Floral induction and flower formation—the role and potential applications of miRNAs

Yiguo Hong1 and Stephen Jackson2,*

1Research Centre for Plant RNA Signalling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, China
2School of Life Sciences, Warwick University, Coventry, UK

Summary

The multiple regulatory pathways controlling flowering and flower development are varied and complex, and they require tight control of gene expression and protein levels. MicroRNAs (miRNAs) act at both the transcriptional and post-transcriptional level to regulate key genes involved in flowering-related processes such as the juvenile–adult transition, the induction of floral competence and flower development. Many different miRNA families are involved in these processes and their roles are summarized in this review, along with potential biotechnological applications for miRNAs in controlling processes related to flowering and flower development.

Introduction

There are a number of different developmental phases through which a plant progresses during its life cycle, these are differentiated by distinct morphological traits and/or the development of new organs (Huijser and Schmid, 2011; Jin et al., 2013). Flowering is an essential part of the reproductive process in angiosperms, and the flowering process involves two developmental phase transitions: the first of these is the transition from the immature juvenile vegetative state (where the plant is unable to flower) to the mature adult vegetative state (where the plant is capable of flowering) (Poethig, 2003; Sgamma et al., 2014). The second developmental transition occurs at floral induction when the plant switches from vegetative growth to reproductive growth and starts to produce flowers. The timing of this transition is tightly controlled by a complex gene regulatory network, this is because the time when a plant flowers affects its reproductive success, for example flowering usually occurs at the optimum time for pollination or seed dispersal. For out-crossing species, this is of particular importance as flowering time needs to be coordinated with other plants of the same species in their vicinity, and for insect pollinated plants with the activity of their pollinators (Huijser and Schmid, 2011; Srikanth and Schmid, 2011). To make this possible, the plant detects environmental cues such as light quality and duration, and temperature and integrates this information with that from endogenous physiological processes such as its circadian clock, phytohormone and carbohydrate levels, and vernalization state to regulate its flowering time to the appropriate time of the year, and even time of the day (Amasino, 2010; Fornara et al., 2010; Jackson, 2009; Kim et al., 2012; Matsoukas et al., 2012; Srikanth and Schmid, 2011).

As the timing of flowering and floral organogenesis both significantly affects 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). Many research has therefore been conducted on the molecular mechanisms controlling the flowering process, and numerous genes that contribute to the different environmental and endogenous regulatory pathways have been identified. In Arabidopsis, it is now well known that the main pathways controlling flowering in response to environmental signals are the photoperiod, ambient temperature and vernalization pathways, which respond to day length, surrounding temperature and prolonged cold exposure, respectively (Fornara et al., 2010; Jackson, 2009). Other endogenous factors such as phytohormones and carbohydrate status also regulate flowering through the autonomous, gibberellic acid (GA), nutrient-responsive and ageing pathways (Kim et al., 2012; Matsoukas et al., 2012; Srikanth and Schmid, 2011; Yamaguchi and Abe, 2012). All these different regulatory pathways converge on a set of floral pathway integrator genes, namely FLOWERING LOCUS T (FT) and its parologue TWIN SISTER OF FT (TSF), as well as SUPPRESSOR OF CONSTANS 1 (SOC1) and AGAMOUS-LIKE 24 (AGL24). These act to control the expression of a small set of meristem identity genes at the shoot apical and lateral meristems including LEAFY (LFY), APETALA 1 (API) and FRUITFUL (FUL). Once the expression of these genes reaches a certain level they induce the expression of floral organ identity genes and flowers are produced (Amasino, 2010; Huijser and Schmid, 2011; Posé et al., 2012; Srikanth and Schmid, 2011).

Over the past decade, there have been numerous reports demonstrating that a number of different microRNA (miRNA) families play important roles in the regulation of flowering time and floral development, which they do by targeting and...
down-regulating transcription factors involved in these processes (Chuck et al., 2009; Jones-Rhoades et al., 2006; Luo et al., 2013; Spanudakis and Jackson, 2014). Much of this evidence has been obtained from research on the model plant Arabidopsis, although a high level of conservation of miRNAs and their regulatory pathways/target genes has been demonstrated from results obtained from tomato, potato, maize, rice and other species (Luo et al., 2013; Sunkar and Jagadeeswaran, 2008; Willmann and Poethig, 2007). There are at least eleven different miRNA families that have been shown to affect different stages in the induction of flowering and flower development in plants, and these are miR156, miR159, miR160, miR164, miR166/165, miR167, miR169, miR172, miR319, miR390 and miR399 (see Figure 1).

Regulation of floral induction

As mentioned above, a plant has to go through two developmental phase transitions before it produces flowers. The first is the juvenile–adult phase transition after which the plant is competent to be induced to flower, the second is the vegetative–reproductive phase transition which occurs once a plant has been induced to flower and is followed by the production of floral organs.

The main miRNA families involved in regulating flowering time are the miR156, miR172 and miR390 families which are involved in the juvenile-to-adult vegetative phase transition, and the miR159, miR169 and miR399 families which along with miR172 are involved in the vegetative-to-reproductive phase transition (Huijser and Schmid, 2011; Jin et al., 2013; Jones-Rhoades et al., 2006; Kim et al., 2011; Rubio-Somoza and Weigel, 2011; Wang, 2014; Zhu and Helliwell, 2011).

Interactions between miR156, miR172 and miR390 in the juvenile–adult phase transition

In Arabidopsis, the miR156 family is encoded by the loci MIR156a-j (Yamaguchi and Abe, 2012). The miR156 family acts together with the miR172 family within the ageing pathway to regulate the time when a plant becomes mature and reproduc-

tively competent (Huijser and Schmid, 2011; Wang et al., 2009; Wu et al., 2009; Yamaguchi and Abe, 2012). MiR156 indirectly regulates the levels of miR172 such that they have temporally opposite expression patterns, miR156 is highly expressed in the embryo and early seedling stage and declines with increasing plant age, whereas miR172 accumulates over time in the leaves and floral buds (Fahlgren et al., 2006; Wu et al., 2009; Zhu and Helliwell, 2011). This forms the basis for the regulation of the juvenile-to-adult phase change and, in addition to flowering time, other morphological traits representative of the juvenile and adult vegetative phases such as leaf morphology and trichome distribution are also affected by the activity of these miRNAs (Huijser and Schmid, 2011).

Transgenic plants constitutively overexpressing miR156 exhibited delayed-flowering and a prolonged juvenile phase, as shown by the increased production of juvenile leaves and lack of abaxial trichomes (Huijser and Schmid, 2011; Wu and Poethig, 2006). This effect was more pronounced at 16 °C than at 23 °C leading to the suggestion that ambient temperature may influence the effect of miR156 overexpression (Kim et al., 2012). An evolutionary conserved role of miR156 in the control of flowering is supported by the fact that delayed flowering is also observed in rice, tomato and maize lines overexpressing miR156 (Chuck et al., 2007; Xie et al., 2006; Zhang et al., 2011). On the other hand, expression of a target mimic of miR156 (MIM156), which sequesters the available miR156 and down-regulates miR156 activity, resulted in early flowering after producing very few leaves that all had adult features (Franco-Zorrilla et al., 2007; Todesco et al., 2010). Thus, high levels of miR156 early in plant development suppress flowering and are necessary for the expression of the juvenile phase. (Huijser and Schmid, 2011).

The targets of the miR156 family are the SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors, miR156 down-regulating the expression levels of 11 of the 17 SPL genes in Arabidopsis (Franco-Zorrilla et al., 2007; Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). The decline in miR156 levels over time with increasing age is therefore accompanied by a corresponding increase in the expression level of SPL transcription factors which promote flowering through the induction of FT, LFY...
and MADS-box gene expression (Wu and Poethig, 2006; Yamaguchi and Abe, 2012). There is functional redundancy within the SPL gene family, and the loss of one SPL protein often has no effect (Yamaguchi and Abe, 2012). The SPL genes have therefore been grouped together into four clades, two of which (clade VI and clade VIII) have a significant influence on flowering (Guo et al., 2008; Huijser and Schmid, 2011; Wu et al., 2009). Clade VIII comprises SPL9 and SPL15 which act redundantly, with spl9 spl15 double loss-of-function mutants exhibiting a similar phenotype to plants overexpressing miR156 (Guo et al., 2008; Schwarz et al., 2008). On the other hand, plants expressing miR156-resistant forms of SPL9 or SPL15 (rSPL9 or rSPL15), that are therefore not down-regulated by miR156, flower very early due to the induction of miR172 expression (Wu et al., 2009; Zhu and Helliwell, 2011). SPL9 has been shown to directly bind to, and activate the transcription of, miR172, and it has also been shown to be a direct regulator of SOC1, AGL24, FUL and AP1 (Wang et al., 2009).

Clade VI consists of the SPL3, SPL4 and SPL5 genes (Huijser and Schmid, 2011). The expression of miR156-resistant rSPL3, rSPL4 or rSPL5 genes, which lack the miR156 binding site in the 3' UTR, resulted in increased levels of SPL proteins and premature appearance of adult leaf traits and early flowering (Guo et al., 2008; Huijser and Schmid, 2011; Wu et al., 2009). SPL3 has been shown to bind the promoter and intragenic elements of the floral meristem identity genes LFY, FUL and AP1 and so appears to target the same floral meristem identity genes as SPL9 (Yamaguchi et al., 2009).

The main role of miR156 is thus to prevent flowering until plants have reached a certain developmental stage, that is until they have completed their juvenile phase. This is evidenced by the fact that miR156 has been shown to prevent precocious flowering (Wang et al., 2009). It inhibits flowering by i) repressing the expression of SPL genes which are direct activators of floral integrator and floral meristem identity genes and ii) repressing the induction of miR172 and in doing so maintaining high levels of expression of AP2-like floral repressors which repress flowering (see Figure 2 and below).

In this way, the plant is not ‘competent’ to respond to inductive environmental signals and cannot be induced to flower whilst high levels of miR156 are present (i.e. during the juvenile phase). As the plant grows older and the levels of miR156 decline, then this repression of flowering is gradually reduced and the plant becomes capable of responding to environmentally inductive signals (i.e. the plant has become mature).

What causes the reduction in miR156 levels with age is not well understood. The findings that metabolically active sugars such as glucose and sucrose, or the levels of trehalose-6-phosphate (T6P) which serves as a signal for carbohydrate availability in the plant, selectively regulate the expression of miR156 genes, however, suggests that nutritional status may serve as a signal for the age or developmental stage of the plant (Wahl et al., 2013; Yang et al., 2013; Yu et al., 2013). Sugar accumulation reduces miR156 levels and conversely sugar deprivation increases miR156 levels with a corresponding increase or decrease in SPL levels, respectively (Figure 2). At least some of the effects of sugar levels on miR156 levels are mediated by the glucose-sensing enzyme HEXOKINASE 1 (HXK1) which up-regulates miR156 levels in low sugar conditions (Yang et al., 2013). Another link between carbohydrate levels and miR156 expression has been shown to be mediated by T6P (Wahl et al., 2013). In transgenic lines with reduced levels of T6P, due to knock-down of Trehalose-6-phosphate synthase 1 (TPS1) expression, flowering was delayed, and in younger plants, miR156 levels were higher and SPL3, SPL4 and SPL5 expression were correspondingly reduced. The levels of miR156 still decreased with age in these plants, but as they were initially at a higher level they took longer to decline (Wahl et al., 2013).

MiR156 levels have also been shown to be affected by cold temperatures in the perennial plant Arabis alpina, a relative of Arabidopsis that requires vernalization to be induced to flower. Exposing A. alpina seedlings to prolonged periods of cold delayed the age-dependent decline of miR156 levels thus increasing the age at which the plants could respond to vernalization, the cold temperature was shown to regulate transcription of the MiR156 gene (Bergonzi et al., 2013).

The miR172 family, which is coded by the MiR172a-e loci, has the opposite effect on flowering time to miR156; overexpression of miR172 (35S::miR172b) results in an extremely early flowering phenotype in both inductive long day (LD) and noninductive short day (SD) conditions (Aukerman and Sakai, 2003; Wu et al., 2009; Zhu and Helliwell, 2011). MiR172 acts downstream of miR156 and the SPL9, 10, 11 and 15 genes, the latter acting redundantly to regulate miR172 levels (Wu et al., 2009). There are multiple copies of SPL binding elements in the promoter of the MiR172b gene, and chromatin immunoprecipitation showed that at least one is strongly bound by SPL9, furthermore, plants overexpress-

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**Figure 2.** The changes associated with the transition from juvenility to maturity. MiR156 levels reduce with age influenced by sugar signalling pathways, this results in an increase in the levels of SPL proteins and miR172 which reduces the level of AP2-like floral repressors. The loss of the AP2-like repressors allows flowering to occur in inductive environmental conditions. AP2-like, APETALA 2-like; CO, CONSTANS; FLC, FLOWERING LOCUS C; SPL, SQUAMOSA PROMOTER BINDING-LIKE; T-6-P, TREHALOSE-6-PHOSPHATE.
ing SPL9 were found to have increased levels of miR172. The increase in miR172 levels with age (a temporally opposite expression pattern to miR156) is thus a direct consequence of reducing miR156 levels and increased expression of SPL genes (Wu et al., 2009).

In Arabidopsis, miR172 targets the expression of a family of AP2-like transcription factors including APETELA 2 (AP2), TARGET OF EARLY FLOWERING 1 (TOE1), TOE2, TOE3, SCHLAFMUTZE (SMZ) and SCHNARCHZAPFEN 1 (SNZ) (Aukerman and Sakai, 2003; Chen, 2004; Yamaguchi and Abe, 2012). These AP2-like proteins are floral repressors and delay flowering by inhibiting expression of FT and floral meristem identity genes AP1, LFY, FUL and SOC1 (Mathieu et al., 2009; Yant et al., 2010; Zhu and Hellwell, 2011). Overexpression of AP2-like genes such as SMZ and SNZ results in a late flowering phenotype (Mathieu et al., 2009; Yamaguchi and Abe, 2012). AP2-type protein levels are high in the early seedling and juvenile stage 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 Hellwell, 2011). AP2 acts in a feedback loop which up-regulates miR156 levels and down-regulates miR172 levels and is thought to fine-tune the flowering response (Huijser and Schmid, 2011; Yant et al., 2010). Furthermore, AP2-type proteins regulate the expression of other AP2-type genes which adds another layer of feedback regulation (Zhou and Wang, 2013; Zhu and Hellwell, 2011).

MiR390 is involved in multiple developmental processes including leaf morphogenesis, lateral root development and flowering time control. Its effects on flowering time are because it prolongs the juvenile phase and thus delays the acquisition of the competence to flower (Fahlgren et al., 2006; Rubio-Somoza and Weigel, 2011). MiR390 mediates its effects on flowering time not by directly targeting protein-coding mRNAs but by triggering the production of trans-acting siRNAs from the TAS3 locus, which in turn target and repress mRNA levels of the transcription factors AUXIN RESPONSE FACTORS 3 (ARF3) and ARF4 (Endo et al., 2013; Garcia, 2008; Montgomery et al., 2008; Rubio-Somoza and Weigel, 2011). ARF3 and ARF4 activity promotes the juvenile-to-adult vegetative phase transition and mutants defective in tasiRNA biogenesis have a shorter juvenile phase as a result of elevated levels of ARF3 and ARF4. Transgenic plants expressing tasiRNA-insensitive ARF3 (ARF3:ARF3mut) also exhibited a shorter juvenile phase (Fahlgren et al., 2006; Garcia, 2008). MiR390 thus delays flowering by repressing ARF3 and ARF4 activity and prolonging the juvenile phase (Fahlgren et al., 2006; Garcia, 2008; Rubio-Somoza and Weigel, 2011).

ARF3 and ARF4 may affect the juvenile-to-adult transition by affecting the expression of miR156-regulated SPL genes (Rubio-Somoza and Weigel, 2011), and as AP2 (a target of miR172) has been shown to directly bind to the ARF3 promoter to repress its expression (Yant et al., 2010), there is therefore a link between miR390 and the miR156/miR172 feedback interaction in the regulation of juvenile phase length and the juvenile-to-adult transition (Rubio-Somoza and Weigel, 2011).

The role of the miR159, miR169, miR172 and miR399 families in the vegetative–reproductive phase transition

MiR159 affects the time of flowering through its role in the GA regulation of floral induction, and is also part of a network involving two other miRNAs, miR319 and miR167, that controls floral organ development (see Figure 3 and below). In Arabidopsis, the miR159 family is encoded by three loci (MiR159a-c). They are involved in the GA flowering pathway which promotes flowering under noninductive SD conditions through the action of GAMYB transcription factors that bind to GA-response elements in the LFY promoter to induce its transcription (Terzi and Simpson, 2008; Yamaguchi and Abe, 2012). MiR159 targets MYB33, MYB65 and MYB101, which are homologues of the rice and barley GAMYB transcription factors (Achard et al., 2004; Allen et al., 2007). The role of miR159 in controlling flowering time has been demonstrated by overexpression of miR159 which causes a decrease in MYB33 and LFY transcript levels and a delay in the onset of flowering in both Arabidopsis and Gloxinia (Achard et al., 2004; Li et al., 2013). Overexpression of miR159 was also shown to delay flowering in rice (Tsujii et al., 2006) with some lines being more delayed than others, most likely due to different levels of overexpression of the transgene with higher levels causing a more significant delay. Schwab et al. (2005), however, did not see any delay in flowering time in their Arabidopsis lines overexpressing miR159, and they speculated that one reason for this may be that their lines were not expressing sufficiently high enough levels of miR159 to cause a reduction in GAMYB levels and delay flowering.

The response to GA is repressed by DELLA proteins, which repress the expression of miR159 and its target GAMYB genes. GA treatment causes the degradation of DELLA proteins with a resulting increase in levels of both miR159 and GAMYB transcription factors, the latter inducing LFY transcription to promote flowering (Achard et al., 2004; Jin et al., 2013). As putative GA-response elements have also been identified in the miR159 promoter, it is possible that the GAMYB factors may also enhance expression of miR159 in a feedback loop that will then down-regulate GAMYB expression to provide a level of homeostatic regulation of the GA response.

Interestingly, whilst overexpressing miR159 reduces GAMYB levels and delays flowering, several studies have shown that abolishing the miR159 inhibition of GAMYB expression either in miR159 mutants, or through expression of an miR159-resistant MYB33 gene, did not cause early flowering (Achard et al., 2004; Alonso-Peral et al., 2010). This could be due to the fact that DELLA proteins are also acting to repress GAMYB expression and could be maintaining relatively normal GAMYB levels. The miR169 family is the largest miRNA family in Arabidopsis as it is made up of 14 members MiR169a-d (Li et al., 2010), and they target the NF-YA transcription factor gene family which is involved in the transcriptional regulation of a large number of genes (Jones-Rhoades and Bartel, 2004; Zhao et al., 2009). MiR169 is an example of a miRNA involved in both abiotic stress response and the control of flowering time, most members of the miR169 families in Arabidopsis, maize, and soyabean are up-regulated by abiotic stress. It has been shown that overexpression of miR169 in Arabidopsis results in early flowering, and conversely overexpression of a miR169-resistant version of NF-YA2, which is a miR169d target gene, causes late flowering (Xu et al., 2013). The effect on flowering time was shown to be mediated through the regulation of FLOWERING LOCUS C (FLC) expression by NF-YA2, which binds to the promoter and first intron of the FLC gene to induce its expression. The reduced levels of NF-YA2 in miR169d overexpressing plants result in reduced FLC expression and increased expression of FT and LFY and therefore early flowering. The opposite happens in plants overexpressing the miR169d-resistant NF-YA2 where FLC expression is increased and FT and LFY expression levels are reduced resulting in late flowering (Xu et al., 2013). These results suggest that miR169
miR172 is also involved in flowering pathways that regulate the vegetative–reproductive phase transition such as the photoperiod, ambient temperature and GA-regulated flowering pathways (Jung et al., 2007; Lee et al., 2010; Yamaguchi and Abe, 2012). In the photoperiodic pathway, GI up-regulates miR172 in a co-dependent manner (Yamaguchi and Abe, 2012; Zhou and Wang, 2013). GI affects the processing of miR172 rather than its transcription because in the gi mutant levels of miR172 are reduced despite increased levels of the primary MIR172 (pri-miR172) transcript (Jung et al., 2007). In the ambient temperature pathway, the floral repressor SHORT VEGETATIVE PHASE (SVP) and the RNA-binding protein FCA both repress the expression of miR172 (Kim et al., 2012; Zhu and Helliwell, 2011). Low ambient temperatures increase the levels of SVP and FCA therefore resulting in decreased miR172 levels. Loss of SVP results in flowering that is less sensitive to changes in ambient temperature (Kim et al., 2012; Yamaguchi and Abe, 2012). In the GA pathway, DELLA proteins repress flowering in part through binding to SPL proteins and repressing miR172 levels, and thus the induction of FT, in leaves (Galvão et al., 2012; Yu et al., 2012). MiR172 and its target genes are thus involved in several different flowering time pathways, and this miRNA therefore represents a hub for the integration of these separate pathways. There is some evidence that miR172 can move across graft unions and throughout the plant, suggesting that it may play a role as a systemically mobile developmental signal (Kasai et al., 2010; Martin et al., 2009).

In Arabidopsis, the miR399 family is encoded by the MIR399a-f loci. It is a key player in phosphate homeostasis, which it affects through its down-regulation of the expression of PHOSPHATE 2 (PHO2) (Kim et al., 2011; Kruszka et al., 2012). PHO2 is an E2 ubiquitin-conjugating enzyme that targets proteins involved in phosphate uptake in the roots (Kim et al., 2011; Liu and Vance, 2010). MiR399 activity is up-regulated under phosphate starvation to increase phosphate uptake and down-regulated under higher phosphate conditions to avoid phosphate toxicity, its activity being tightly controlled to prevent excessive phosphate accumulation and tissue necrosis (Chiou et al., 2006; Kruszka et al., 2012; Liu and Vance, 2010).

A potential role for miR399 as an ambient temperature-responsive flowering time regulator has been suggested (Kim et al., 2011). Plants flower earlier when grown at 23 °C than 16 °C, and miR399 levels were shown to be higher in plants grown at 23 °C than those grown at 16 °C. Furthermore, both miR399 overexpressors and PHO2 loss-of-function mutants flowered earlier than wild type when grown at 23 °C, whereas this difference was not observed when they were grown at 16 °C. The earlier flowering of these plants at 23 °C could be explained by increased levels of TSF expression (Kim et al., 2011). MiR399 is thus another example of a miRNA involved in both abiotic stress response and the control of flowering time.

Figure 3 An overview summarizing the action of miRNAs and their target genes in setting boundaries and regulating floral organ gene expression. MiR159 and miR319 and their target GAMYB and TCP proteins interact to regulate miR167 and the levels of ARF6 and ARF8. GA relieves the repression of both miR159 and GAMYB by DELLA proteins. GAMYB activates LFY to promote flowering and possibly also might up-regulate miR159. AP2, APETELA 2; ARF, AUXIN RESPONSE FACTOR; CUC, CUP-SHAPED COTYLEDON; GA, Gibberellic acid; GOB, GOBLET; LFY, LEAFY; LUG, LEUNIG; PHAB, PHABULOSA; PHAV, PHAVOLUTA; NAM, NO APICAL MERISTEM; REV, REVOLUTA; SOC1, SUPPRESSOR OF CONSTANS 1.

Regulation of floral patterning and floral organ development

Once the plant is induced to flower the floral meristem genes induce the expression of floral organ identity genes which direct...
the formation of floral organ primordia at the meristem. The formation of floral organs occurs in successive whorls which in Arabidopsis is a whorl of four sepal primordia, a whorl of four petal primordia, a whorl of six stamen primordia and finally, two carpel primordia. These distinct whorls require boundaries to be established that limit the expression of floral organ identity genes as per the ABC model (Causer et al., 2010). miRNAs such as miR164, miR169 and miR172 are involved in setting up such boundaries. Other miRNAs are involved in the subsequent development of floral organs such as petals, sepals, anthers and carpels. These include miR159, miR160, miR165, miR166, miR167, and miR319, some of which act together in regulatory modules (Figure 3; Rubio-Somoza and Weigel, 2013).

The setting of boundaries of floral organ development by miR164, miR169 and miR172

The miR164 family is made up of three members miR164a-c. This family down-regulates the expression of members of the NAC-domain family of transcription factors such as the Arabidopsis CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 genes, and the palm NO APICAL MERISTEM genes Engrailed1 and 2 (En1 and En2) (Adam et al., 2011; Takada et al., 2001; Vroemen et al., 2003). These transcription factors are involved in defining morphogenetic boundaries in the establishment of the floral meristem. Reducing the activity of the CUC1 and CUC2 genes changes the sepal boundary and results in fused sepals and a reduced number of petals, and miR164c has been shown to control petal number by regulating the expression levels of CUC1 and CUC2 (Baker et al., 2005; Laufs et al., 2004). MiR164 has also been shown to be involved in setting boundaries between the SAM and leaf primordia in tomato, where it has a complimentary pattern of expression in the SAM to its NAC transcription factor target GOBEL (GO8). Misexpression of miR164 or expression of a miR164-insensitive GOB gene results in defective leaf development (Berger et al., 2009). In addition to its role in setting the sepal-petal and leaflet boundaries, miR164 has been shown to be involved in carpel development, with Arabidopsis mutants of miR164 exhibiting defects in carpel closure (Baker et al., 2005; Sieber et al., 2007).

In Petunia and Antirrhinum, miR169 family members target the NF-YA transcription factor gene family which are involved in the activation of the homeotic C-class genes in flower development (Jones-Rhoades and Bartel, 2004; Zhao et al., 2009). These miR169 encoding genes are called BLIND (BL) in Petunia and FISTULATA (FIS) in Antirrhinum, and they regulate NF-YA gene expression to restrict the activity of the C-class genes to the inner two floral whorls during flower development. Loss-of-function fis and bl mutations result in conversion of petals to stamens in the second whorl, indicating that there is abnormal C-gene activity in the second whorl due to the lack of miR169 (Cartolano et al., 2007). Whilst such a role for miR169 in floral development has been shown in Petunia and Antirrhinum, this does not appear to be the case in Arabidopsis; however, where miR169 does not regulate C-gene activity (Cartolano et al., 2007).

MiR172 plays a role in floral patterning because it determines the domain of expression of the floral organ identity gene AP2. MiR172 acts at the post-transcriptional level to restrict AP2 expression to the two outer whorls of the floral meristem which give rise to sepals and petals. The domains of expression of miR172 in the inner two whorls and AP2 expression in the outer two whorls are largely complimentary as shown by in situ hybridization (Chen, 2004; Wollmann et al., 2010; Zhao et al., 2007); however, at some timepoints, an overlap in expression domains has been observed suggesting that other factors in addition to miR172 may be regulating the extent of AP2 expression (Wollmann et al., 2010). There is also evidence of a positive feedback loop whereby AP2 is involved in the repression of miR172 expression in the outer whorls. Yant et al. (2010) identified MiR172b as a target of AP2 using ChiP-seq, and Grigorova et al. (2011) showed that the transcriptional repressor LEUNIG (LUG) directly represses miR172 expression in petals and that this repression requires the presence of AP2. It is proposed that AP2 is responsible for recruiting the LUG transcriptional repressor complex to the MiR172 genes to repress miR172 levels in the outer floral whorls and thus maintain its own expression in those whorls (Grigorova et al., 2011).

MiR172 has been shown to be involved in floral patterning in cereals such as rice and barley where it targets the AP2-like transcription factor genes SUPERNUMLARY BRACT (SNB) and Oryza sativa INDETERMINATE SPIKELET1 (OsIDS1) in rice, and CLEISTOGAMY1 (Cly1) in barley, to regulate lodicule development (Lee and An, 2012; Nair et al., 2010; Zhu et al., 2009).

MiR159, miR167 and miR319 form a regulatory circuit controlling flower development

MiR159 and miR319 have overlapping roles in controlling floral development as the target genes they regulate, the MYB and TCP transcription factors, respectively, interact with each other and both act to regulate levels of miR167 (Figure 3). MiR159, miR319 and miR167 therefore act together forming a regulatory circuit. Despite the fact that miR159 and miR319 evolved from a common ancestor and have 17 identical nucleotides (Li et al., 2011), these miRNAs do not cross-regulate each other’s target genes. MiR159 cannot bind TCP transcripts, and whilst miR319 is capable of binding MYB transcripts, it has a much more limited spatial and temporal expression pattern compared with miR159. Because of this, in addition to their overlapping functions, miR159 and miR319 can also play distinct regulatory roles in plant development (Jones-Rhoades et al., 2006; Palatnik et al., 2007).

As mentioned previously, miR159 targets GAMYB-related genes which, in addition to regulating LFY transcript levels and thus impacting on flowering time, are also involved in anther development (Achard et al., 2004). MiR159 restricts the expression of GAMYB genes such as MYB33 and MYB65 just to anthers (Alonso-Peral et al., 2010). Overexpressing miR159 in Arabidopsis decreases the levels of MYB33 causing defects in anther development and male sterility (Achard et al., 2004; Schwab et al., 2005). In rice as in Arabidopsis, OsGAMYB expression is also restricted by miR159 to anthers (Aya et al., 2009; Tsuji et al., 2006). OsGAMYB loss-of-function mutants have defective anthers and pollen (Kaneko et al., 2004).

The miR319 family is encoded by the MiR319a-c loci and targets a subset of TCP transcription factor genes (TCP2, TCP3, TCP4, TCP10 and TCP24) that are involved in multiple aspects of plant growth, including flower production, leaf and gametophyte development (Schommer et al., 2012). Overexpression of miR319 affects leaf and cotyledon development, but also causes stamen defects and male sterility similar to what is observed in miR159 overexpressing plants (Palatnik et al., 2007). A loss-of-function miR319 mutant exhibited defects in petal and stamen development, such as narrower and shorter petals and abnormal anther formation (Nag et al., 2009).
The defects in the maturation of sepals, petals and anthers that are observed in plants where miR159 and miR319 activities are reduced through expression of target mimics resemble the defects observed in auxin response factor 6 (arf6) arf8 double mutants (Rubio-Somoza and Weigel, 2011). ARF6 and ARF8 regulate the expression of auxin homeostatic genes and are involved in limiting the extent of cytokinin activity in the meristem, and thus some of the floral defects in the meristems of arf6/8 double mutants can be attributed to reduced auxin activity and increase cytokinin activity in the meristem (Rubio-Somoza and Weigel, 2011).

In Arabidopsis, the patterns of expression of ARF6 and ARF8 in specific floral organs are regulated by miR167 (Wu et al., 2006), and increased miR167 levels result in the same floral phenotypes as reduced ARF6/8 expression, that is underdeveloped floral organs and reduced fertility (Ru et al., 2006). The expression of miR167 is up-regulated independently by both TCP4 and MYB33 which are themselves regulated by miR319 and miR159, respectively (Figure 3). Thus, the overlapping functions of miR319 and miR159 can be attributed to the interaction between their own targets (TCP and MYB) as well as a common downstream target in miR167 and its target genes ARF6 and ARF8 (Rubio-Somoza and Weigel, 2011). There is added complexity due to the fact that different isoforms of miR67 differ in their expression patterns and in their ability to repress ARF6/8 expression, and also due to the fact that there is cross-regulation between miR167 genes such as between miR167a and miR167c (Rubio-Somoza and Weigel, 2011).

The role of miR160 in flower development

MiR160 is involved in the regulation of other ARF genes, it down-regulates the ARF10, ARF16 and ARF17 genes (Liu et al., 2010; Mallory et al., 2005). In the floral organs in carpel (foc) mutant, there is a transposon insertion in the 3′ regulatory region of miR160 which results in reduced expression of this miRNA in flowers. As a result of ARF10, ARF16 and ARF17 expression levels are elevated in the foc mutant and the mutant displays defects in floral organ formation such as reduced fertility, the appearance of floral organs inside siliques, and irregular flower shape, as well as aberrant seeds, and viviparous seedlings (Liu et al., 2010). The foc mutant is deficient in its response to auxin as might be expected as ARF10, ARF16 and ARF17 are involved in auxin signalling. Interestingly, however, auxin was also found to control the levels of miR160a and up-regulates its expression through auxin response elements in the 3′ regulatory region of the MiR160 gene (Liu et al., 2010).

The roles of miR165 and miR166 in flower development

In Arabidopsis, miR165 and miR166 differ by only one nucleotide and both target the same HD-ZIP III genes ATHB15, ATHB8, REVOLUTA, PHABULOSA and PHAVOLUTA (Floyd and Bowman, 2004; Reinhart et al., 2002; Zhou et al., 2007), although they exhibit distinct temporal and spatial expression patterns suggesting that they might regulate these target genes in different ways (Jung and Park, 2007). Overexpression of miR165 in the Arabidopsis meristem enlargement 1 (men1) mutant results in reduced the levels of all of these HD-ZIP III target genes and causes developmental defects in the SAM such as an enlarged apical meristem and short and sterile carpels (Kim et al., 2005). MiR166 has also been shown to control embryonic SAM development in rice and maize (Nagasaki et al., 2007; Nogueira et al., 2007).

MiR165 and miR166 act in parallel to the WUSCHEL-CLAVATA pathway to regulate SAM development (Jung and Park, 2007), and they do this through an interaction with ARGONAUTE 10 (AGO 10) (Zhu et al., 2011). Whilst miR165/166 would normally bind to AGO 1 to form an active RNA-induced silencing complex (RISC), AGO 10 specifically interacts with miR165/166 with a higher binding affinity than AGO 1. The expression of AGO 10 in the SAM thus sequesters the miR165/166 and prevents them forming an active RISC with AGO 1, thus HD-ZIP III expression levels are not repressed allowing normal SAM development (Zhu et al., 2011).

Sex determination in flowers

Maize is monoeocious, with male tassels and female ears. Flowers start as bisexual but later undergo stamen arrest in the ear, and pistil abortion in the tassel. In tassels of the recessive tasselseed4 (ts4) and the dominant Tasselseed6 (Ts6) mutants, pistils fail to abort and male floral organs do not develop, this results in pistils rather than stamens in the tassel. Cloning of the Ts4 gene showed it to be an miRNA of the miR172 gene family, called zma-MIR172e. Sequencing of zma-MIR172e from different ts4 mutants demonstrated that insertion mutations in the promoter region of zma-MIR172e were responsible for the ts4 mutant phenotypes. A target of zma-MIR172e is the AP2-like gene INDETERMINATE SPIKELET1 (IDS1), mutation of which partially suppress the ts4 phenotype (Chuck et al., 2007). Cloning of Ts6 revealed it to be a mutated form of the IDS1 gene, the mutation being in the miR172 binding site thus preventing regulation by zma-mir172e which explains the similar phenotypes of the ts4 and Ts6 mutants. Deregulated expression of IDS1 in these mutants represses the expression of MADS-box genes in the maize spikelet, resulting in floral meristem indeterminacy and a failure of carpel abortion (Chuck et al., 2007).

Interestingly, as Chuck et al. point out, the wheat domestication gene Q is an AP2-like gene orthologous to IDS1. The mutation that gave rise to the dominant Q allele that is found in cultivated wheats occurred only once and is a C-to-T mutation at the 3′ end of the miR172 binding site (Chuck et al., 2007), which could explain its higher expression levels than the recessive q allele (Simons et al., 2006). It is possible that other mutations, similar to ts4, Ts6 and Q, resulting in altered miRNA regulation of AP2-like genes involved in inflorescence development could have had a role in the domestication of other crop species.

Biotechnological applications of miRNAs in manipulating flowering processes

miRNAs offer the potential to the control of almost every aspect of plant development. Altering the expression levels of specific plant miRNAs, as well as the use of artificial miRNAs, makes it possible to regulate key transcription factors and thus entire downstream gene regulatory networks (Schwab et al., 2006). Economically, important processes such as plant growth and stature, flowering, seed set and yield are obvious targets for the use of this technology to improve agricultural and horticultural productivity, and there is also evidence that miRNAs regulate nodulation (Yan et al., 2013).

Regulating flowering time

Flowering time is an important target for plant breeding as it affects flower, seed and fruit development, ease of harvest and
expressing this amiR-FT/TSF present in both genes. The transgenic Arabidopsis plants designing an amiRNA that would target a common sequence FT plants. to down-regulate this gene to try to delay flowering in transgenic et al. was delayed to a similar extent as in inhibition of flowering was also shown to work when an construct was expressed under the control of the promoter which is expressed specifically in phloem companion cells (Mathieu et al., 2007).

The FT gene acts partially redundantly with a closely related and partially redundant paralog called TWIN SISTER OF FT (TSF), the tsf double mutant is even later flowering than the ft mutant. It is possible to use amiRNAs to down-regulate more than one gene target if the gene targets share enough homologous sequence for an amiRNA binding site (Schwab et al., 2006). Mathieu et al. did this in the case of FT and TSF by designing an amiRNA that would target a common sequence present in both genes. The transgenic Arabidopsis plants expressing this amiR-FT/TSF construct flowered as late as the ft tsf double mutant (Mathieu et al., 2007). Caution needs to be taken with this approach as Schwab et al. have shown that the degree of down-regulation of different target genes by an amiRNA can vary, and this variation is not correlated with the degree of complementarity to the target, or to the level of endogenous expression of the target gene (Schwab et al., 2006).

Li et al. showed that it was possible to both delay and advance the onset of flowering in ornamental Gloxinia (Sinningia speciosa) by up- or down-regulating the levels of miR159, which inhibits flowering by reducing the expression levels of the Gloxinia homologues of GAMYB and LFY. Transgenic lines with increased levels of miR159 were delayed in the onset of flowering, whilst in those lines that had reduced levels of miR159 caused by overexpression of a target mimic of miR159 (MIM159), an early flowering phenotype was observed (Li et al., 2013). In a few transgenic lines where MIM159 levels were very high, and where there was consequently strong repression of miRNA159 and high levels of LFY expression, some petal to sepal conversion was observed. Thus, it is not desirable to severely/completely inhibit the expression of miR159. Using inducible expression systems to regulate the levels of this miRNA could reduce this problem and also have the potential to control the timing of flowering in a very precise manner.

Such an inducible expression approach has been shown to work by Yeoh et al. (2011). They used Arabidopsis plants that were late flowering because they were expressing amiR-FT directed against the Arabidopsis FT gene (originally described in Mathieu et al., 2007), and they transformed these plants with the FTA1 gene from Medicago truncatula, an orthologous gene that has diverged sufficiently from the Arabidopsis equivalent to avoid suppression by the amiR-FT. The expression of the FTA1 gene from Medicago in the amiR-FT Arabidopsis plants rescued the late flowering phenotype. Using an alcohol-inducible promoter to drive the expression of the FTA1 gene enabled them to induce flowering in the transgenic plants in a fairly precise and synchronous manner by exogenous application of ethanol (Yeoh et al., 2011).

Male sterility systems
In addition to manipulating the time of flowering, miRNAs are involved in male sterility (Achard et al., 2004; Toppino et al., 2011; Zhou et al., 2012) and can be used to create male-sterile lines for F1 hybrid production. F1 hybrid production is mainly based on the three-line and two-line systems. The three-line system uses cytoplasmic male sterility lines, maintainer lines and restorer lines. The two-line hybrid system is based on environmentally sensitive (e.g. photoperiod-sensitive (PGMS), or thermo-sensitive, (TGMS)) male-sterile lines, which serve as both the male-sterile lines and maintainer lines under different environmental conditions (Chen and Liu, 2014). The two-line system is much simpler for breeding purposes and makes it easier to exploit hybrid vigour (heterosis).

PGMS and TGMS are widely used in two-line hybrid rice production, but the molecular basis underlying this male sterility was unknown. Zhou et al. (2012) cloned the locus responsible for PGMS in a japonica rice line, and TGMS in an indica rice line (the same locus was responsible for both), and found that it encoded a novel small 21-nt RNA named osa-smR5864W, which was preferentially expressed in young panicles. The PGMS and TGMS phenotypes in the male-sterile lines were due to a C-to-G point mutation in the RNA, the mutated RNA being named osa-smR5864 m. Expression of the wild-type miRNA, osa-smR5864W, restored fertility in both the PGMS and TGMS male-sterile lines (Zhou et al., 2012). If this small RNA is conserved in other crop plants, then it would be a clear target for site-directed mutagenesis to engineer PGMS or TGMS in those crop plants.

Another approach to engineer reversible male sterility using miRNAs was described by Toppino et al. (2011). In this approach, anther-specific promoters were used to drive the expression of amiRNAs designed to target and repress the expression of factors involved in the basal RNA polymerase II transcription complex. Some of these factors are essential for cell viability and are highly conserved, Toppino et al. targeted two of these factors for repression by amiRNAs and by expressing the amiRNAs only in the tapetum or microspores they were able to generate lines in which pollen viability was greatly reduced. Reversibility of the male-sterile phenotype was achieved through expression of amiRNA-insensitive forms of the target genes that had been designed with mismatches in the amiRNA target sequence to make them insensitive to the activity of the amiRNAs. Expressing these amiRNA-insensitive alleles from an alcohol-inducible promoter enabled fertility in these male-sterile lines to be restored by ethanol treatment.

In addition to regulating flowering time and the development of male sterility systems in different crops as outlined above, which have probably the most obvious economic benefits, other potential flowering-related targets for manipulation by miRNA
approaches could be altering flower shape/appearance, and possibly even controlling the sex of the flowers in monocots and dioecious plant species (Chuck et al., 2007; Song et al., 2013). As new discoveries reveal even more roles for miRNAs in flowering-related processes, perhaps some specific to individual species, then the potential biotechnological applications will only increase further.

Conflict of interest

The authors declare that they have no conflict of interest.

References


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