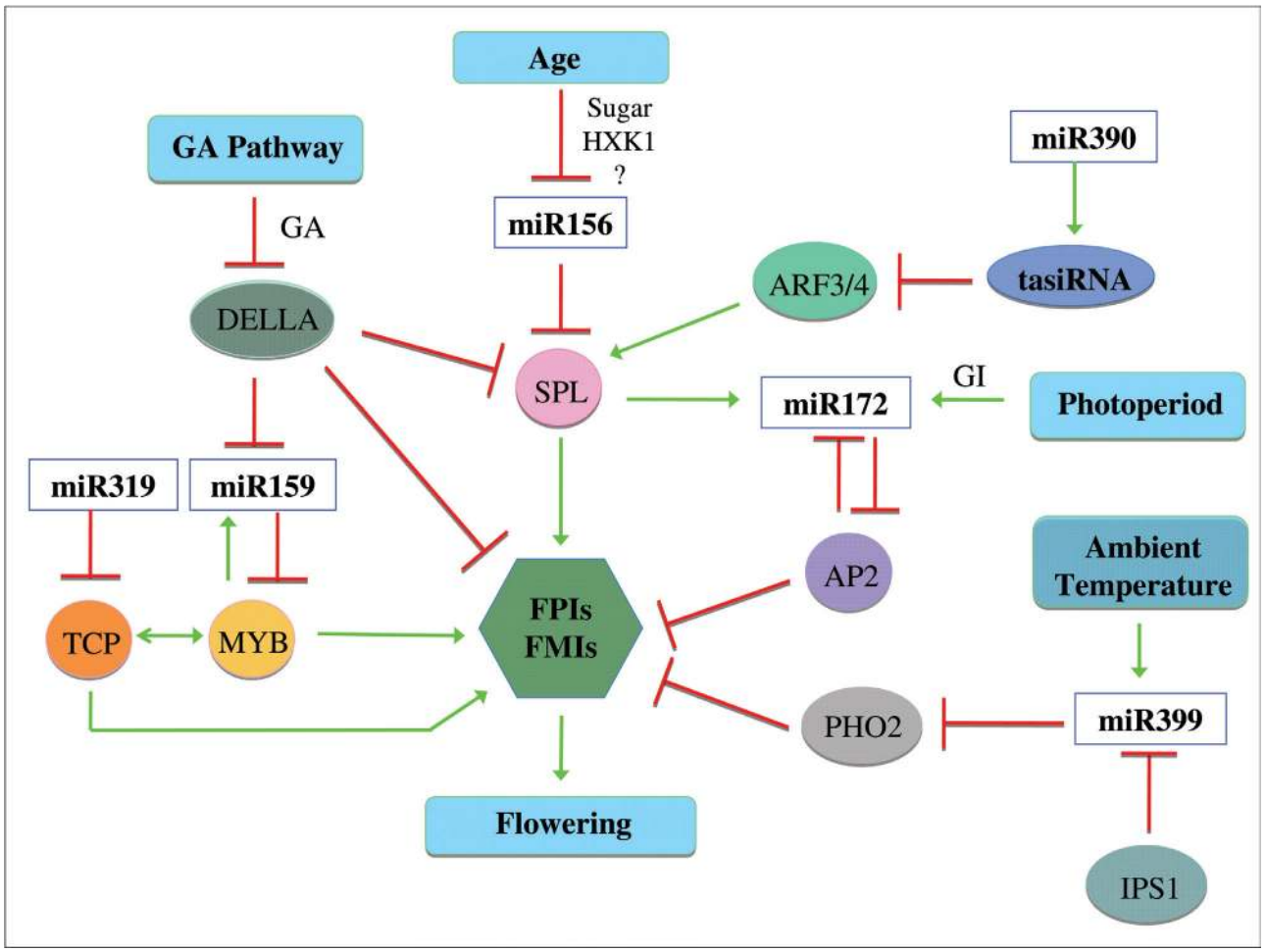

Fig. 4. Summary of the miRNA families involved in the regulation of flowering. The *miR156* family, whose levels decline with increasing plant age due in part to sugar accumulation, is the main regulator of 11 of the 17 *SPL* transcription factors. Rising SPL levels and GI positively regulate *miR172* expression, resulting in the suppression of the AP2-type floral repressors. The closely related *miR319* and *miR159* families in turn regulate the TCP and MYB transcription factors, respectively, which have overlapping functions. *MiR159* levels rise in response to increased GA signalling due to the concomitant decrease in DELLA protein expression. The *miR390* family regulates flowering indirectly by promoting the production of tasiRNAs from the *TAS3* locus, which negatively regulate the ARF3/4 transcription factors. These serve multiple functions, including the induction of the SPL proteins. Finally, the *miR399* family is expressed under both conditions of phosphate starvation and normal ambient temperatures, and regulates the E2 ubiquitin-conjugating enzyme *PHO2*. Overall, miRNA activity serves to either inhibit or promote the expression of the floral pathway integrator (FPI) and/or floral meristem identity (FMI) genes, thereby delaying or promoting the onset of flowering, respectively. (Achard *et al.*, 2004; Alonso-Peral *et al.*, 2010; Huijser and Schmid, 2011; Kim *et al.*, 2011; Rubio-Somoza and Weigel, 2011; Schommer *et al.*, 2012; Yamaguchi and Abe, 2012; Wahl *et al.*, 2013; Yang *et al.*, 2013; Zhou and Wang, 2013). Abbreviations: AP2, APETALA 2-TYPE PROTEIN; ARF3/4, AUXIN RESPONSE FACTOR 3/4; GA, gibberellic acid; GI, GIGANTEA; HXK1, HEXOKINASE 1; IPS1, INDUCED BY PHOSPHATE STARVATION 1; tasiRNA, *trans*-acting small interfering RNA.

flowering, whilst those in which *miR159* activity was attenuated displayed an early flowering phenotype (Li *et al.*, 2013). Coupling these methods of miRNA manipulation to inducible expression systems would enable precise control of plant flowering time.

Artificial miRNAs can be engineered to regulate the expression of highly specific target genes (Schwab *et al.*, 2006). This approach was employed in a study by Yeoh and colleagues, in which the expression of the *Arabidopsis FT* gene was suppressed by the artificial miRNA *amiR-FT*. The delayed flowering phenotype of these transgenic plants was rescued by expressing the *FTa1* gene from *Medicago truncatula*, an orthologuous gene that has diverged sufficiently from the *Arabidopsis* equivalent to avoid suppression by *amiR-FT*. Precise control over flowering time was achieved using an ethanol-inducible *FTa1* expression system (Yeoh *et al.*, 2011).

Summary

In recent years, plant miRNAs have been shown to play a role in almost every aspect of plant growth, development, and stress adaptation. Their involvement in the regulation of the reproductive phase transition is of particular agricultural and

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economic importance, as the onset of flowering, or its prevention, directly influences plant reproductive capacity and yield. The identification of the *miR156–SPL* and *miR172–AP2* nodes of the ageing pathway, as well as the *miR159–MYB*, *miR319–TCP*, *miR390–TAS3-ARF3/4*, and *miR399–PHO2* nodes has significantly advanced our understanding of the role of miRNAs in the mechanisms underlying flowering time control (Fig. 4).

Much still remains to be discovered. In order to exploit properly the gene silencing activity of, for instance, *miR156* to prolong the juvenile vegetative phase and delay flowering, the signals mediating its age-dependent decline must be understood. In general, the multiplicity and differential expression of these *MIR* loci, the diverse effects of miRNA–target interactions, and the complex interplay of these miRNAs with various flowering pathway components are all areas for further research. The pace at which advancements in sequencing technologies, experimental techniques, and computational capabilities are being made, however, means that progress in this area is likely to be rapid.

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