MicroRNAs (miRNAs) and Plant Development

Wang-Xia Wang, *University of Kentucky, Lexington, Kentucky, USA*
Bobby Gaffney, *University of Kentucky, Lexington, Kentucky, USA*
Arthur G. Hunt, *University of Kentucky, Lexington, Kentucky, USA*
Guiliang Tang, *University of Kentucky, Lexington, Kentucky, USA*

microRNAs (miRNAs) are a novel class of 21–24 nucleotide, endogenous, noncoding small ribonucleic acids (RNAs) that play critical roles in diverse biological processes such as: development, cellular differentiation, cell-cycle control, apoptosis and oncogenesis. In plants, roles of miRNAs in development have been extensively investigated in recent years. This review highlights and discusses the current knowledge about the roles of microRNAs in leaf development, patterning and polarity, floral identity and flower development, developmental phase transition and flowering time, shoot and root development, vascular development and hormone signalling for plant development.

Advanced article

Article Contents

- Introduction
- miRNA in Leaf Development, Patterning and Polarity
- miRNA in Floral Identity and Flower Development
- miRNA in Developmental Phase Transition and the Control of Flowering Time
- miRNA in Shoot and Root Development
- miRNA in Vascular Development
- miRNAs in Hormone Signalling for Plant Development
- miRNA Biogenesis and Plant Development
- Conclusion
- Acknowledgements

doi: 10.1002/9780470015902.a0020106

Introduction

microRNAs (miRNAs) are a class of 21–24 nucleotide, endogenous noncoding small ribonucleic acids (RNAs) (Bartel, 2004). It was not until 8 years after the discovery of miRNA that regulatory functions for miRNA were elucidated (Reinhart *et al*., 2002; Ambros *et al*., 2003). Since then, numerous studies have significantly expanded our knowledge of the roles of miRNAs in biology and physiology. As of October 2006, the sequences of 4361 miRNAs have been deposited in the miRNA database (miRbase, release 9.0; http://microrna.sanger.ac.uk/sequences/index.shtml). These miRNAs include 863 plant miRNAs that belong to 42 miRNA families. The list is still expanding as a result of both intensive cloning and computational prediction approaches. Many miRNA families are evolutionarily conserved, while nonconserved miRNA families also exist and are widely spread across different species. The expression of many miRNAs is altered in specific environmental conditions.

Mature miRNAs are derived from stem-loop regions of RNA precursors by Dicer enzymes of the RNAase III family. Mature miRNAs initially exist as double stranded duplex RNA molecules and activate the RNA interference machinery by being recruited to a large protein complex, the RNA-induced silencing complex (RISC). One strand of the miRNA duplex is incorporated into RISC and guides the targeting of mRNA in a sequence-specific manner. miRNAs negatively control gene expression by promoting the cleavage of a target transcript or by the suppression of translation of a target mRNA.

It is now known that miRNAs play critical roles in diverse biological process such as development, cellular differentiation, cell-cycle control, apoptosis and oncogenesis. As in other organisms, in plants miRNAs play crucial roles in various stages of development and maintenance of organ identity (Jones-Rhoades *et al*., 2006). They are involved in many other biological processes such as regulation of miRNA and trans-acting siRNA (ta-siRNA) biogenesis, signal transduction and responses to environmental stress and pathogen invasion.

Plant development is a highly regulated process that is controlled at many levels. The discovery of miRNAs and their roles in plant development has opened a new frontier to further understand the mechanisms underlying this regulation. Major classes of miRNA-targeted genes include those that encode transcription factors and F-box (a motif that was first identified in cyclin F) proteins, which constitute major plant developmental regulatory networks. These transcription factors and F-box proteins regulate numerous plant developmental processes (**Table 1**) including but not limited to: leaf development, patterning and polarity, floral identity and flower development, flowering time, developmental phase transition, shoot and root development, vascular and plastid development and hormone signalling for plant development (**Figure 1**). This review highlights and discusses the current knowledge of the regulatory role of miRNAs in plant development.

miRNA in Leaf Development, Patterning and Polarity

Leaf development, patterning and polarity are controlled by many transcription factors, including leucine zipper family, TCP family and MYB family. Asymmetry is a hallmark of plant leaf structure. The adaxial (upper face) and abaxial (lower face) pattern formation is determined by a group of class-III homeodomain leucine zipper (HD-ZIP) transcription factors. Three closely related *Arabidopsis* HD-ZIP genes, *phabulosa* (*phb*), *phavoluta* (*phv*) and *revoluta* (*rev*),

Table 1 miRNAs and their regulatory roles in plant development

Development events	MiRNA	miRNA targets	Reference
Leaf development, patterning and polarity	miR165/166	HD-ZIP TFs: PHB, PHV, REV	(Juarez *et al*., 2004; Mallory *et al*., 2004b; Zhong and Ye, 2004)
	miR164a	NAC-containing TF: CUC2	(Nikovics *et al*., 2006)
	miR319/JAW	BHLH TFs: TCP2, TCP3, TCP4, TCP10, TCP24	(Palatnik *et al*., 2003)
	miR159	MYB TFs: MYB33, MYB65	(Millar and Gubler, 2005; Palatnik *et al*., 2003)
Floral identity and flower development	miR172	APETALA2-like TFs: AP2, TOE1, TOE2, TOE3	(Aukerman and Sakai, 2003; Chen, 2004; Mlotshwa *et al*., 2006; Schwab *et al*., 2005)
	miR164c	NAC-containing TF: CUC1, CUC2	(Baker *et al*., 2005)
	miR159	MYB TFs: GAMYB, MYB33, MYB65	(Achard *et al*., 2004; Millar and Gubler, 2005; Schwab *et al*., 2005; Tsuji *et al*., 2006)
Flowering time	miR159	MYB TFs: GAMYB	(Achard *et al*., 2004; Schwab *et al*., 2005)
	miR172	APETALA2-like TFs: AP2, TOE1, TOE2, TOE3	(Aukerman and Sakai, 2003; Chen, 2004; Mlotshwa *et al*., 2006; Schwab *et al*., 2005)
	miR156	SBP-like TFs: SPL3	(Schwab *et al*., 2005)
	miR171	SCL TFs: SCL6-II, SCL6-III, SCL-IV	(Llave *et al*., 2002; Reinhart *et al*., 2002)
Developmental phase transition	miR172	APETALA2-like TFs: GL15	(Lauter *et al*., 2005)
	miR156	SBP-like TFs: SPL3, SPL4, SPL5	(Luo *et al*., 2006; Schwab *et al*., 2005; Wu and Poethig, 2006)
Shoot and root development	miR164	NAC-containing TF: CUC1, CUC2, NAC1	(Guo *et al*., 2005; Laufs *et al*., 2004; Mallory *et al*., 2004a; Rhoades *et al*., 2002; Schwab *et al*., 2005)
Vascular and plastid development	miR166	HD-ZIP TFs: ATHB15	(Kim *et al*., 2005; Ochando *et al*., 2006; Rhoades *et al*., 2002; Williams *et al*., 2005)
Hormone signalling for plant development	miR159	MYB TFs: GAMYB	(Achard *et al*., 2004; Schwab *et al*., 2005)
	miR160	ARF TFs: AFR10, AFR16, AFR17	(Mallory *et al*., 2005; Rhoades *et al*., 2002; Wang *et al*., 2005)
	miR167	ARF TFs: AFR6, AFR8	(Rhoades *et al*., 2002; Ru *et al*., 2006; Wu *et al*., 2006)
	miR164	NAC-containing TF: NAC1	(Guo *et al*., 2005)
	miR393	F-box protein: TIR1	(Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004)

have been found to regulate leaf patterning. Dominant mutations of any of these transcription factors result in abnormal adaxialized and radicalized leaf patterning (Emery *et al*., 2003; McConnell *et al*., 2001). Using computational approaches, miR165/miR166 have been implicated in the functioning of these transcription factors. In *phb-d*, *phv-d* and *rev-d* mutants, the mutations are found to be in the region of miR165/miR166-complementary sites and decrease the efficiency of miRNA-directed cleavage *in vitro* (Tang *et al*., 2003). Subsequent studies confirmed that miR165/miR166 target these transcription factors and are therefore involved in the regulation of leaf patterning (Juarez *et al*., 2004; Mallory *et al*., 2004b; Zhong and Ye, 2004). More recently, another member of class III HD-ZIP transcription factors, INCURVATA4/ATHB15/CORONA, was found to participate in the control of leaf polarity, as well as in shoot and root apical meristem patterning and stem vascular differentiation (Ochando *et al*., 2006; Prigge *et al*., 2005; Williams *et al*., 2005). Gain-of-function *icu4* mutants bear the same nucleotide substitution similar to *phabulosa*, *phavoluta* and *revoluta* mutants. This mutation affects the miR165/166 complementarity in target mRNAs, and consequently, resulting in impaired cleavage of the mutant transcripts. Overexpression of miR166 g in *jba*-1D mutants, in which miR166 g is activated by 35S promoter, causes morphological defects in shoot apical meristems, stem vasculature, rosette leaves and gynoecia. Importantly, like their target genes (HD-ZIP), the miR165 and

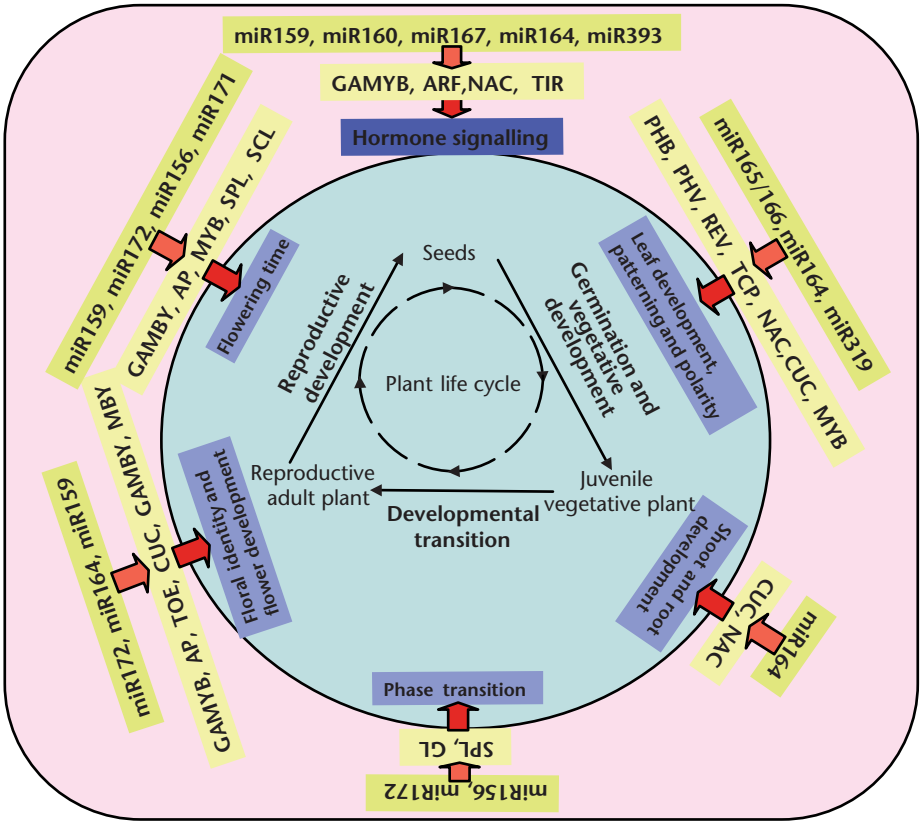

Figure 1 miRNAs regulate various aspects of plant development. miRNAs regulate plant development via their posttranscriptional control on the expression of many transcription factors and F-box proteins. This control that fine-tunes the regulatory network of plant development is an important level of regulation, which ensures a proper development of a plant.

miR166 gene families are evolutionarily conserved in land plants. It is likely that the role of miR165/miR166 in leaf development is conserved in all land plants.

A recent study revealed that miR164 is another regulator of leaf patterning (Nikovics *et al.*, 2006). These authors showed that the balance between the miR164a and the transcription factor CUP-SHAPED COTYLEDON2 (CUC2) gene controls leaf margin serration in *Arabidopsis*. Although the pattern of serration is determined first independently of CUC2 and miR164, the balance between coexpressed CUC2 and miR164a then determines the extent of leaf serration.

The regulatory role of miR319 (also called JAW) in leaf development has come to light in a developmental mutant screen in *Arabidopsis thaliana*. A dominant *jaw-d* mutant exhibits a phenotype of uneven leaf shape and curvature. In these mutant plants, the expression of the miR319a/JAW gene is elevated. In addition, these plants show a reduced accumulation of five TCP mRNAs, each of which has complementary sites to miR319 (Palatnik *et al.*, 2003). Furthermore, overexpression of wild-type and miRNA-resistant TCP variants demonstrated that miRNA-guided TCP mRNA cleavage is largely sufficient to restrict TCP function to its normal domain of activity. As with HD-ZIP genes, TCP genes and the miR319/JAW family are found in a wide range of plant species, suggesting that miRNA-mediated control of leaf morphogenesis is conserved across different plant species with very different leaf forms (Palatnik *et al.*, 2003). miR159 shares sequence similarity with miR319/JAW. The targets of miR159 include two members of MYB transcription factors, MYB33 and MYB65. Palatnik *et al.* reported that overexpression of a miRNA-resistant version of MYB33 in *Arabidopsis* causes leaves to curl upwards (Palatnik *et al.*, 2003). Similarly, *Arabidopsis* transformed with MYB33 containing the mutated miRNA target site show dramatic pleiotrophic developmental defects, including abnormal leaves that were more rounded and upturned at the sides (Millar and Gubler, 2005).

miRNA in Floral Identity and Flower Development

The reproductive phase and formation of flowers is of vast importance in the plant life cycle. The development of the floral organ may be described by the so-called ABC model. In this model, the origination of the four whorls (sepal, petal, stamen and carpel) of a flower is controlled by three classes of transcription factors: A, B and C. The combination and

interaction of different classes of transcription factors play essential roles in floral identity and flower timing. Class A genes are required in sepal and petal development, class B genes are required in petal and stamen development and class C genes are required for stamen and carpel development. During the formation of a flower, the products of these gene classes interact combinatorially or cadastrally to produce the four whorls of a mature flower. For example, class A gene products repress expression of class C genes in whorls 1 and 2, while class C gene products repress expression of class A genes in whorls 3 and 4. Members of these gene classes have been demonstrated to be regulated by miRNAs. *apetala2 (ap2)*, a class A gene, is a target of miR172. Overexpression of miR172 in *Arabidopsis* causes early flowering and disrupts the specification of floral organ identity (Aukerman and Sakai, 2003). Overexpression of an *ap2*-like target of miR172, TARGET OF EAT1 (TOE1), causes late flowering. This result, in conjunction with loss-of-function analyses of TOE1 and another miR172 target gene, TOE2, indicates that at least some of the *ap2*-like genes targeted by miR172 normally function as floral repressors. The role of miR172 in floral identity and flowering has also been demonstrated in *Nicotiana benthamiana* (Mlotshwa *et al*., 2006). These authors showed that the expression of the *Arabidopsis ap2* gene is repressed at the mRNA level by an endogenous tobacco miR172 homologue. Consistent with the role of miR172 in *Arabidopsis*, transgenic *N. benthamiana* lines over-expressing *Arabidopsis* miR172a-1 also exhibit early flowering. It was shown that miR172 acts as a translational repressor rather than through mRNA cleavage (Aukerman and Sakai, 2003; Chen, 2004), but more recent work (Schwab *et al*., 2005) revealed that miR172 can also function through RNA cleavage, and that the miR172 target gene may subject to autoregulation to maintain a stable level of *ap2* transcripts.

The predominant phenotype of *Arabidopsis* mutant *eep1* (early extra petals 1) is the formation of extra petals in early-arising flowers (Baker *et al*., 2005). It was demonstrated that *eep1* is a loss-of-function allele of miR164c, one of the three known members of the miR164 family. The other two members of the miR164 family are miR164a and miR164b, which negatively regulate several members of the NAC family of transcription factors that are required for plant organ formation and boundary establishment (Jones-Rhoades and Bartel, 2004; Reinhart *et al*., 2002; Rhoades *et al*., 2002). Analyses of miR164c function and *eep1*/ *mir164b* double mutants revealed that miR164c controls petal number in a nonredundant manner by regulating the accumulation of mRNAs encoding the transcription factors CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 (Baker *et al*., 2005). The results of this study suggest that closely related miRNA family members that are predicted to target the same set of genes can have different functions in plant development.

The gibberellin-modulated MYB transcriptional activator GAMYB is a major modulator of gibberellin (GA) signalling. It has been recently shown that GAMYB has an important role in flower development (Achard *et al*., 2004; Millar and Gubler, 2005). Barley (*Hordeum vulgare*) GAMYB (HvGAMYB) was first identified in barley aleurone cells and was found to be upregulated by GA. In transgenic barley, increasing levels of HvGAMYB result in a progressive decrease in anther size and a lightening of colour. Overexpression of HvGAMYB causes male sterility. Depletion of rice GAMYB (OsGAMYB) results in defects in internode elongation and floral organ development, especially in anther and pollen. *Arabidopsis* GAMYB-like genes *atmyb*33 and *atmyb*65 are expressed in flowers and are regulated by GA. Double mutants deficient in both of these genes display conditional male sterility (Millar and Gubler, 2005). Several studies suggest that expression of *gamyb* genes are regulated by miR159. Thus, overexpression of miR159 in *Arabidopsis* results in a decreased level of expression of *gamyb*-like genes as well as anther defects, male sterility and delays in flowering (Achard *et al*., 2004; Schwab *et al*., 2005). More recently, Tsuji *et al*. reported that rice *gamyb* and *gamyb*-like genes are coexpressed with miR159 in anthers (Tsuji *et al*., 2006), and the mRNA levels for *gamyb* and *gamyb*-like genes are inversely correlated with miR159 levels during anther development. Thus, *osgamyb* and *osgamyb*-like genes are regulated by miR159 in flowers.

miR171 is complementary to an internal region of three *scarecrow*-like (*scl*) mRNAs, which encode a family of putative transcription factors (Reinhart *et al*., 2002). miR171 directs specific cleavage of the SCL target mRNAs. miR171 accumulates to relatively high levels in inflorescence and flower tissues, and to lower or non-detectable levels in leaf and stem tissue of *Arabidopsis*, rice and *N. benthamiana* (Llave *et al*., 2002). This suggests that miR171 may have a role in floral development.

miRNA in Developmental Phase Transition and the Control of Flowering Time

In contrast to animals, plant postembryonic development is highly plastic. Most plants undergo three major developmental transitions during their life cycle: germination, vegetative phase change and floral transition. All of these developmental transitions are regulated by external environmental signals such as light, temperature and nutrients, as well as by endogenous signals transmitted by plant regulators. Accumulating data indicate that miRNAs and other endogenous small RNAs have regulatory roles in developmental transitions in plants.

The shoot apex houses the growing point of the plant, the shoot apical meristem. During plant development, the shoot apex progresses through juvenile and adult phases of vegetative development before floral induction, and afterwards undergoes floral transition prior to flower development. Several miRNAs have been demonstrated to affect the phase transitions.

miR156 targets members of the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) family, which encodes a class of plant-specific transcription

factors. Ten of the 16 *spl* genes in *Arabidopsis* have target sites for miR156. All of these genes were specifically down-regulated in plants that constitutively over-express miR156b (Schwab *et al*., 2005). Consequently, the phase transition from vegetative phase to reproductive phase was affected, resulting in severe decreases of apical dominance, accelerating initiation of rosette leaves and a delay in flowering. Wu and Poethig recently showed that three closely related members of *spl* gene, *spl3*, *spl4* and *spl5*, have overlapping functions in the regulation of vegetative phase change and floral induction in *Arabidopsis*, thus, demonstrating that miR156 is responsible for the temporal change in *spl3* expression during vegetative development (Wu and Poethig, 2006). As discussed in the previous section, miR172 targets AP2 transcription factors. In maize, miR172 targets *glossy15 (gl15)*, an *ap2*-like gene, which is usually expressed in maize juvenile leaves. Overexpression of miR172 decreases the levels of *gl15* mRNAs, resulting in a delayed phase change from the vegetative to the reproductive stage (Lauter *et al*., 2005).

Over the last decade, genetic and molecular analyses have revealed several signalling pathways that control flowering time. Activation of these pathways is determined by either environmental conditions such as light and temperature, or metabolic state and plant growth regulators. These various pathways converge on a small set of genes that function as pathway integrators. These genes are *flowering locus t (ft), suppressor of overexpression of co 1 (soc1) and leafy (lfy)*. In *Arabidopsis*, long days activate the photoperiod pathway that is mediated by the zinc finger transcriptional factor CONSTANS (CO). Short photoperiod induces flowering in a gibberellin-dependent manner. One of the important mediators in this GA-dependent pathway is GAMYB. AP1 and LEAFY, which acts downstream of CO and GAMYB, together activate homeotic genes such as *apetala3 (ap3)* and *agamous (ag)*, which specify the identity of the different floral organs and control flower timing.

miRNAs that play roles in controlling flowering time include miR159, miR156 and miR172. miR159 guides the cleavage of mRNAs encoding GAMYB-like proteins and further affects the GA-dependent activation of LEAFY (Achard *et al*., 2004). Overexpression of miR159 leads to a late flowering phenotype which is similar to the phenotype seen in miR156 overexpressors (Schwab *et al*., 2005). Another miRNA that has a role in the control of flower timing is miR172. The early flowering mutant, *eat-D*, and the late flowering mutant, *toe1-D*, identified in an *Arabidopsis* activation tagging population, exhibit increased levels of miR172b and the miR172 target TOE1, respectively (Aukerman and Sakai, 2003). Overexpression of miR172 resulted in early flowering in transgenic *Arabidopsis* and *N. benthamiana* (Achard *et al*., 2004; Chen, 2004; Mlotshwa *et al*., 2006; Schwab *et al*., 2005).

miRNAs have been recently suggested to play regulatory roles in plant post-embryonic development (Luo *et al*., 2006). Several miRNAs show distinct changes during the transition from undifferentiated to differentiated calli. Among these miRNAs, the expression level of miR156 clearly increases in differentiated calli compared to that of undifferentiated calli. In contrast, the expression of miR397 is significantly decreased during this transition. Along with miR156 function in regulation of SBP-containing transcription factors and the possible function of miR397 in plant lignification enzyme laccase, the authors suggested that these miRNAs might be important for meristem maintenance. Direct evidence of these miRNA actions in plant post-embryonic development is needed to confirm these suggestions.

miRNA in Shoot and Root Development

Plant shoots and roots develop from the shoot apical meristems and the root apical meristems, respectively. *cuc* genes encode members of the NAC family of transcription factors and are known to be involved in shoot apical meristem formation and cotyledon separation during embryogenesis in *Arabidopsis*. Loss-of-function mutations in these genes result in abnormal floral and shoot development. Members (e.g. CUC1, CUC2 and NAC1) of the NAC family are targets of the miR164 family (Guo *et al*., 2005; Laufs *et al*., 2004; Mallory *et al*., 2004a; Schwab *et al*., 2005). Plants overexpressing miR164 show a range of developmental abnormalities similar to *nac* gene family mutants. For example, plants carrying a 35S-miR164 transgene exhibit embryonic and floral organ fusion (Mallory *et al*., 2004a; Schwab *et al*., 2005), which is similar to the *cuc1*/*cuc2* lost-of-function double mutants. These plants also show vegetative organ fusion and decreased root branching (Guo *et al*., 2005) as found in plants with reduced levels of NAC1. In contrast, loss-of-function miR164 mutants accumulate higher levels of *nac* mRNA, resulting in more lateral root formation (Guo *et al*., 2005). Moreover, miR164 also helps to control organ boundaries (Guo *et al*., 2005). Thus, in the *Arabidopsis* meristem, miR164 constrains the expansion of the boundary domain by degrading *cuc1* and *cuc2* mRNAs during organ initiation.

miRNA in Vascular Development

Plant vascular development is regulated by a variety of genetic components and growth hormones. The HD-ZIP family of transcription factors plays a role in many of these developmental processes including lateral organ polarity, apical and lateral meristem formation and vascular development. ATHB15 is one of the members of the class III HD-ZIP transcription factor family. ATHB15 is predicted to be the target of miR166 (Rhoades *et al*., 2002). Indeed, overexpressing miR166a results in a reduced level of *athb15* mRNA and leads to an abnormal vascular development phenotype (Kim *et al*., 2005). The overexpressor exhibits an altered vascular system with expanded xylem tissue and an interfascicular region, indicative of accelerated vascular cell

differentiation from cambial/procambial cells. A similar phenotype is observed in *Arabidopsis* plants with reduced *athb15* expression but was reversed in transgenic plants overexpressing a miR166-resistant *athb15*. The study reveals that miR166-mediated *athb15* mRNA cleavage is a principal mechanism for the regulation of vascular development.

The vascular system of woody plants is particularly dramatic in forming a networked system within the plants and generating wood, a valuable and renewable resource for lumber, paper and energy production. Ko *et al*. recently reported that the *Populus* class III HD-ZIP transcription factor PtaHB1 is the target of Pta-miR166 (Ko *et al*., 2006). The expression of PtaHB1 was closely associated with wood formation and was regulated both developmentally and seasonally. This observation suggests that the functions of Pta-miR166 may be associated with control of the vascular cambium function.

miRNAs in Hormone Signalling for Plant Development

Plant hormones, such as auxin, GA and ABA, play critical roles in regulating developmental processes such as embryogenesis, cell division, elongation, differentiation and organ formation. Several miRNAs have been shown to respond to plant hormones; these include miR159, miR160, miR164, miR167 and miR393 (Sunkar and Zhu, 2004; Zhang *et al*., 2005).

Auxins are growth regulators involved in virtually all aspects of plant development. Auxin signalling is initiated by the binding of auxin to its receptor, a Skp1-Cullin-F-box (SCF) E3 ligase, also termed transport inhibitor response 1 (TIR1). The binding of auxin to TIR1 promotes the interaction between TIR1 and a large family (29 members in *Arabidopsis*) of short-lived nuclear transcriptional regulators termed Auxin/Indole-3-acetic acid (AUX/IAA) proteins, leading to the degradation of these proteins by an ubiquitin-related machinery. AUX/IAA proteins are repressors of auxin response factors (ARFs); the degradation of the AUX/IAA proteins releases ARFs from this repression, permitting the formation of hetero- or homodimers that subsequently control the expression of auxin-responsive genes by binding to the auxin response elements (AREs) in the promoter region.

ARFs, TIR1 and other closely related F-box proteins are predicted or have been shown to be the targets of miRNAs in plants (Mallory *et al*., 2005; Jones-Rhoades and Bartel, 2004; Rhoades *et al*., 2002; Sunkar and Zhu, 2004). More than 20 ARFs have been identified in *Arabidopsis*, and several of these have been predicted to be targets of miRNAs (Rhoades *et al*., 2002). ARF10, ARF16 and ARF17 are predicted targets of miR160, and ARF6 and ARF8 are targets of miR167. Disruption of miR160 leads to increased levels of ARF10, ARF16 and ARF17, altered expression of auxin early responsive genes and consequently severe developmental defects (Mallory *et al*., 2005). miR160 regulates root cap formation by targeting ARF10 and ARF16, both of which control root cap cell differentiation (Wang *et al*., 2005). miR160 overexpressors, in which the expression of ARF10 and ARF16 is repressed, display the same root-tip defect as *arf10-2*/*arf16-2* double mutants. In the root distal region of these mutants, cell differentiation is blocked and a tumour-like root apex and loss of gravity-sensing is seen.

Two recent reports reveal the regulatory role of miR167 in plant reproductive development (Ru *et al*., 2006; Wu *et al*., 2006). ARF6 and ARF8 regulate gynoecium and stamen development in immature flowers. It was shown that miR167 causes the degradation of ARF8- and ARF6-encoded mRNAs (Wu *et al*., 2006; Ru *et al*., 2006). miR167 may also repress ARF6 expression at the level of translation. *Arabidopsis* plants that overexpress miR167 mimic *arf6*/*arf8* double mutant phenotypes, in which stamens of *arf6*/*arf8* flowers are short and anthers do not dehisce to release pollen. Mutations in the miR167 target sites of *arf6* or *arf8* cause ectopic expression of these genes in domains of both ovules and anthers where miR167 is normally present. As a result, ovule integuments have arrested growth, and anthers grow abnormally and fail to release pollen (Wu *et al*., 2006). Similar phenotypes are observed in plants overexpressing miR167b (Ru *et al*., 2006). In flowers overexpressing miR167b, filaments are abnormally short, anthers do not properly release pollen and pollen grains do not germinate. Moreover, there is a defective development in all four whorls of floral organs (Wu *et al*., 2006). These studies demonstrate that miR167, via its action on *arf6* and *arf8*, is an essential regulator for correct gene expression pattern, and for fertility of both ovules and anthers.

ARFs are not only the direct targets of miRNAs, but are also regulated indirectly by miRNAs through miRNA-induced trans-acting siRNAs (ta-siRNAs) (Allen *et al*., 2005; Fahlgren *et al*., 2006). In *Arabidopsis*, miR390 guides the production of ta-siRNA (TAS3) to target *arf3*/*ettin* and *arf4* mRNAs for downregulation (Allen *et al*., 2005). While the regulation by miRNAs requires general miRNA biosynthesis and function components (e.g. AGO1 and DCL1), miRNA390 regulation of ARF3 and ARF4 also requires the RNA-dependent RNA polymerase 6 (RDR6), Dicer-like 4 (DCL4) and Argonaute 7 (AGO7) (Allen *et al*., 2005; Yoshikawa *et al*., 2005). These latter characteristics reflect another complexity regulation by miRNAs in the plant developmental processes.

miRNA Biogenesis and Plant Development

Given these numerous roles for miRNAs in developmental processes, it is to be expected that impaired function of major components in miRNA biogenesis pathways should result in developmental abnormalities, and this is indeed seen to be the case (Park *et al*., 2002; Bohmert *et al*., 1998; Telfer and Poethig, 1998; Chen *et al*., 2002). For example, *dcl1*, *ago1*,

hen1 and *hyl1* mutants were first identified as a consequence of their associated developmental defects. In plants, miRNAs are processed from precursors by the Dicer-like enzyme DCL1 in conjunction with the dsRNA binding protein, HYL1. The 3′ ends of the miRNA duplex in plants are methylated by HEN1 in the nucleus. The methylated miRNA duplex is then transported by HASTY, an orthologue of Exportin 5, to the cytoplasm. The miRNA strand of miRNA duplex is preferentially loaded into the Argonaute-containing RISC and guides sequence-specific mRNA cleavage or translation repression (**Figure 2**). Impaired function or loss-of-function mutation of the components involved in miRNA biogenesis severely affect the general plant architecture, and may even be lethal to the

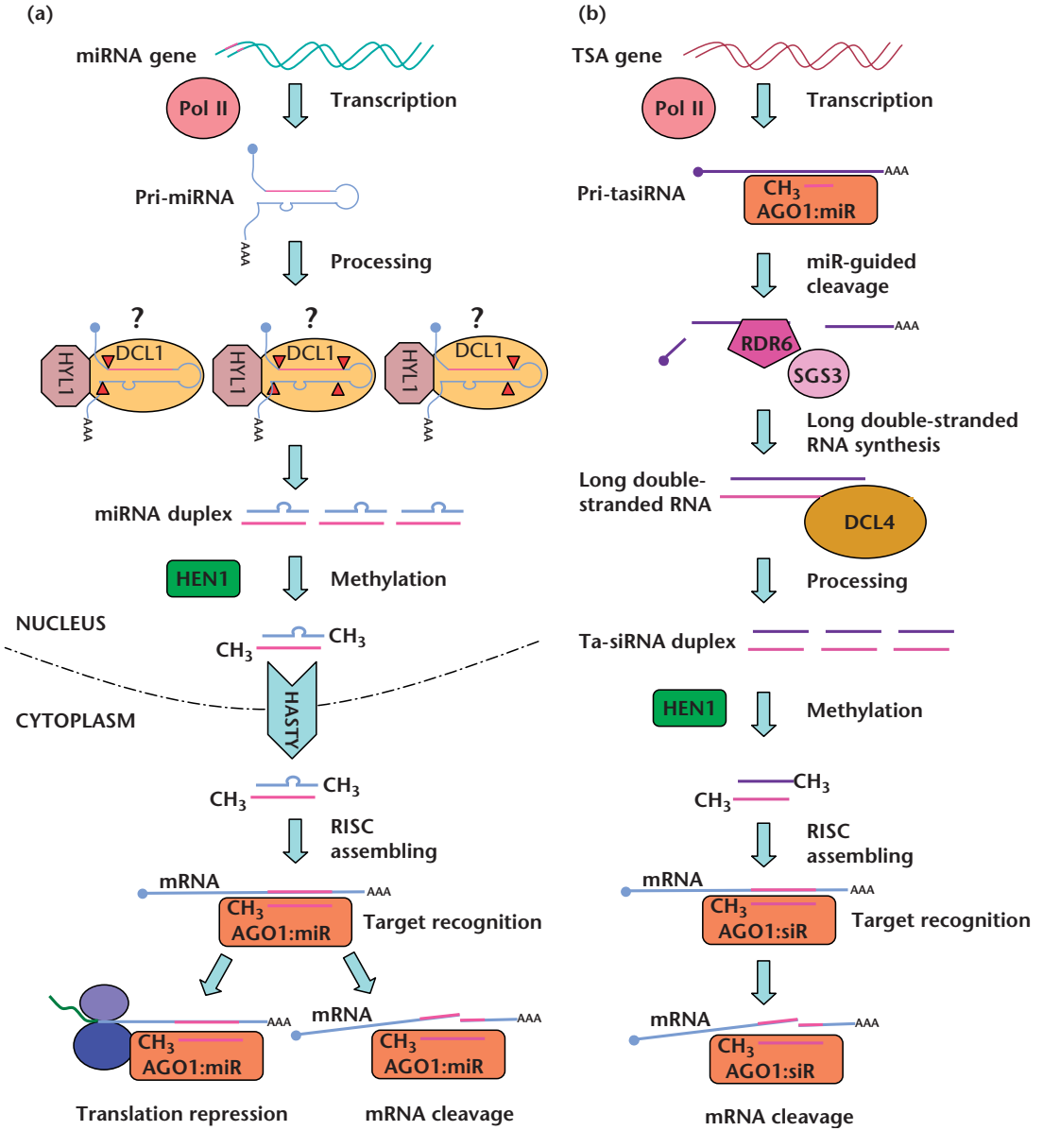

Figure 2 Biogenesis of miRNA and ta-siRNA in the RNA-silencing pathways.

(a) miRNA genes is transcribed by Pol II to produce fold-back structure of pri-miRNA transcripts. Following transcription, the pri-miRNA is processed by DCL1 with the aid of HYL1 and probably other unknown factors. In contrast to animal miRNA biogenesis, in which miRNA precursor (pre-miRNA) is generated by a different set of enzymes, the detail for the production of plant pre-miRNAs by DCL1 complex is unclear. In plants, miRNA duplex are first methylated by HEN1 in nucleus and then exported to the cytoplasm by HASTY. Mature methylated miRNAs are assembled into AGO1-containing complex or RISC that target complementary mRNAs for cleavage or translation repression. (b) pri-tasiRNA transcripts are transcribed in the same way as primary miRNAs (pri-miRNAs). miRNA-directed AGO1-associated RISC guides double cleavage on the pri-tasiRNA to generate templates of RNA fragments for RDR6, an RNA dependent RNA polymerase, to produce long double stranded RNAs (dsRNAs). DCL4 initiates processing of the dsRNA to produce phased ta-siRNAs that are also methylated by HEN1. ta-siRNAs are subsequently assembled into RISC to cleave homologous mRNAs.

plant. For example, the *Arabidopsis dcl1* null mutant is embryo lethal. Partial loss-of-function hypomorphic mutants of *dcl1* result in drastic developmental abnormalities, including defects in floral organogenesis, leaf morphology and axillary meristem initiation. These pleiotropic developmental abnormalities have also been observed in the partial or complete loss-of-function mutants of *hyl1*, *hen1*, *hst* and *ago1* (Bohmert *et al*., 1998; Park *et al*., 2002; Telfer and Poethig, 1998; Chen *et al*.,2002). The developmental abnormalities in these mutants are a direct consequence of disruption of miRNA production (Schauer *et al*., 2002), which results in misregulation of miRNA targets (Vazquez *et al*., 2004).

Conclusion

Plant development is a tightly controlled process that is regulated at various levels of gene expression. The discovery of miRNAs has revealed a level of posttranscriptional control that is important for fine-tuning the regulatory processes (**Figure 1**). In particular, plant miRNAs regulate the expression of many transcription factors that control aspects of plant development ranging from the development of various organs (leaf, shoot, root, flower), their identity, patterning, polarity and developmental timing (flowering time, developmental phase transition), to specific tissue or cellular organelle (vascular and plastid) development. Various plant hormone signalling pathways intersect with sets of transcriptional factors frequently via plant miRNAs to ensure a proper development of a plant.

In humans, more than one third of the human genes may be subject to direct control by miRNAs. In plants, the number of the known miRNAs and their predicted target genes is smaller. However, predictions of plant miRNA targets are usually predicated on the assumption that miRNA targets in plant are perfectly or extensively paired to the miRNAs. Considering that the mechanism of miRNA-directed downregulation of gene expression is generally conserved between plants and animals, it is very likely that plant miRNAs can also act as translational repressor (Aukerman and Sakai, 2003; Chen, 2004) via a partial base pairing to their targets. Thus, there may be many as yet undiscovered miRNA targets. On the other hand, there may also be many new miRNAs yet to be discovered. This speculation is supported by the observation that, beside the conserved miRNA families, the numbers of reported nonconserved, species-specific or tissue-specific miRNAs are continuing to increase. Continued efforts at large-scale cloning and high-throughput sequencing, along with additional computational analyses, should continue to yield new miRNAs and their target genes. These unidentified miRNAs will likely include those that are associated with specific tissue/organs, developmental stages and biotic and abiotic environmental responses.

In the past several years, genetic and transgenic analyses have greatly helped to understand regulatory roles of miRNA. However, the functional roles of many identified miRNAs remains unknown. Functional characterization of the continuously expanding numbers of miRNA is an ongoing challenge to plant biologists. To better understand the coordinated roles of several or a group of miRNAs, global analysis of miRNAs and the expression of their target genes will identify more regulatory links between miRNAs and their direct or indirect targets (Axtell and Bartel, 2005). Furthermore, transgenic plants that express a specific miRNA or miRNA-resistant-target genes, as already implemented in many studies, will continue to serve as powerful tool to analyse each particular regulatory event. It is reasonable to expect that the growing insight into the roles of miRNAs in plant development will provide deep insight into the life cycle of plants.

Although not all aspects are completely clear, our knowledge of miRNA regulation in plant development has grown rapidly. The complex relationships between miRNAs and overlapping sets of potential targets (**Table 1**) indicate that the miRNA regulatory network has intricate complexities that are comparable to those seen in transcriptional regulatory networks. Just as an understanding of the dynamics of the transcriptional regulatory network informs us on processes important in growth, development and evolution (Davidson and Erwin, 2006), it is to be expected that a delineation of the miRNA regulatory network will bring a clearer understanding of processes and events associated with miRNAs. Moreover, a more refined picture of growth, development and evolution will result from a superimposition of the transcriptional and miRNA regulatory networks into a single conceptual model.

Acknowledgements

G.T. is supported by the USDA-NRI grants 2006-35301-17115 and 2006-35100-17433.

References

Achard P, Herr A, Baulcombe DC and Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131**: 3357–3365.

Allen E, Xie Z, Gustafson AM and Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**: 207–221.

Ambros V, Lee RC, Lavanway A, Williams PT and Jewell D (2003) MicroRNAs and other tiny endogenous RNAs in C. elegans. *Current Biology* **13**: 807–818.

Aukerman MJ and Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* **15**: 2730–2741.

Axtell MJ and Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. *Plant Cell* **17**: 1658–1673.

Baker CC, Sieber P, Wellmer F and Meyerowitz EM (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in *Arabidopsis*. *Current Biology* **15**: 303–315.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281–297.

Bohmert K, Camus I, Bellini C *et al*. (1998) AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. *The EMBO Journal* **17**: 170–180.

Chen X (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. *Science* **303**: 2022–2025.

Chen X, Liu J, Cheng Y and Jia D (2002) HEN1 functions pleiotropically in *Arabidopsis* development and acts in C function in the flower. *Development* **129**: 1085–1094.

Davidson EH and Erwin DH (2006) Gene regulatory networks and the evolution of animal body plans. *Science* **311**: 796–800.

Emery JF, Floyd SK, Alvarez J *et al*. (2003) Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes. *Current Biology* **13**: 1768–1774.

Fahlgren N, Montgomery TA, Howell MD *et al*. (2006) Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in *Arabidopsis*. *Current Biology* **16**: 939–944.

Guo HS, Xie Q, Fei JF and Chua NH (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. *Plant Cell* **17**: 1376–1386.

Jones-Rhoades MW and Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Molecular Cell* **14**: 787–799.

Jones-Rhoades MW, Bartel DP and Bartel B (2006) MicroRNAS and their regulatory roles in plants. *Annual Review of Plant Biology* **57**: 19–53.

Juarez MT, Kui JS, Thomas J, Heller BA and Timmermans MC (2004) microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature* **428**: 84–88.

Kim J, Jung JH, Reyes JL *et al*. (2005) microRNA-directed cleavage of ATHB15 mRNA regulates vascular development in *Arabidopsis* inflorescence stems. *Plant Journal* **42**: 84–94.

Ko JH, Prassinos C and Han KH (2006) Developmental and seasonal expression of PtaHB1, a Populus gene encoding a class III HD-Zip protein, is closely associated with secondary growth and inversely correlated with the level of microRNA (miR166). *New Phytology* **169**: 469–478.

Laufs P, Peaucelle A, Morin H and Traas J (2004) Micro RNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* **131**: 4311–4322.

Lauter N, Kampani A, Carlson S, Goebel M and Moose SP (2005) microRNA172 down-regulates glossy15 to promote vegetative phase change in maize. *Proceedings of the National Academy of Sciences of the USA* **102**: 9412–9417.

Llave C, Xie Z, Kasschau KD and Carrington JC (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* **297**: 2053–2056.

Luo YC, Zhou H, Li Y *et al*. (2006) Rice embryogenic calli express a unique set of microRNAs, suggesting regulatory roles of microRNAs in plant post-embryogenic development. *FEBS Letters* **580**: 5111–5116.

Mallory AC, Bartel DP and Bartel B (2005) MicroRNA-directed regulation of *Arabidopsis* AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* **17**: 1360–1375.

Mallory AC, Dugas DV, Bartel DP and Bartel B (2004a) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Current Biology* **14**: 1035–1046.

Mallory AC, Reinhart BJ, Jones-Rhoades MW *et al*. (2004b) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. *The EMBO Journal* **23**: 3356–3364.

McConnell JR, Emery J, Eshed Y *et al*. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature* **411**: 709–713.

Millar AA and Gubler F (2005) The *Arabidopsis* GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* **17**: 705–721.

Mlotshwa S, Yang Z, Kim Y and Chen X (2006) Floral patterning defects induced by *Arabidopsis* APETALA2 and microRNA172 expression in Nicotiana benthamiana. *Plant Molecular Biology* **61**: 781–793.

Nikovics K, Blein T, Peaucelle A *et al*. (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in *Arabidopsis*. *Plant Cell* **18**: 2929–2945.

Ochando I, Jover-Gil S, Ripoll JJ *et al*. (2006) Mutations in the microRNA complementarity site of the INCURVATA4 gene perturb meristem function and adaxialize lateral organs in *Arabidopsis*. *Plant Physiology* **141**: 607–619.

Palatnik JF, Allen E, Wu X *et al*. (2003) Control of leaf morphogenesis by microRNAs. *Nature* **425**: 257–263.

Park W, Li J, Song R, Messing J and Chen X (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Current Biology* **12**: 1484–1495.

Prigge MJ, Otsuga D, Alonso JM *et al*. (2005) Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. *Plant Cell* **17**: 61–76.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B and Bartel DP (2002) MicroRNAs in plants. *Genes Development* **16**: 1616–1626.

Rhoades MW, Reinhart BJ, Lim LP *et al*. (2002) Prediction of plant microRNA targets. *Cell* **110**: 513–520.

Ru P, Xu L, Ma H and Huang H (2006) Plant fertility defects induced by the enhanced expression of microRNA167. *Cell Research* **16**: 457–465.

Schauer SE, Jacobsen SE, Meinke DW and Ray A (2002) DICER-LIKE1: blind men and elephants in *Arabidopsis* development. *Trends in Plant Science* **7**: 487–491.

Schwab R, Palatnik JF, Riester M *et al*. (2005) Specific effects of microRNAs on the plant transcriptome. *Developmental Cell* **8**: 517–527.

Sunkar R and Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* **16**: 2001–2019.

Tang G, Reinhart BJ, Bartel DP and Zamore PD (2003) A biochemical framework for RNA silencing in plants. *Genes Development* **17**: 49–63.

Telfer A and Poethig RS (1998) HASTY: a gene that regulates the timing of shoot maturation in *Arabidopsis thaliana*. *Development* **125**: 1889–1898.

Tsuji H, Aya K, Ueguchi-Tanaka M *et al*. (2006) GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. *Plant Journal* **47**: 427–444.

Vazquez F, Gasciolli V, Crete P and Vaucheret H (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not post-transcriptional transgene silencing. *Current Biology* **14**: 346–351.

Wang JW, Wang LJ, Mao YB *et al*. (2005) Control of root cap formation by MicroRNA-targeted auxin response factors in *Arabidopsis*. *Plant Cell* **17**: 2204–2216.

Williams L, Grigg SP, Xie M, Christensen S and Fletcher JC (2005) Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166 g and its AtHD-ZIP target genes. *Development* **132**: 3657–3668.

Wu G and Poethig RS (2006) Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. *Development* **133**: 3539–3547.

Wu MF, Tian Q and Reed JW (2006) *Arabidopsis* microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. *Development* **133**: 4211–4218.

Yoshikawa M, Peragine A, Park MY and Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. *Genes & Development* **19**: 2164–2175.

Zhang BH, Pan XP, Wang QL, Cobb GP and Anderson TA (2005) Identification and characterization of new plant microRNAs using EST analysis. *Cell Research* **15**: 336–360.

Zhong R and Ye ZH (2004) Amphivasal vascular bundle 1, a gain-of-function mutation of the IFL1/REV gene, is associated with alterations in the polarity of leaves, stems and carpels. *Plant Cell Physiology* **45**: 369–385.