Floral induction and flower formation -the role and potential applications of miRNAs.

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Abstract

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, eg. 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 co-ordinated 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 vernalisation state in order 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; Srikanth and Schmid, 2011; Matsoukas et al., 2012).

As the timing of flowering and floral organogenesis both significantly affect 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). A lot of 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 aging pathways (Kim et al., 2012; Srikanth and Schmid, 2011; Matsoukas et al., 2012; 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 paralogue *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* (*AP1*) 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; Pose 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 (Willmann and Poethig, 2007; Sunkar and Jagadeeswaran, 2008; Luo et al., 2013). 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, these are *miR156, miR159, miR160, miR164, miR166/165, miR167, miR169, miR172, miR319, miR390* and *miR399* (see Fig.1).

Regulation of the time of flowering

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 *miR156* and *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).

The role of *miR156*

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 reproductively competent (Huijser and Schmid, 2011; Yamaguchi and Abe, 2012; Wang et al., 2009; Wu et al., 2009). *MiR156* 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, 2010). 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^{o}C than at 23^{o}C leading to the suggestion that ambient temperature may influence the effect of *miR156* over-expression (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 over-expressing *miR156* (Xie et al., 2006; Zhang et al., 2011; Chuck et al., 2007). 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 out 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 *SPL*9 or *SLP15* (*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, 2010). 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, ie. 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 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 (ie. 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 (ie. 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. 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

levels 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 shown to be affected by cold temperatures in the perennial plant *Arabis alpina*, a relative of *Arabidopsis* that requires vernalisation 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 role of *miR172*

The *miR172* family, which are coded by the *MIR172a-e* loci, have 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 non-inductive short day (SD) conditions (Aukerman and Sakai, 2003; Wu et al., 2009; Zhu and Helliwell, 2010). *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 over-expressing *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 EAT 1 (TOE1), TOE2, TOE3, SCHLAFMÜTZE (SMZ) and SCHNARCHZAPFEN (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 Helliwell, 2010). 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 Helliwell, 2010). 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 (Yant et al., 2010; Huijser and Schmid, 2011). 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 Helliwell, 2010).

In addition to the aging pathway *miR172* is also regulated by other flowering pathways 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-independent 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, 2010). Low ambient temperatures increase the levels of SVP and FCA therefore resulting in decreased *miR172* levels. Loss of *SVP* results in ambient temperature insensitive flowering (Kim et al., 2012; Yamaguchi and Abe, 2012). In the GA pathway DELLA proteins repress flowering in part through repressing SPLs and *miR172* levels, and thus the induction of *FT*, in leaves (Galvao 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 (Martin et al., 2009; Kasai et al., 2010).

The role of *miR390*

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 *miR159*

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 Fig.3 and below). In *Arabidopsis* the *miR159* family is encoded by three loci (*MIR159a-c*), and are involved in the GA flowering pathway, which promotes flowering under non-inductive SD conditions (Terzi and Simpson, 2008; Yamaguchi and Abe, 2012). DELLA proteins, which repress the GA response, also repress the expression of *miR159* and its target genes, *MYB33, MYB65* and *MYB101*, which are homologues of the rice and barley GAMYB transcription factors

(Achard et al., 2004; Allen et al., 2007). GA treatment results in the degradation of DELLA proteins with a resulting increase in both *miR159* levels and levels of the GAMYB transcription factors which then are able to bind to GA-response elements in the *LFY* promoter to induce its transcription (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 (Fig 3). This is supported by the fact that overexpression of *miR159* causes a decrease in *MYB33* and *LFY* transcript levels and a delay in the onset of flowering (Achard et al., 2004; Li et al., 2013).

The role of *miR169*

The *miR169* family is the largest miRNA family. In Arabidopsis is made up of 14 members *miR169a-n* (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 soybean are up-regulated by abiotic stress. It has been shown that over-expression of *miR169d* in Arabidopsis results in early flowering, and conversely over-expression of a *miR169d*-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 *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* over-expressing plants results in reduced *FLC* expression and increased expression of *FT* and *LFY* and therefore early flowering. The opposite happens in plants over-expressing 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* might play a key role in the promotion of early flowering by abiotic stresses.

The role of *miR399*

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 high phosphate conditions to avoid phosphate toxicity, its activity being tightly controlled to prevent excessive phosphate accumulation and tissue necrosis (Chiou et al., 2006; Liu and Vance, 2010; Kruszka et al., 2012).

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.

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 (Causier 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 (Fig.1; Rubio-Somoza and Weigel, 2013).

<u>The role of *miR164* in flower development</u>

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 *EgNAM1* and *PdNAM1* (Takada et al., 2001; Vroemen et al., 2003; Adam et al., 2011). 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* (Laufs et al., 2004; Baker et al., 2005). *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 *GOBLET* (*GOB*). Mis-expression 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).

The role of *miR169* in flower development

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* 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).

The role of *miR172* in flower development

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; Zhao et al., 2007; Wollmann et al., 2010), 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 sepals 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 *SUPERNUMARY 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).

The roles of *miR159, miR167* and *miR319* in 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* (Fig.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 to *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). Over-expressing *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). Over-expression of *miR319* affects leaf and cotyledon development, but also causes stamen defects and male sterility similar to what is observed in *miR159* over-expressing 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 increased 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 results in the same floral phenotypes as reduced *ARF6/8* expression, ie. under-developed 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 (Fig. 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 (Mallory et al., 2005; Liu et al., 2010). 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 *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 signaling. 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 role 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). Over-expression 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 monoecious, 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 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. De-regulated 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 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 also have had a role in the domestication of other crop species.

Biotechnological applications of miRNAs in manipulating the 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, 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 marketing (Jung and Muller 2009). Flowering in those crops and forages where the vegetative tissues are the important parts that are harvested has negative impacts on yield and nutritional quality, therefore inhibiting

flowering in these crops would be beneficial (although flowering is needed to enable seed production). On the other hand being able to induce early flowering would be of benefit to breeding companies enabling them to speed up their breeding programmes and develop new varieties more quickly, this is particularly true in tree species where it would be necessary to overcome their naturally long juvenile phases.

The activity of miRNAs can be reduced by the expression of specific target mimics that sequester the miRNA (Franco-Zorrilla et al., 2007). Conversely it is possible to increase miRNA activity generally throughout the plant, or in specific tissues, by overexpressing *MIR* genes or artificial miRNAs (amiRNAs) using constitutive or tissue-specific promoters (Li et al., 2013; Schwab et al., 2006). Schwab et al. expressed amiRNAs directed against different sequences of the Arabidopsis *FT* gene, *amiR-ft-1* or *amiR-ft-2*, to down-regulate this gene to try to delay flowering in transgenic plants. *FT* mRNA levels were reduced below RT-PCR detection levels in the *amiR-ft* expressing plants and flowering of these lines was delayed to a similar extent as in *ft* null mutants. This inhibition of flowering was also shown to work when an *amiR-FT* construct was expressed under the control of the *SUC2* 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 *ft tsf* double mutant is even later flowering 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 over-expression of a target mimic of *miR159* (*MIM159*), an early flowering phenotype was observed (Li et al., 2013). In a few transgenic lines where *MIM159* expression was 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 (eg. 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 was due to a C-to-G point mutation in the RNA, the mutated RNA being named osa-smR5864m. 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 mis-matches 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 monoecious 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.

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Figures

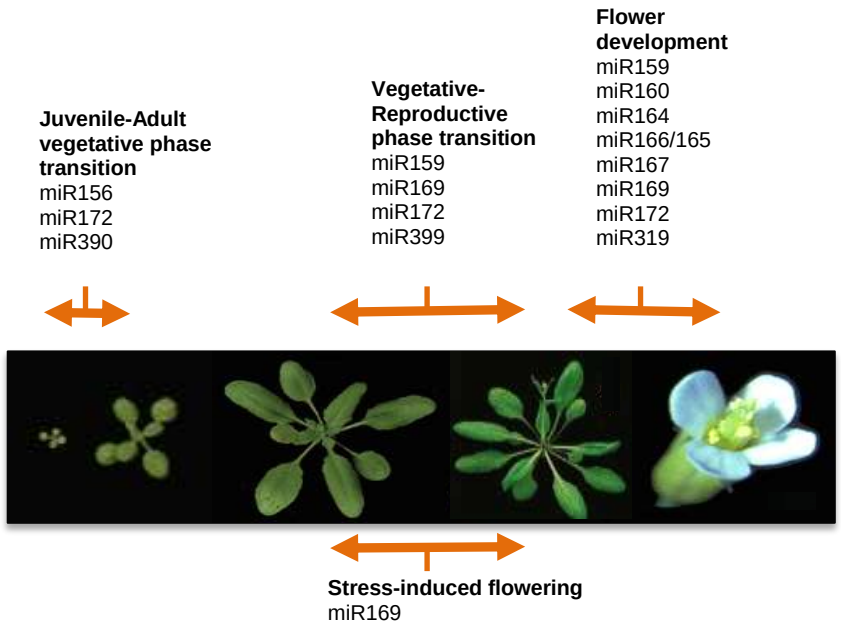

Fig.1

The involvement of various miRNAs in the different flowering-related phases of plant development, from the transition from juvenility to maturity, to floral induction and development.

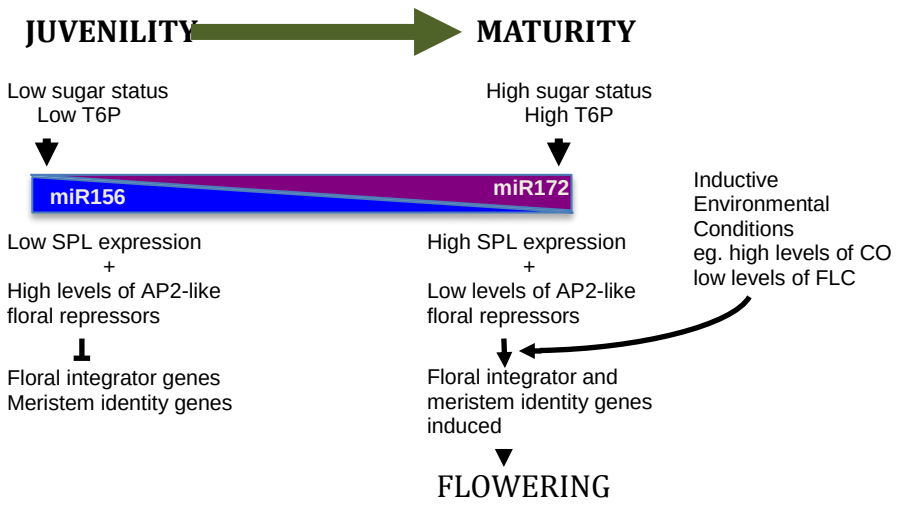

Fig.2

The changes associated with the transition from juvenility to maturity. MiR156 levels reduce with age influenced by sugar signaling 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: APETELA 2-like, CO: CONSTANS, FLC: FLOWERING LOCUS C, SPL: SQUAMOSA PROMOTER BINDING-LIKE, T-6-P: TRHALOSE-6-PHOSPHATE.

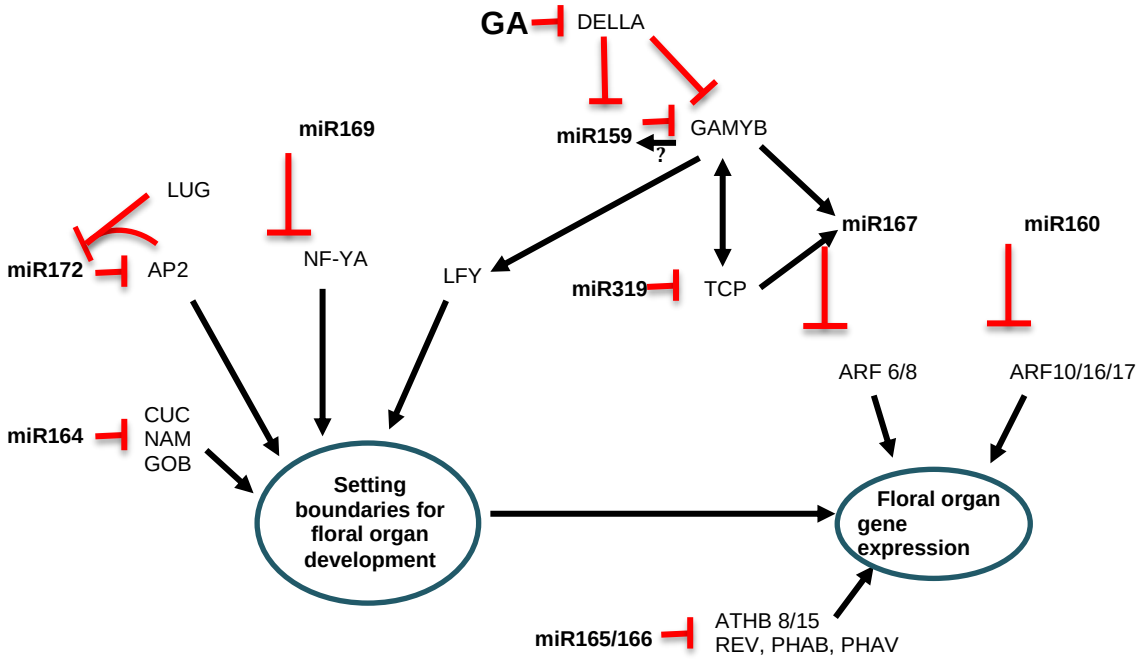

Fig.3

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