Evolutionary conservation of microRNA regulatory programs in plant flower development

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A B S T R A C T

MicroRNAs (miRNAs) are post-transcriptional regulators of growth and development in both plants and animals. Flowering is critical for the reproduction of angiosperms. Flower development entails the transition from vegetative growth to reproductive growth, floral organ initiation, and the development of floral organs. These developmental processes are genetically regulated by miRNAs, which participate in complex genetic networks of flower development. A survey of the literature shows that miRNAs, their specific targets, and the regulatory programs in which they participate are conserved throughout the plant kingdom. This review summarizes the role of miRNAs and their targets in the regulation of gene expression during the floral developmental phase, which includes the floral transition stage, followed by floral patterning, and then the development of floral organs. The conservation patterns observed in each component of the miRNA regulatory system suggest that these miRNAs play important roles in the evolution of flower development.

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Introduction

It is normally agreed that plant development can be divided into vegetative and reproductive growth phases. Flowering is critical for the reproduction of angiosperms. During flowering, plants acquire reproductive competence, and can be induced by environmental cues to form floral organs. Flower development can be divided into three developmental phases: the floral transition phase, the floral patterning phase, and the phase of development of floral organs. The floral transition is the transition from the vegetative growth phase to the reproductive phase. During this transition, the vegetative shoot apical meristem (SAM) or lateral meristem (LM) is converted to floral meristems (FMs) (Liu et al., 2009a, 2009b; Barton, 2010). After completion of the floral transition, FMs give rise to different floral organ primordia during the floral patterning phase. Finally, the floral organ primordia develop in successive whorls, and then grow and differentiate into floral organs.

Recently, significant progress has been made towards understanding the molecular mechanisms that control flowering. Flowering processes are regulated by a very complex set of pathways controlled by numerous genes. These include floral meristem identity genes, floral organ identity genes, and some evolutionary conserved miRNAs (Amasino, 2010; Irish, 2010; Nag and Jack, 2010; Poš et al., 2012; Srikanth and Schmid, 2011). MiRNAs are small (20–25 nucleotides), endogenous, non-coding, single-stranded RNAs, which are found in many organisms, where they regulate gene expression at the post-transcriptional level (Bartel, 2004). MiRNAs negatively regulate certain genes involved in flower development by directing RNA cleavage or inhibiting translation of the target transcripts (Jones-Rhoades et al., 2006).

MiRNAs are not only regulators of gene expression patterns, but also play an essential role in the coordination of complex developmental processes through their extensive integration within genetic networks (Huijser and Schmid, 2011; Nag and Jack, 2010; Srikanth and Schmid, 2011; Sun, 2012; Yamaguchi and Abe, 2012; Zhu and Hellwell, 2011).

This review first discusses the conservation of miRNAs and their targets. We then summarize their contribution to three developmental phases of flower development, including the floral transition, the floral patterning, and the development of floral organs. Some miRNAs regulate members of the florigen and integrators involved in flowering, and thus participate in complex genetic networks at floral transition phase. Other miRNAs target and restrict the action of various genes that control different flower-related processes. We also discuss the evolutionary...
conservation of both of these miRNAs and the regulatory pathways in which they participate in various plant species.

**Phylogenetic distribution and conservation of nine miRNAs**

**Phylogenetic distribution**

At least nine conserved miRNA families have been reported to play key roles during flower development in plants. These include miR156, miR159, miR160, miR164, miR166/165, miR167, miR169, miR172, and miR319 (Fig. 1). These miRNAs regulate flower development by targeting various transcription factors involved in flower developmental processes. For example, miR172 regulates floral organ identity and flowering time by translational repression or target cleavage of members of the APETELA2 (AP2) transcription factor genes (Aukerman and Sakai, 2003; Chen, 2004; Glaziska et al., 2009; Jung et al., 2007; Schwab et al., 2005; Varkonyi-Gasic et al., 2012). MiR156 targets SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factor gene family to control the transition from the vegetative phase to the floral phase in Arabidopsis, rice, and maize (Chuck et al., 2007a; Gandikota et al., 2007; Jiao et al., 2010; Miura et al., 2010; Yang et al., 2010). MiR159 is required for normal anther development, which it controls through regulating the expression of genes that encode MYB transcription factors (Achar et al., 2004; Tsuji et al., 2006). Some rice miRNAs are more abundant in pollen than in leaves (Wei et al., 2011). MiR156, miR164, miR166, miR172, and miR167 display marked differences in their levels expressed during the development of female flowers of Carya cathayensis (Wang et al., 2012). Most studies about the roles of miRNAs in flowering were conducted using the model plant Arabidopsis, although results obtained from maize, rice, tomato, potato, and other non-model plants have indicated a high level of conservation of these miRNAs and the regulatory interactions with their targets across the plant kingdom (Axtell and Bartel, 2005; Willmann and Poethig, 2007; Sunkar and Jagadeeswaran, 2008; Zhang et al., 2006).

Based on the recently released miRBase (Release 19.0: August 2012, Kozomara and Griffiths-Jones, 2011) and some recently published reports, at least 1505 miRNA sequences have been identified, which belong to nine miRNA families (Supplementary Table S1). These 1505 miRNAs were obtained from 58 plant species, which range from angiosperms to bryophytes and include 10 monocotyledonous species, 41 dicotyledonous species, 4 gymnosperms, 2 pteridophytes, and one bryophyte. These 58 species represents 23 families, including some families that occupy key phylogenetic positions, such as Ranunculacea (basal eudicots), Magnoliaceae (basal angiosperms), Pinaceae (gymnosperms), and Funariae (mosses). The phylogenetic distribution of the nine miRNA families across various species from all major lineages of land plants (Fig. 2) indicates the high levels of their conservation. Of these miRNA families, miR164 is found in both angiosperms and gymnosperms, and thus appears to be conserved across all spermatophytes. Members of the miR159, miR169, and miR172 families are found in angiosperms, gymnosperms, and ferns, and thus appear to be conserved across all tracheophytes. However, the five remaining families (miR156, miR160, miR166/165, miR167, and miR319) are found not only in dicots and monocots but also in gymnosperms, lycopsids, and mosses, and thus appear to be conserved across all embryophytes.

**Sequence conservation**

The alignments and sequence similarities of these mature miRNAs from various species are shown in the supplementary figure. The sequence alignment of mature miRNAs reveals an overall well-conserved consensus with a few variations. For example, whereas members of the miR164 family are completely identical through 28 species, the mature sequences of miR172 from 37 angiosperm species and miR166 from 36 species have 90% similarity. Mature sequences of miR159, miR160, miR167, miR169, and miR319 are more than 80% similar. Mature miR156 sequences from 40 species are 75% similar (Supplementary Fig.). Members of the miR156 family from gymnosperms display greater sequence diversity than those from angiosperms. However, moss miR156 is

![Fig. 1](image-url) (The flower development in Arabidopsis ((a1), (b1) and (c1)) and rice ((a2), (b2), (c2)). The involvement of miRNAs and miRNA-target genes at various phases is indicated. (A) Floral transition: (a1) In Arabidopsis, three sets of miRNA-mediated module, miR156-SPLs, miR172-AP2-like, miR159-MYBs, affect floral meristem formation. (a2) In rice, miR156-4sPLs14 and miR172-SNB/oldD51 modules are involved in spikelet meristem converting into floral meristem. (B) Floral patterning: (b1) In Arabidopsis, miR172 functions to restrict AP2 in the center of the flower, which expresses in whorls 3 and 4 (indicating by black stars). AP2 transcripts are present in whorls 1 and 2 (indicating by blue stars) to form sepal and petal primordia. miR164 negatively regulates CUC1, CUC2 expression to establish boundaries between and within whorls of organs. (b2) In rice, miR172 and its targets SNB and oldD51 participate in regulating lodicules development. (C) Floral organs development: (c1) In Arabidopsis, miR159 functions to negatively regulate MYB23 and MYB65 in stamens, miR166/165 functions to negatively regulate the HD-ZIP III genes in various floral organs, miR172 targets ARF6 and ARF8 in stamens, miR160 targets ARF6 and ARF77 in reproductive organs, and miR319 targets TCP in the petal. (c2) In rice, miR159 targets OsGAMYB in stamens. Abbreviation used in this figure: SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; AP2, APETALA2; CUC, CUP-SHAPED COTYLEDON; ARF, AUXIN RESPONSE FACTOR; HD-ZIP III, class III homeodomain-leucine zipper; SNB, SUPERNUMERARY BRACT; IDS1, INDETERMINATE SPIKELET1; L, Lodicule; S, Sepal; P, Petal; St, Stamen; C, Carpel.)
almost identical to miR156 of the most angiosperms, with a different nucleotide found at only a single position.

Conservation of targets

The observation that the target genes of these nine miRNA families are also conserved across different plant families indicates that these miRNAs are conserved in function as well as in sequence. The targets of the nine miRNA families all encode transcription factors (Table 1), which are known to affect flowering. Although there are many nucleotide changes among the targets of different plant species, the sequences of complementary sites are highly conserved (Axtell and Bartel, 2005).

One of the best known miR172 targets is AP2. Homologs and orthologs of AP2 have been found in a variety of gymnosperms and angiosperms (Shigyo et al., 2006), and have also been detected in the fern Ceratopteris thalictroides (Axtell and Bartel, 2005). Phylogenies of both gymnosperm and angiosperm members of the AP2 family suggest significant conservation of the target site of miR172 (Shigyo et al., 2006). Very few nucleotides differ between the miR172 binding sites of AP2 genes from Arabidopsis, rice, maize and barley (Zhu and Helliwell, 2011). Moreover, AP2 orthologs in these species have very specific expression patterns in inflorescences and floral primordia (Aukerman and Sakai, 2003; Chuck et al., 1998; Nair et al., 2010; Wollmann et al., 2010; Zhu et al., 2009).

The SPL transcription factors, which were first identified in Antirrhinum majus, contain an SPB-box region, bind to the promoter of the floral identity gene SQUAMOSA and were originally proposed to function on specifying flowers by activating genes that control floral meristem identity (Klein et al., 1996). Most members of SPL genes contain a site targeted by miR156. In Arabidopsis, 11 of the 17 SPL genes are targets of miR156, whereas in rice, 11 SPL genes are targeted by miR156 (Cuperus et al., 2011; Xie et al., 2006). SPB-box genes have been also found to be targeted by miR156 in the moss (Riese et al., 2007).

The targets of members of the miR166/165 family are Class III HOEMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) transcriptional factor genes (Floyd and Bowman, 2004). The HD-ZIP III genes are highly-conserved in land plants, having been identified in dicotyledonous, monocotyledonous, gymnosperm, and moss species (Floyd and Bowman, 2004; Prigge and Clark, 2006). In addition, all of these homologs have the same miR166/165 binding site, indicating that miR166/165-mediated HD-ZIP III gene regulation is conserved in all land plants (Sakaguchi and Watanabe, 2012).

Members of the miR164 family are potentially capable of targeting the NAC family of transcription factor genes (Laufs et al., 2004). Currently, at least five NAC transcription factor genes are targeted by miR164 in Arabidopsis, including CUP-SHAPED COTYLEDON1 (CUC1) and CUC2, both of which regulate plant development (Laufs et al., 2004; Kim et al., 2009; Raman et al., 2008). Other miR164 target genes have also been found in a variety of species, including EgNAM1 and PdNAM1 from palms (Adam et al., 2011) and PaNAC01 from gymnosperms (Larsson et al., 2012).

Several auxin response transcription factors (ARFs) are regulated by miR160 and miR167. Five of the 23 ARF genes in Arabidopsis are targeted by these two miRNAs, with ARF10, ARF16, and ARF17 targeted by miR160, and ARF6 and ARF8 targeted by miR167 (Nagpal et al., 2005; Wu et al., 2006a, 2006b). Both ARF6 and ARF8 are also targeted by miR167 in rice (Yang et al., 2006).

The targets of miR319 are a subset of TEOSINTE BRANCHED/CYCLOIDEA/PCF (TCP) family of transcription factor genes. The targets of miR319 have been identified in Arabidopsis, rice, and sugarcane (Nag et al., 2009; Thiebaut et al., 2012).

Evolutionary conservation of miRNA regulatory programs during the floral transition

The floral transition is the most important phase during plant development because the proper timing of this transition is critical.
### Table 1

<table>
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<th>Targets</th>
<th>Functions</th>
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<th>References</th>
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<td>SBP1</td>
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<td></td>
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<td>Zea mays</td>
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<td></td>
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<td></td>
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<td>FvGAMYB</td>
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<td>miR164</td>
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<td></td>
<td></td>
<td>Establish flower primordia boundaries</td>
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<td></td>
<td></td>
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<td></td>
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to ensure reproductive success (Huijser and Schmid, 2011). Flower initiation occurs in the SAM, which acts as a floral inducer and triggers the floral transition network. In Arabidopsis, when signals from flower-promoting pathways reach the SAM, the vegetative meristem first acquires an inflorescence meristem (IM) identity. The IM then gives rise to the reproductive organs after generating the FM on its flank. In monocotyledonous species, the primary IM produces several lateral branches. Each branch meristem gives rise to spikelet meristems (SMs), each of which eventually differentiates into the FM (Liu et al., 2009a, 2009b; Barton, 2010).

Genetic and molecular analyses in Arabidopsis and other plants have identified multiple interdependent genetic pathways that control the timing of the floral transition. Some key components are thought to be very important in the floral transition network. One class of key components is the group of floral meristem identity genes, such as LEAFY (LFY) and APETALA1 (API), which promote the differentiation of SAM primordia into FMs (Liljegren et al., 1999; Wigge et al., 2005). The other class comprises floral integrator genes, such as SUPPRESSOR OF OVEREXPRESSION OF CO (SOC1), which together with AGAMOUS-LIKE 24 (AGL24), promote the expression of the floral meristem identity genes LFY and API (Amasino, 2010; Schmid et al., 2003; Wigge et al., 2005). Moreover, the small mobile protein Flowering Locus T (FT) acts as a florigen, and plays a key role by activating SOC1 to promote flowering together with the meristem-specific bZIP transcription factor FD (Abe et al., 2005; Amasino, 2010; Srikanth and Schmid, 2011).

The key components of floral transition networks mentioned above are regulated by various environmental and endogenous cues, such as photoperiod, temperature, gibberellins (GAs), and vernalization. Recent studies have shown these key genes or proteins are also regulated by endogenous miRNAs (Poethig, 2009; Nag and Jack, 2010). It has become increasingly clear that three miRNAs with a high degree of evolutionary conservation – miR156, miR172, and miR159 – directly or indirectly regulate those components that are critical to the regulation of flowering (Fig. 1A).

**miRNA regulatory programs in leaves and the SAM in floral transition of Arabidopsis**

The floral transition occurs in both leaves and the SAM. Leaves receive inductive environmental cues, and the subsequent production of the floral promoter FT triggers the establishment of the regulatory network that governs the timing of flowering. After being transported to the SAM, FT prompts a genetic network to activate the floral meristem identity genes, which culminates in differentiation of the SAM into the FM. Different regulatory circuits, which comprise miR156, miR172, and miR159 together with their targets, control the floral transition in leaves and the SAM (Fig. 3).

In leaves, miR156 and miR172 regulate the production of FT through a complex regulatory circuit (Fig. 3). Expression of the FT gene is activated by GIGANTEA (GI) and CONSTANTS (CO) in leaves following photoperiodic induction in Arabidopsis adult plants (Amasino, 2010). However, the FT protein is also regulated by the targets of miR156 and miR172. MiR172 and its AP2-like targets use a CO-independent mechanism to regulate FT (Jung et al., 2007). AP2-like genes, such as TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHLAMMUTZE (SMZ), and SCHNARCHZAPFEN (SNZ) act as repressors of FT (Aukerman and Sakai, 2003; Jung et al., 2007; Schmid et al., 2003). Elimination of all AP2-like genes results in extremely early flowering, similar to the phenotype caused by overexpression of miR172 (Yant et al., 2010). Chromatin immuno-precipitation (ChIP) analysis revealed that FT is directly targeted by SMZ, and that changes in the levels of FT accumulation caused by alteration of the levels of either SMZ or miR172 occur independently of CO (Mathieu et al., 2009). Furthermore, the expression pattern of miR172 is correlated with that of miR156. The miR156

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*Fig. 3. A schematic diagram of the molecular mechanisms of miR156, miR172, miR159 and their targets regulating floral transition in leaves and the apical shoot meristem in Arabidopsis. A regulatory circuit consisting miR156, miR172 and their targets exist in leaves to control the expression of the florigen FT. AP2-like genes targeted by miR172 mediate the induction of flowering by photoperiod. MiR172 represses expression of AP2-like genes, which negatively regulate FT. MiR172 expression is positively regulated by certain SPL genes, which is repressed by miR156. MiR156-SPL module is also positive regulators of FT through temperature pathway. The FT protein is transported through the phloem to the shoot apical meristem. In the shoot apical meristem, miRNAs can be involved in floral meristems initiation by directly regulating the floral meristem identity genes through different pathway. FT interacts with the bZIP transcription factor FD and coordinately they upregulate SOC1 and API, then give rise to identity of floral meristem. SPLs targeted by miR156 can active SOC1 and LFY, through age pathway. MYBs targeted by miR159 regulate LFY through gibberellins pathway. AP2 gene also participates in SOC1 repression in the shoot apical meristem. Green lines with an arrow represent promotion, and red ones with a perpendicular bar represent repression. Ovals represent genes or proteins involved in floral transitions, and purple ones represent targets of miRNAs. Abbreviations used in the figure: FT, Flowering Locus T; SOC1: SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1; AGL24, AGAMOUS-LIKE 24; LFY, LEAFY; API, APETELA1; AP2-like, APETALALA2-like; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE.*
target SPL9 promotes expression of miR172 (Wu et al., 2009). In addition, the miR156-SPLs module can directly regulate FT (Kim et al., 2012; Lee et al., 2010). Overexpression of miR156 in leaves delays flowering at a lower ambient temperature (16 °C), and this is associated with a reduced abundance of FT transcripts (Kim et al., 2012; Lee et al., 2010). The SPL3 protein binds directly to FT to regulate flowering (Kim et al., 2012). However, the miRNA-mediated temperature-sensitive pathway appears to be independent of known temperature-sensitive pathways. SHORT VEGETATIVE PHASE (SVP), FLOWERING LOCUS C (FLC), FLOWERING LOCUS M (FLM) and other components in known temperature-sensitive pathways (Balasubramanian et al., 2006; Lee et al., 2007a, 2007b), have no correlation with miR156 or SPLs. These findings suggest the existence of a feedback loop in the miR156/miR172-mediated module that controls flowering.

In the SAM, the targets of miR156, miR172 and miR159 participate in the activation of floral meristem identity genes (Fig. 3). The miR156-SPL module promotes the floral transition through an age-dependent pathway. MiR156 targets a subset of SPLs, which were originally proposed to promote flowering (Klein et al., 1996). In young Arabidopsis seedlings, miR156 levels are high and levels of SPLs are low. As development proceeds, the decline of miR156 levels and the increase of SPLs in the SAM lead to the activation of floral meristem identity genes (Wang et al., 2009; Wu et al., 2009). SPLs activate expression of the floral meristem identity genes LFY and AP1 and the floral integrator gene SOC1 (Yamaguchi et al., 2009; Wang et al., 2009). With decreasing miR156 activity, flowering can be promoted through the photoperiod-dependent FT/FD pathway, and increasing levels of SPLs might trigger the floral switch (Schmid et al., 2003; Wang et al., 2009). A continuing increase in the activities of SPLs will cause plants to flower even without activation of the FT/FD pathway (Wang et al., 2009). This change in levels of miR156 and SPLs appears to be independent of several known floral regulators, such as temperature, vernalization, and GAs. The miR156-SPL module thus seems to define an age-dependent pathway to induce flowering. MiR159-MYB modulates the regulation of the floral transition by GA through targeting the GA-specific transcriptional regulator CAMYB. The GA signaling pathway plays an important role in promoting flowering during short-day photoperiods (Wilson et al., 1992; Simpson and Dean, 2002). Arabidopsis AtMYB33 is a GA-specific transcriptional regulator that regulates panicle branching in the in monocotyledonous species. Recent studies from rice and maize have shown miR156 and miR172 control the conversion of SMs to FMs to ensure the initiation of floral organ primordia (Fig. 1a2). maize contains at least 17 genes that encode SBP-box proteins and are potential targets of miR156 (Hultquist and Dorweiler, 2008). The maize Conrrass1 (Cg1) mutant, which carries a STONER retrotransposon insertion in the upstream regulatory region of the miR156 gene, causes overexpression of miR156 and reduces the number of branches in tassels and increased tilling (Chuck et al., 2007a). The teosinte glume architecture1 (tga1) gene, which encodes an SBP-box-containing protein, is also expressed at a lower level in the Cg1 mutant than in its wild-type counterpart, and mutation of tga1 has been implicated in the domestication of maize (Chuck et al., 2007a; Hultquist and Dorweiler, 2008; Wang et al., 2005). Another SBP-box gene, tasselsheath4 (tsh4), is also a target of miR156, and is known to regulate the development of bracts and meristem boundaries (Chuck et al., 2010). Some AP2-like orthologous genes are targets of miR172. These include Indeterminate Spikelet1 (Ids1) and Sister of Indeterminate Spikelet 1 (Sid1), which control spikelet meristem fate in maize (Chuck et al., 1998, 2007b, 2008). Additional spikelets and florets are formed on branches in the maize mutant tassel seed 6 (ts6), which carries a mutation in the miR172 binding site of Ids1. A very similar phenotype is observed in ts4, which carries a mutation in miR172e (Chuck et al., 2007b). Reduced expression of Sid1 delays the conversion of SMs to FM identity. An ids1 and sid1 double mutant forms many bracts from SMs, and florets are never initiated (Chuck et al., 2008). The inflorescence and floret defects caused by mutations in these miRNA genes or target genes suggest a central role for miR156 and miR172 in determining the identities of maize inflorescence or FMs.

In rice, 11 OsSPL genes are putative targets of miR156. Overexpression of miR156 causes severe dwarfism, strongly reduced panicle size, and delayed flowering (Xie et al., 2006). The miR156 target OsSPL14 gene regulates panicle branching in the inflorescences and directly affects grain yield in rice (Jiao et al., 2010; Miura et al., 2010). Overexpression of miR172 delays the transition from SM to FM, changing the numbers and identities of floral organs (Zhu et al. 2009). The wheat domestication gene Q, which is also a miR172 target and is orthologous to maize Ids1, affects several domestication-related traits, including spike compactness and glume shape (Simons et al., 2006).

From these results it appears that miR156 and miR172 also participate in the floral transition in monocotyledonous species. This indicates that miRNA-mediated pathways that regulate the floral transition are conserved in flowering plants.

The role of miRNAs in the floral patterning

After completion of the floral transition, different floral organ identity genes are activated by floral meristem identity genes, and thus convert FMs into floral organ primordia. The formation of floral organs relies on the effects of floral organ identity genes, and the establishment of boundaries. To date, two miRNAs are known to be involved in floral organ formation. MiR172 controls the inner
whorl organ formation by restricting the expression of the AP2 gene, whereas the miR164 gene targets NAM-related genes to establish boundaries between whorls of organs, and also between organs within whorls, which define the sizes of the primordia and the resulting floral organs.

The role of miR172 in perianth identity in Arabidopsis

The floral organ primordia occur in successive whorls in most dicotyledonous species. In Arabidopsis, these comprise a whorl of four sepals primordia, a whorl of four petals primordia, followed by a whorl of six stamens primordia, and finally two carpel primordia. The activation of ABC model genes specifies floral organ identity (Causier et al., 2010).

AP2 is an A-class gene that is well known for its role in specifying perianth development through interacting with other A- or B-class genes, and acts mutually antagonistic with the C-class gene AGAMOUS (AG) (Jofuku et al., 1994). It is puzzling that AP2 transcripts were reported to accumulate throughout all of the floral whors, rather than being limited to the outer two whors, and this is not consistent with the role of AP2 in specifying perianth identity (Jofuku et al., 1994). However, it has recently been reported that AP2 expression is actually restricted to the outer two whors (Chen, 2004; Zhao et al., 2007; Wollmann et al., 2010). The key regulator for this event is referred to miR172, which repress AP2 expression at the post-transcriptional level (Chen, 2004).

In situ hybridization experiments show that miR172 and AP2 expression are largely complementary, with miR172 in the inner floral whors and AP2 in the outer floral whors (Fig. 1b1) (Chen, 2004; Zhao et al., 2007; Wollmann et al., 2010). Therefore the function of AP2 protein is restricted to the outer whors and it specifies the formation of the primordia that give rise to sepal and petals. However, there is transient overlap between miR172 and AP2 in second and third whorl primordia from stage 3 onwards. It would appear that miR172 is not sufficient to fully restrict AP2 activity, and thereby specifying the boundary between perianth and reproductive organs (Wollmann et al., 2010).

Two upstream regulators of miR172, LELNING (LLUG) and SEUSS (SEU), were identified recently. These proteins bind to the AG cis-regulatory elements to repress AG transcription in the two outer whors (Franks et al., 2002; Sridhar et al., 2004). The SEU-LLUG corepressor complex also negatively regulates miR172 expression in the outer whors, cooperating with AP2 (Grigorova1 et al., 2011).

The role of miR172 in the development of lodicules in monocotyledonous species

Lodicules in the grasses appear to be structurally homologous to petals in Arabidopsis (Bommert et al., 2005). Lodicules are small structures at the base of the carpels that act to open the palea and lemma for anthesis. The involvement of miR172 in the development of lodicule was reported from rice and barley (Fig. 1b2).

Overexpression of miR172 in rice increases the number of lodicules and results in the enlargement of lodicules, which prevent the closing of spikelets after flowering (Zhu et al., 2009). The SUPERNUMBRARY BRACT (SNB) and Oryza sativa INDETERMINATE SPIKELET1 (OsIDS1) genes are involved in the development of lodicules. They are homologous to AP2 and are targeted by miR172. The snb or ids1 single mutant, as well as their double mutants, have abnormal lodicules (Lee and An, 2012; Lee et al., 2007a, 2007b). SNB and IDS1 might regulate the development of lodicules by either activating lodicule-specifying genes or repressing lemma/palea-specifying genes (Lee and An, 2012).

In barley, CLEISTOGAMY1 (Cly1) is an AP2 transcription factor gene regulated by miR172 (Nair et al., 2010). Mutation within the miR172 binding site of Cly1 causes defective lodicule development, which prevents the lemma and palea from opening owing to the failure of lodicules to swell, which in turn causes self-pollination (Nair et al., 2010).

Establishment of boundaries by miR164

The NO APICAL MERISTEM (NAM)-related genes encode members of the plant-specific NAC family of transcription factors, which help to define morphogenetic boundaries (Aida et al., 1997; Souer et al., 1996). The Arabidopsis genes CUC1, CUC2, and CUC3 (Takada et al., 2001; Vroemen et al., 2003) belong to the NAM gene family, and are involved in the establishment and maintenance of the shoot apical and axillary meristem during floral development to control floral organ formation (Aida and Tasaka, 2006). The CUC1 and CUC2 genes are targets of miR164 (Larue et al., 2009; Laufs et al., 2004; Mallory et al., 2004; Sieber et al., 2007), whereas CUC3 is not (Vialette-Guiraud et al., 2011). Reduced expression of the CUC1 and CUC2 genes causes abnormal cellular proliferation within the boundaries of sepal primordia during the early phases of the sepal boundary development. In general, the enlargement of the sepal boundary causes defective flower phenotypes, such as the fusion of sepal and reductions of petals (Laufs et al., 2004). MiR164c can act independently to control the petal number by regulating the accumulations of CUC1 and CUC2 transcripts at the boundaries between petal primordia (Baker et al., 2005). RABBIT EARS (RBE), which encodes a C2H2 zinc finger transcriptional repressor, was recently identified as an upstream of regulator of miR164 that controls floral organogenesis (Huang et al., 2012).

Besides their roles in sepal and petal boundary development, miR164 and its targets are thought to be involved in carpel fusion (Jasinski et al., 2010; Larue et al., 2009; Nahar et al., 2012; Sieber et al., 2007). Carpel fusion is an important event in the evolution of angiosperms. MiR164 mutants show some defects in carpel fusion in Arabidopsis (Baker et al., 2005; Sieber et al., 2007). The ANA grade angiosperm, including Amborellales, Nymphaeales and Austrobaileyales, represents the three earliest lineages of extant angiosperm (Bremer et al., 2009). Jasinski et al. (2010) have shown that miR164 and CUC genes are associated with carpel closure in the ANA grade species Amborella trichopoda and Cabomba aquatica, indicating a potential mechanism for the initial evolution of closed carpels in early flowering plants.

The NAC-domain genes of many dicotyledonous species contain a potential miR164-binding site. Most Petunia nam mutants lack the SAM and die at the seedling stage (Souer et al., 1996). Antirrhinum Cupuliformis (CUP) is homologous to the Petunia NAM and Arabidopsis CUC proteins. The CUP mutants are defective in the SAM formation (Weir et al., 2004). In tomato, the NAM ortholog GOBLET and miR164 are expressed in complementary domains in the SAM (Berger et al., 2009). Moreover, the NAM-related genes from some monocotyledonous species (e.g., rice and palm) and gymnosperm species also possess a miR164-binding site (Adam et al., 2011; Larsson et al., 2012; Li et al., 2010). The homologous NAM gene from oil palm (Elaeis guineensis) is EgNAM1. The observation that transgenic 35S-EgNAM1 Arabidopsis plants have similar leaf and inflorescence phenotypes to those of plants that overexpress CUC2 indicates that the NAM-related genes of palm are functionally equivalent to AtCUC2 (Adam et al., 2011). Moreover, the observation that the expression of PaNAC01, a CUC-like homolog in Picea, increases dramatically as early embryos start to differentiate (Larsson et al., 2012), indicates that miR164-dependent regulation of NAM-related genes is associated with SAM differentiation in gymnosperms.

The conservation of the miR164 and NAM-related gene expression patterns observed in dicotyledonous, monocotyledonous, and gymnosperm species suggests that the regulation of organ
boundary establishment and SAM differentiation is likely to be evolutionarily conserved throughout seed plants. MiR164 and their target genes are identified in some species that occupy key phylogenetic positions, and the phylogeny of the miR164 family indicates that miRNAs and their targets may play key roles in the evolution of carpel closure (Jasinski et al., 2010; Vialette-Guiraud et al., 2011).

**The role of miRNAs in the development of floral organs**

The evolutionary conserved miRNAs miR159, miR160, miR166/165, miR167, miR169, and miR319 regulate the development of floral organs, including the growth and differentiation of sepal petals, anthers, and carpels (Fig. 1c1 and c2).

**MiR159 regulates anther development**

Angiosperms share a conserved program that directs anther development. MiR159 and its targets the GAMYB-related genes are required for normal anther development in Arabidopsis, rice, and barley (Achard et al., 2004).

GAMYB was first identified as a positive regulator of GA signaling in barley aleurone cells during seed development (Gubler et al., 1999). Subsequent studies revealed that GAMYB also functions in flower development (Murray et al., 2003). In barley, overexpression of HvGAMYB results in decreased anther length and causes male sterility (Murray et al., 2003). GAMYB genes were also identified in other species, such as Arabidopsis, rice, Avena sativa, and Lolium temulentum (Achard et al., 2004; Tsuji et al., 2006; Woodger et al., 2003). The expression and function of GAMYB genes appear to be highly conserved (Achard et al., 2004; Kaneko et al., 2004; Millar and Gubler, 2005). The GAMYB and GAMYB-like genes from Arabidopsis, rice, A. sativa, and L. temulentum share a conserved miR159-binding site (Achard et al., 2004; Millar and Gubler, 2005; Tsuji et al., 2006).

**Arabidopsis MYB33 and MYB65 belong to the GAMYB-like family.** The expression of AtMYB33 is restricted to young anthers. Tapetum hypertrophy and pollen abortion are found in the Arabidopsis myb33 myb65 double mutants (Millar and Gubler, 2005). Overexpressing miR159 decreases levels of AtMYB33, and causes anther defects, male sterility, and delayed flowering (Achard et al., 2004; Schwab et al., 2005). Alonso-Peral et al. (2010) have found that miR159 acts as a molecular switch that restricts the expression of MYB33 and MYB65 to anthers.

In rice, three genes that encode GAMYB-like transcription factors were found to contain a miR159-binding site (Tsuji et al., 2006). As in Arabidopsis, OsGAMYB expression is also anther-specific and is negatively correlated with miR159 expression (Aya et al., 2009; Tsuji et al., 2006). Loss-of-function mutations of OsGAMYB caused defects in anthers and pollen (Kaneko et al., 2004). The expression of OsGAMYB::GUS in both the tapetum and young microspores suggests that GAMYB may also directly regulate microspore development (Aya et al., 2009). More recently, the interaction between miR159 and GAMYB was reported in strawberry, where their functions correlate with receptacle development (Csukasi et al., 2012).

Strong similarities in anther development between Arabidopsis, rice, and barley, and the homologies between the transcription factors that regulate the process in different species, suggest the existence of a conserved anther developmental program within angiosperms.

**MiR166/165 regulates the SAM and floral organ polarity**

The miR166/165 group includes two miRNAs, miR166 and miR165. Both miRNAs were identified in Arabidopsis, but only miR166 has been identified in other species (Jones-Rhoades and Bartel, 2004). Mature miR165 and miR166 sequences in Arabidopsis differ by only a single nucleotide (Reinhart et al., 2002), and both miR166 and miR165 target the same set of the HD-ZIP III genes that perform the same functions (Zhou et al., 2007). MiR166/165 targets the HD-ZIP III transcription factor genes ATHB15, ATHB8, REVOLUTA (REV), PHABULOSA (PHB), and PHAVOLUTA (PHV) (Floyd and Bowman, 2004). Comprehensive genetic studies in Arabidopsis have shown miR166/165 and their targets play an important role in shoot apical and lateral meristem formation, organ polarity, and vascular development (Kim et al., 2005). MiR166-mediated repression of ATHB15 may have a role in apical meristem formation as well as in vascular development in inflorescence stems. Overexpression of miR166 or miR165 resulted in reduced floral organs. The gain-of-function miR166a mutant *meristem enlarged1* (men1) and a miR166 activation-tagged mutant *jабba-1D* (*jabba-1D*) have substituted inflorescence stems, drastically reduced levels of *ATHB15* compared with wild-type plants, and defective vascular differentiation and radial patterning (Kim et al., 2005; Williams et al., 2005). Overexpression of miR165 reduces levels of all five HD-ZIP III genes, and causes developmental defects in the SAM (Zhou et al., 2007). The HD-ZIP III family genes are also required for organ polarity. The REV gene is expressed in developing vascular tissues and adaxial cells of floral organs, the FM, and at presumptive FM initiation sites (Pridge et al., 2005). MiR166/165 genes regulate SAM formation and floral development by acting in parallel with the WUSCHEL (WUS)–CLAVATA (CLV) pathway (Jung and Park, 2007). The individual MiR166/165 gene expression showed distinct temporal and spatial expression patterns in different plant organs, suggesting that miR166/165 might differentially regulate target genes during plant development (Jung and Park, 2007).

Recent studies have shown miR166/165 regulate HD-ZIP III transcription through ARGOUSA (AGO10) proteins (Liu et al., 2009a, 2009b; Zhu et al., 2011). AGO10 modulates the SAM maintenance and the establishment of organ polarity by genetic repression of miR165/166 expression (Liu et al., 2009a, 2009b). The prolonged floral meristem maintenance associated with the ago10 loss-of-function mutation suggests that AGO10 competes with AGO1 for binding to miR166/165 (Zhu et al., 2011). AGO10 and miR166/165 are also involved in floral stem cell termination. The reduction of the HD-ZIP III genes by rendering them resistant to miR166/165 can prolong the activity of floral stem cell (Ji et al., 2011).

Downregulation of the HD-ZIP III genes of rice and maize by miR166 affects leaf polarity and defects in the embryonic SAM (Nagasaki et al., 2007; Nogueira et al., 2007). There are three copies of miR166 genes but no miR165 in the lycoperd Selaginella (Axtell et al., 2007). The homologous HD-ZIP III genes in *Selaginella* are expressed adjacent to the shoot apical cell region (Pridge and Clark, 2006).

The miR166/165 complementary sequence is highly conserved in mRNAs of HD-ZIP III genes and their homologs from dicotyledons, monocotyledons, and moss species (Floyd and Bowman, 2004; Pridge and Clark, 2006). It is therefore likely that miR166-mediated HD-ZIP III gene repression is highly conserved in all land plants.

**MiR167 and miR160 regulate ARFs to control the formation of floral organs**

ARFs regulate the expression of a large set of auxin-responsive genes by binding to the auxin-response elements in their promoters (Guilfoyle and Hagen, 2007; Mockaitis and Estelle, 2008). Some ARF proteins have been shown to regulate floral organ formation and to be targeted by miRNAs (Chapman and Estelle,
2009; Mallory et al., 2005; Wu et al., 2006a, 2006b). Of the known ARF genes, ARF6 and ARF8 are targeted by miR167, whereas ARF10, ARF16, and ARF17 are targeted by miR160 (Mallory et al., 2005; Wu et al., 2006a, 2006b).

ARF6 and ARF8 are essential for both ovule and anther development (Nagpal et al., 2005; Wu et al., 2006a, 2006b). Flowers of arf6 arf8 double loss-of-function mutants show some defects in anther and gynoecium development, such as short stamens, anthers indehiscence, and defective ovule integuments (Ru et al., 2006; Wu et al., 2006a, 2006b; Yang et al., 2006). Overexpression of miR167 causes defects in anther dehiscence and failure to release pollen owing to reduced levels of ARF6 and ARF8 transcripts. These results suggest that the downregulation of ARF6/ARF8 by miR167 is critical for pollen development. In rice, miR167 accumulates to high levels during the late stage of anther development, indicating its role in regulating anther development is conserved (Fujikoa et al., 2008).

Several studies have shown that miR160 negatively regulates ARF10, ARF16, and ARF17 (Mallory et al., 2005; Liu et al., 2010). A loss-of-function mutant floral organs in carpel (foc) with a Ds transposon insertion in the 3′ regulatory region of miR160, exhibits some defects of flowering, such as the formation of irregularly shaped flowers and floral organs inside siliques, as well as reduced fertility (Mallory et al., 2005; Liu et al., 2010). In the foc mutant, ARF10, ARF16, and ARF17 transcripts are all present at levels higher than those in wild-type plants owing to reduced expression of miR160.

MiR169 regulates the C-class gene to control the development of reproductive organs

The expression of C-class genes is repressed by miR169. Cartolano et al. (2007) demonstrated that FISTULATA (FIS) in Antirrhinum majus and BLIND (BL) in Petunia hybrida encode miR169. FIS and BL restrict C-class gene activity to the inner two floral whorls to specify the identities of the reproductive organs in the flower. Loss-of-function mutants of FIS and BL produce stamenoid petals in their second whorls, which is indicative of abnormal C function in the second whorl. Nonetheless, there is no miR169 target site in C-class genes. MiR169 targets members of the NF-YA transcription factor gene family (Davies et al., 1999; Jones-Rhoades and Bartel, 2004). Given that NF-YA transcription factors can activate C-class genes, miR169 are anticipated to repress the expression of C-class genes by post-transcriptional repression of NF-YA members. Although a miR169/NF-YA module exists in Arabidopsis, the function of restricting class C gene expression has not been detected (Cartolano et al., 2007).

MiR319 regulates petal development

It is interesting that miR159 and miR319 share 17 identical nucleotides in Arabidopsis, and that the two miRNAs evolved from a common ancestor (Li et al., 2011). However, given that miR159 and miR319 have distinct expression patterns and target genes, they have different functions (Palatnik et al., 2007). As mentioned above, miR159 targets several GAMYB transcription factor genes involved in floral initiation and anther development, whereas miR319 targets a subset of TCP transcription factor genes that control leaf and flower growth (Nag et al., 2009; Palatnik et al., 2007). A loss-of-function mutant of miR319 in Arabidopsis showed some defects in petal and stamen development, such as narrower and shorter petals and impaired anther formation (Nag et al., 2009). Plants with high TCP activity also suffer impaired development of floral organs in Arabidopsis (Koyama et al., 2007; Nag et al., 2009; Sarvepalli and Nath, 2011).

Conclusions and future perspectives

This review summarizes recent insights into the essential roles that miRNAs play in flower development. Nine highly conserved miRNA families have been implicated in regulating flower development. Most show considerable conservation among all most land plants at the levels of sequence conservation and target identities. Analysis of the level of conservation of miRNA-regulated systems in a variety of plant species reveals that miRNAs and their targets affect a broad spectrum of flower developmental programs, and that their regulatory modules are also broadly conserved across all angiosperms (Fig. 1). MiRNAs and their targets are key components in complex networks that control flower development. The interactions between miRNAs, their targets, regulatory factors that act upstream of miRNAs, and other genes involved in flower development have been extensively characterized in Arabidopsis, rice, and maize. However, the conserved miRNA-target modules that are believed to control flowering require further investigation in other species. Several questions about the evolution of these miRNAs remain to be established. The origin(s) and evolution of miRNA-target regulatory systems are not understood, and even less is known about the phylogenetic aspects of miRNA functions, the evolutionary relationships between miRNAs and their targets, and the factors that drive the co-evolution of miRNAs and their targets.

We anticipate that future studies will identify more miRNAs and their targets in non-model species, especially those that belong to key evolutionary lineages that include basal angiosperms, gymnosperms, ferns and mosses. Moreover, it is important to examine the molecular mechanisms that support the regulatory roles of miRNAs, and to obtain a more comprehensive understanding of the evolution of miRNA-mediated regulatory pathways. Research in this area will undoubtedly help us to unravel the evolutionary history and level of conservation of the miRNA-target pathways that regulate growth and development.

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Appendix A. Supporting information

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