Review

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MicroRNA networks and developmental plasticity in plants

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Plant microRNAs (miRNAs) are embedded in regulatory networks that coordinate different gene expression programs in support of developmental plasticity. Modification of miRNA-target nodes during evolution might in turn underlie morphological and physiological diversity. A survey of the literature indicates that miRNA-target nodes themselves are organized in networks, and here we discuss some of the developmental traits they control along with possible interactions between miRNA and their targets. Because miRNAs and their interactions are not only at the heart of regulating many aspects of developmental plasticity, but because they also have an inherently quantitative mode of action, they present important targets for biotechnology applications.

miRNA nodes

Small RNAs (sRNAs) are key regulators of gene expression in many eukaryotic organisms. These molecules, mostly ranging from about 20 to 30 nucleotides in length, affect all levels of genetic information in plants. A special class of sRNAs, known as microRNAs (miRNAs), can regulate both the chromatin state of their targets and the availability of the encoded transcripts for translation into functional proteins [1–3]. Compared to animal miRNAs, which have often hundreds of targets, plant miRNAs tend to have fewer targets, often with regulatory function, such as transcription factors and F-box proteins [4–6]. This places miRNAs in a central position within gene expression programs underlying plant development. Thus, miRNA-target nodes (miRNA nodes for short, see Box 1) play a pivotal role in governing plastic behaviour during development, such as phase change and plant architecture, and in responses to the biotic and abiotic environment. Furthermore, evolutionary well-conserved miRNAs are likely to contribute to proper plant growth and morphogenesis [7,8]. Nevertheless, while mutant and transgenic analyses have demonstrated the general importance of specific miRNA families, there are still important aspects that need to be tackled in order to fully understand the functional complexity of miRNAs. Examples are: how important are miRNA target interactions compared to other types of regulatory interactions? Which miRNAs are particularly likely to cooperate in specific processes? And how are miRNA networks integrated into cellular and physiological events throughout the plant life cycle? In this review we will first highlight the different miRNA nodes that have been already described to interact with each other, and then discuss additional miRNA nodes that potentially participate in miRNA networks that regulate dynamic developmental events.

Developmental timing

Developmental progression throughout the plant life cycle

The first miRNA described, *lin-4*, in concert with another miRNA, *let-7*, drives developmental transitions in the worm *Caenorhabditis elegans* through mutually antagonistic action [9–11]. Similarly, one of the best understood and extensively reviewed miRNA network is one that regulates developmental timing and involves the antagonistic miR156 and miR172 nodes. The interaction of these two miRNA nodes plays a highly conserved role in promoting the progression through different developmental phases in both monocots and dicots [12–18]. After seed germination the plant life cycle advances from the juvenile to the adult stage of vegetative growth, and later enters the reproductive, flowering phase. Each of these stages is characterized by different morphological features. In *Arabidopsis* (*Arabidopsis thaliana*), the onset of the adult phase is reflected in the production of trichomes on the lower side (abaxial) of leaves, which also become narrower, longer and more serrated, with a concomitant decrease in cell size [19–21]. In maize (*Zea mays*), the transition from juvenile to adult leaves is marked by changes in cell shape, the production of epidermal wax deposits and of specialized cell types like leaf hairs, and a change in the identity of organs that grow from their axillary meristems. In both plant species, developmental transitions are coordinated by the antagonistic activities of miR156 and miR172 [12,22–25]. miR156 expression levels decrease with leaf age, while miR172 increases (Figure 1a). Their targets, Squamosa Promoter Binding Protein-Like (SPL) and Apetala 2 (AP2) transcription factors, respectively, are expressed in complementary patterns. Increased levels of miR156, as well as reduced miR172 activity, restrain developmental transitions, prolong juvenile features (neoteny) and delay flowering. In turn, release of SPLs from miR156 regulation leads to premature acquisition of adult leaf features and early flowering, resembling effects observed in plants with reduced activity of AP2-like miR172 targets. The miR156 targets SPL can be divided into two groups: the small SPL3, SPL4 and SPL5 proteins and the large SPL proteins such as SPL9, SPL10 and SPL15. Larger SPLs seem to play a more important role in leaf

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Box 1. miRNA-target node (miRNA node)

A miRNA node is an interface that comprises the regulatory relationship between a specific locus from a miRNA family and the direct targets under its control. In specific cases, a node reflects the interaction between one miRNA locus and one target (e.g. miR167c–ARF8 node). In other cases, it might include all miRNA loci of a family and all its potential targets (e.g. miR319–TCP). This concept was previously introduced and further explained in [80].

patterning, while both groups contribute to trichome production and the promotion of flowering [16,17,26,27]. SPL9, and likely other large SPLs, regulate the transition to the reproductive phase by directly binding to the promoter of at least one of the five *MIR172* genes, *MIR172b*. An increase in miR172, which reverses the delay in abaxial trichome formation observed in plants overexpressing miR156, reduces activity of the potent floral repressors SCHLAFMÜTZE (SMZ), SCHNARCHZAPFEN (SNZ), TARGET OF EAT 1 and TARGET OF EAT 2 (TOE1, TOE2) [16,17,22,23,28,29]. This sequence of genetic events places miR172 action downstream of miR156 (Figure 1a).

A third set of sRNA nodes involved in regulating the vegetative phase transition includes trans-acting siRNAs (tasiRNAs) from the *TAS3* locus. In this case, a miRNA, miR390, does not target a protein-coding mRNA, but rather triggers the production of tasiRNAs, which in turn cause in a miRNA-like fashion destruction of the *AUXIN RESPONSE FACTOR 3 (ARF3) and ARF4* mRNAs [30–32]. Mutant plants impaired in tasiRNA biogenesis, such as those lacking RNA-dependent RNA POLYMERASE 6 (*rdr6*), DICER-LIKE 4 (*dcl4*) and ARGONAUTE 7 (*ago7*), have elevated levels of ARF3 and ARF4. As a consequence, they enter precociously into the adult phase, as evidenced by premature initiation of abaxial trichomes and changes in leaf shape [30–34]. A potential connection with miR172–AP2 and miR156–SPL nodes is supported by the findings that the miR172 target AP2 directly binds to the *ARF3* promoter [29] along with the presence of higher levels of SPL3 and SPL4 in *ago7* mutant plants [24,35]. In the absence of *TAS3*-derived tasiRNAs, higher levels of ARF3 might promote expression of SPL3 and/or SPL4, likely in a miR156-independent fashion. SPLs directly activate TRICHOMELESS1 (TCL1) and TRIPTYCHON (TRY) [26], thus this might explain premature abaxial trichomes in plants with deficient *TAS3* function. Other effects of ARF3, such as changes in leaf patterning, are, however, not likely to be mediated by SPL9 or SPL15. Based on the abovementioned common phenotypic consequences of reducing the activity of miR172 targets and increasing ARF3 activity with respect to phase transition, we speculate that AP2-like proteins directly repress ARF3 expression. In such a scenario, it seems plausible that ARF3 contributes to the miR156-independent regulation of SPL3 by miR172 targets [17,29]. Hence, we propose that the miR172 targets AP2 and/or TOE2 and TOE3, diminish SPL3 levels through downregulation of its activator ARF3 (Figure 1a).

Both miR156 and the miR390–TAS3 node are conserved in *Physcomitrella patens* [18,36–39], but only the TAS3

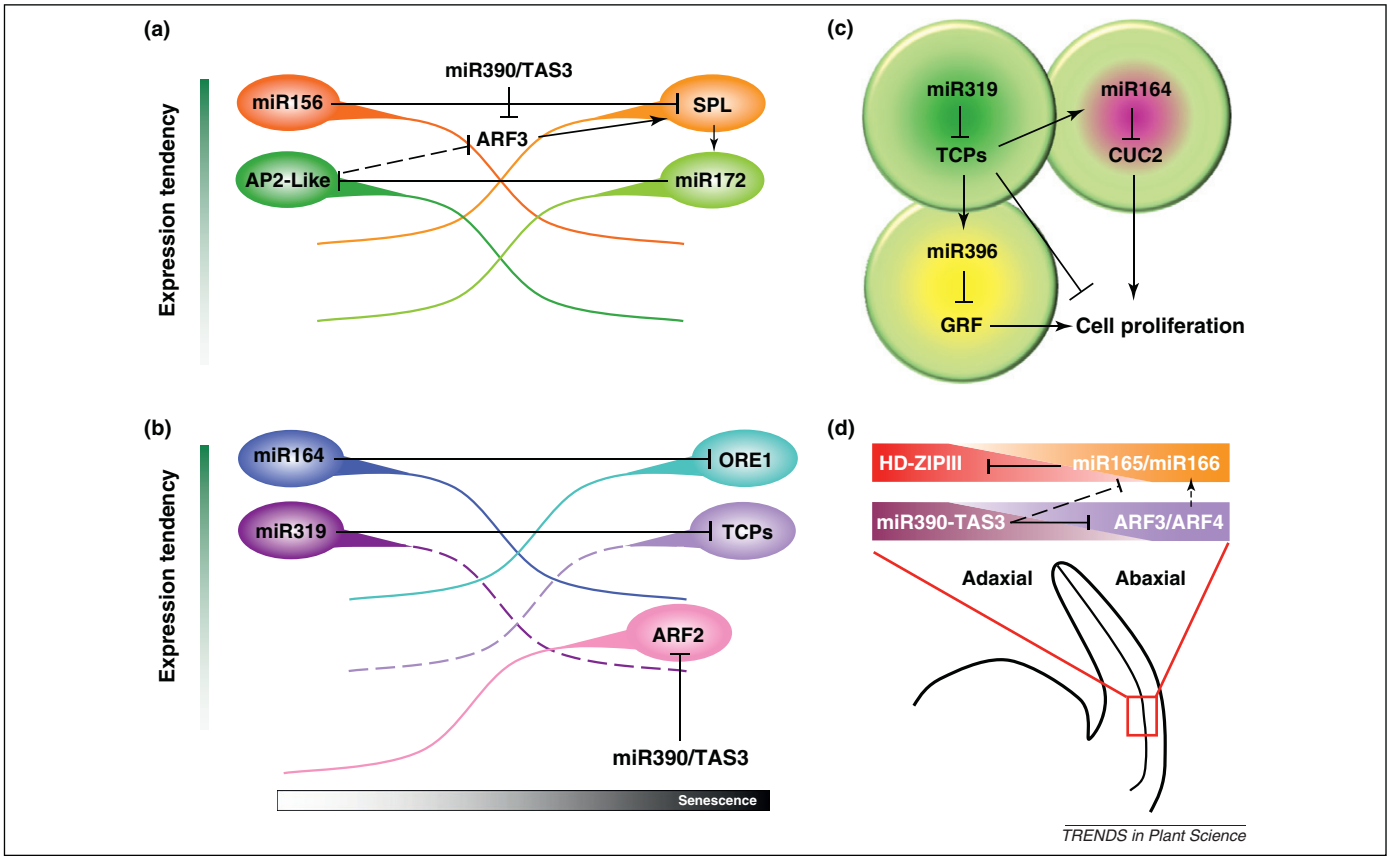

Figure 1. Coordinated action of miRNA nodes in developmental timing and tailoring leaf shape. **(a)** Expression dynamics of interacting miRNA nodes driving developmental phase transitions. **(b)** miRNA nodes related to leaf senescence in *Arabidopsis*. **(c)** Interplay of miRNA nodes regulating cell proliferation in leaves. **(d)** miRNA–target interactions in the determination of abaxial–adaxial leaf polarity. Lines emanating from ovals show tendency of gene expression. Dashed lines denote speculative behaviours and interactions. Arrows indicate positive and T-lines negative regulation.

pathway has so far been related to the regulation of phase transition. Interestingly, apart from targeting *ARF3* and *ARF4*, *TAS3*-tasiRNAs target members of the AP2-like family of transcription factors. It would be particularly interesting to compare the interaction of these sRNA nodes in mosses and angiosperms.

The miR156–SPL node regulates an additional aspect of developmental timing in both *Arabidopsis* and rice (*Oryza sativa*), plastochron length, which is the time interval that elapses between the initiation of two successive leaves [40–42]. So far, there is no evidence that miR172 is an antagonist of miR156 in this process.

Senescence

In many leaf-like organs, including cotyledons, true leaves and sepals, the end point of development leads to controlled extraction of resources during senescence. Nutrients and cellular components are remobilized from leaves to support seed development, or for storage in rhizomes [43]. The control of senescence involves two miRNAs, miR164 and miR319. In an ethylene-dependent manner, which relies on the function of the EIN2 (ETHYLENE INSENSITIVE 2) regulator, the expression of miR164 declines with leaf age, leading to increasing levels of its targets NAC1, ORE1 (AtNAC2) and At5g61430 (Figure 1b) [44]. Accordingly, miR164 ectopic expression and/or lack of ORE1 activity promote leaf longevity, as indicated by the delayed appearance of phenotypic and genetic senescence markers such as chlorophyll breakdown and *SAG12* expression [44]. Another miRNA, miR319, has several activities, among them the repression of the onset of senescence [45]. miR319 overexpression causes plants to stay green much longer while compromised miR319-dependent regulation of one of its main targets, TCP4, results in increased expression of genes that are normally active in older leaves. It is therefore tempting to speculate that during normal development, miR319 levels gradually decline, although this has not yet been investigated (Figure 1b). The miR319 targets of the TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) transcription factor family affect senescence by increasing levels of jasmonic acid (JA), through activation of LIPOXYGENASE2 (LOX2), one of the main JA biosynthetic enzymes [45]. However, this cannot be the only downstream pathway, as manipulation of the miR319 node has much more dramatic effects on senescence than alteration of the JA biosynthesis pathway. Although there is no experimental evidence for interactions between the miR164 and miR319 nodes in a senescence context, miR319–TCP3 nodes seems to regulate miR164 expression during early leaf development (see below). Apart from ethylene and JA, the hormone auxin plays a role in the regulation of plant longevity, delaying degenerative processes like leaf senescence or floral organ abscission [43,46]. The *TAS3* target ARF2 is a negative regulator of auxin responses, and is involved in controlling the onset of leaf senescence and floral organ abscission [46–48]. It is striking that despite reports that these three miRNA nodes, miR319–TCP, miR164–NAC and miR390–TAS3–ARF2, regulate similar processes, it has not been explored whether they form a miRNA regulatory network. Moreover, leaf senescence can be regarded as an aspect of developmental timing, it would thus be interesting to investigate whether it is affected by the miR156 and miR172 nodes.

Variation in leaf morphogenesis

Regulation of cell proliferation

The impressive variety of leaf shapes found in nature has been intriguing naturalists for centuries, especially as it is still unclear what adaptive function, if any, this variation serves. Specific and systematic reduction of miRNA activity in *Arabidopsis* has revealed the prominent role of miRNAs in leaf patterning, accounting for the majority of phenotypic alterations in the aerial parts of plants in which different miRNAs have been suppressed [8]. Leaves emerge from undifferentiated pools of cells in the shoot apical meristem (SAM), with local auxin maxima dictating where leaf primordia are formed. The boundary between meristematic, undifferentiated cells and those that are competent for differentiation is established by the miR164 targets CUP-SHAPED COTYLEDON1 (CUC1) and CUC2. Plants compromised in CUC1 and CUC2 activity have fused cotyledons, closely resembling mutants affected in miR319–TCP4 regulation [49,50]. The phenotypic connection between miR164–CUC and miR319–TCP nodes is further supported by the finding that TCPs can directly bind to miR164a regulatory sequences modulating its expression levels. In addition, *CUC* genes function in the growth of the leaf front, a process in which TCPs have an important role as well (Figure 1c) [51–53]. Accordingly, *Arabidopsis* leaf margins proliferate excessively in the absence of miR319-regulated TCPs or upon release of CUC2 from miR164a regulation; both leading to crinkled and more serrated leaves [8,50,52–55]. Cell proliferation at the leaf margin determines leaf complexity, which is expressed in margins being entire or dissected, or even producing secondary leaflets, resulting in compound leaves. In agreement, both miR319 and miR164 nodes are essential for leaflet formation in compound leaves because of their ability to regulate cell proliferation. Tomato (*Solanum lycopersicum*) plants in which the TCP protein LANCEOLATE (LA) escapes negative regulation by miR319 suffer from striking, dose-dependent impairment of compound leaf development. In *la* homozygous individuals, only simple leaves are produced, because increased TCP activity arrests cell proliferation and promotes precocious cell differentiation [56]. Similarly, the miR164 targets of the *CUC* family, along with closely related *CUC* genes that are not subject to miRNA regulation, form a partially evolutionarily conserved regulatory network for the control of leaf complexity in different species [55,56]. Lack of CUC activity leads to simple leaves because of a premature cessation of leaf margin growth. Additionally, the ability of the miR319–TCP nodes to regulate cell proliferation in *Arabidopsis* is partially mediated by the conserved miR396–GRF (Growth Regulator Factor) node [57]. Compromised miR319–TCP4 regulation leads to reduced GRF expression and a subsequent reduction in mitotic activity (Figure 1c) [57]. Finally, the miR390–TAS3–ARF nodes have been invoked in the control of leaf margin growth in both *Arabidopsis* and *Lotus japonicus*, a specie with compound leaves [31,34]. Again,

how these four miRNA nodes might interact has not yet been studied, but it will be interesting to determine at what level the function of the miR164, miR319 and miR396 nodes converges on the regulation of the cell cycle.

Organ polarity

In addition to leaf complexity, two important aspects of leaf morphology are organ symmetry and polarity. The adaxial–abaxial, proximo–distal and medio–lateral axes, which persist during leaf development, are established soon after a primordium appears. Abaxial/adaxial differentiation is essential for leaf performance, because it structures the leaf for optimal photosynthesis and gas and water exchange. The specification of abaxial/adaxial polarity was one of the first developmental processes in plants found to be intimately associated with sRNAs [58–60]. The MYB R2R3 protein AS1 (ASYMMETRIC LEAF1) specifies adaxial fate in concert with members of the miR165/miR166 regulated HD-ZIPIII (class III HOMEODOMAIN LEUCINE ZIPPER) family of transcription factors, whereas KANADI (KAN) and YABBY proteins along with ARF3/ARF4, the outputs of the miR390–TAS3 node, are abaxial determinants. When miR165/miR166 control of HD-ZIPIII proteins such as PHAVOLUTA, PHABULOSA and REVOLUTA is alleviated, these proteins are no longer confined to the adaxial leaf side, leading to loss of abaxial identity [4,58,59,61–63]. On the other hand, accumulation of ARF3/ARF4 on the abaxial side depends on non-cell autonomous action of *TAS3*-tasiRNAs produced after miR390 targeting of *TAS3* on the adaxial face of the leaf [64–67]. The interplay between polarly localized factors sets the precise boundaries between the abaxial and adaxial domains. Thus, the interaction between miR165 and miR166 and miR390–TAS3 relies on ARF3 and ARF4 having a positive role in miR165/miR166 regulation [60,66]. In addition, differential contribution from miR166 loci to specific miR165/miR166–HD-ZIPIII nodes suggests specialization within this node in maize [66].

Vascular development

Leaf development, shape and function are dependent on the vasculature, which not only provides mechanical support, but also conveys metabolites and signalling molecules [68]. Elements of the miR165/miR166 and miR159 nodes have essential roles in the development of leaf vasculature. ATHB8, a miR165/miR166 target, promotes progression in vasculature differentiation [69]. Similarly, defects in vasculature development in *hyl-1* mutants, which are defective in miRNA processing, as in *phb-d* mutants, can be at least partially attributed to REVOLUTA and PHABULOSA escaping miR165/miR166 regulation [63,70]. Related leaf growth (hyponasty) and vasculature defects are found when miR159 action is inhibited [8,50,71–73]. Unless miR159a and miR159b exclude their targets MYB33 and MYB65 from developing vasculature, it will not become properly functional [8,71,74]. Interestingly, another member of the MYBR2R3 subfamily, AS1, negatively regulates miR165/miR166 expression in the polarization process mentioned above [60,75]. It will be interesting to investigate a potential interplay between both miRNA nodes in leaf vasculature development based on contributions of AS1 and miR159–targets to downregulation of miR165/miR166 expression.

Reproductive development

Inflorescence architecture, flower organogenesis, sex determination and organ maturation are key traits for seed yield and overall fitness. Several miRNA nodes guide various aspects of reproductive development, with prominent roles for the antagonistic miR156 and miR172 nodes. In maize inflorescences, spikelet meristem specification and organogenesis are regulated by these two nodes. Increased levels of miR156 in the dominant *Congrass1* mutant (*Cg1*) or inactivation of its targets *tga1* and *tassel-sheath4* (*ZmSBP6*) cause similar phenotypic alterations as seen in *tasselseed4* (*ts4*) mutants. In *ts4* mutants reduced expression of miR172e increases the expression of at least two of its targets, *ids1* (*indeterminate spikelet1*) and *sid1* (*sister of indeterminate spikelet1*) [12–15]. The role of miR172 nodes in regulating shoot meristem activity in *Arabidopsis* is mediated by its contribution to patterning the expression of the stem cell regulator *WUSCHEL* (*WUS*) [76,77]. Although the miR172 target AP2 directly binds to *MIR156e* cis-regulatory sequences in *Arabidopsis* leaves, it can additionally regulate the expression of the *MIR172a* locus in inflorescences [29]. Likewise, the miR172 target AP2 directly contributes to *ARF3* patterning, constituting a possible unidirectional link between miR172 and miR390–TAS3–ARF3 nodes [29]. Finally, late stages of flower development in *Arabidopsis*, rice and *L. japonicus* plants rely on regulated ARF3 function [31,34,78].

Modulation of root architecture

The root system is at least as plastic as the aerial part of the plant, as it interacts with a highly structured physical environment. Root system plasticity mainly relies on secondary growth of lateral roots and root hairs, which enable the plant to forage efficiently for nutrients. The periodicity with which lateral roots emerge along the main root is set by two oscillating, opposite waves of gene expression that correlate with pulses of auxin-induced transcription [79]. Lateral root development starts when single pericycle cells turn into founder cells, which proliferate to form a lateral root primordium. Depending on environmental cues, the primordium continues to develop, breaking through the surface of the primary root. Given the key role of auxin in lateral root development, and the fact that several miRNAs are linked to auxin signalling (reviewed in [80]), it is not surprising that miRNAs play an important role in lateral root development. While the miR164–NAC1 and miR160–ARF10/ARF16/ARF17 nodes affect their initiation [41,81,82], miR390–TAS3–ARFs together with two nitrate-regulated miRNA nodes, miR167–ARF8 and miR393–AFB3, act subsequently during emergence and elongation of the lateral root [83–86].

The miR167 and miR393 nodes constitute a beautiful example of how miRNAs can coordinate the integration of metabolic state, soil nutrient availability and contribute to dose-dependent, auxin-based plasticity of the root system. Nitrate is an important source of nitrogen, which is often limiting for plant growth and agricultural productivity.

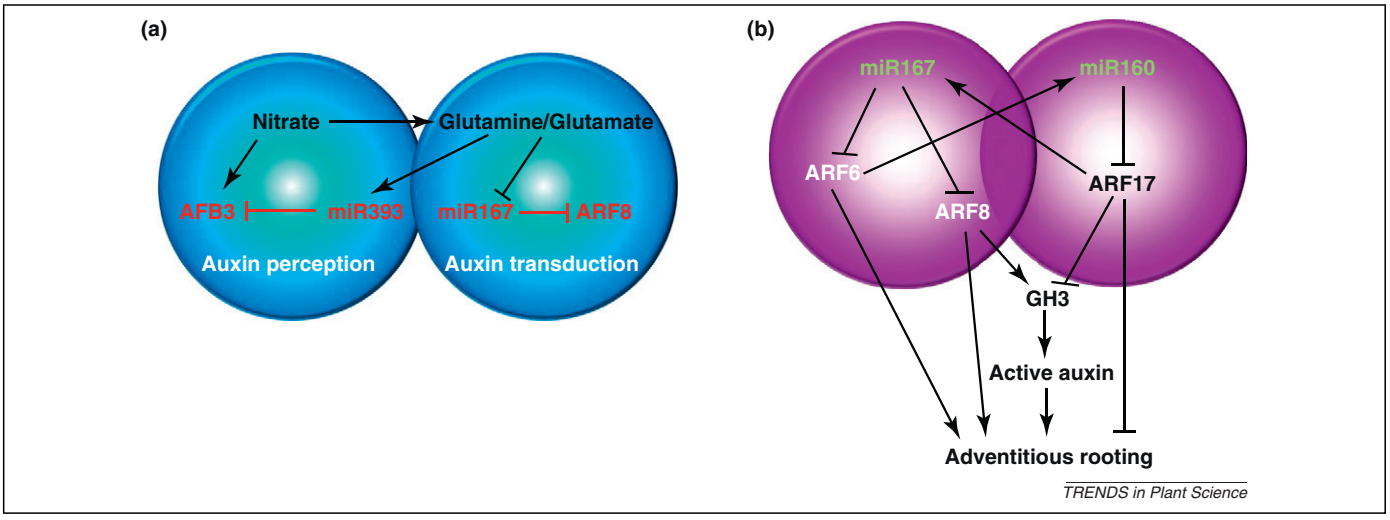

Figure 2. miRNA–target nodes in root architecture plasticity. **(a)** miRNA nodes coordinating nitrate- and auxin-dependent formation of lateral roots. Nitrogen metabolic pathway and effect on the expression of node components are shown in black. Elements from the miRNA nodes and their regulatory relation are depicted in red letters and lines. White letters refer to the relevant point of the auxin signalling pathway in which the abovementioned interactions occur. **(b)** miRNA nodes in the regulation of auxin homeostasis and adventitious rooting.

Auxin competes with nitrate for transport by the NTR1.1 transmembrane protein [87]. NTR1.1 thus prevents outgrowth of lateral roots at low nitrate concentration in the soil by pumping out auxin and therefore preventing the formation of local auxin maxima. NTR1.1 has, however, a higher affinity for nitrate than for auxins, and when the root system meets a soil fraction rich in nitrate, auxin maxima can form. These auxin maxima then trigger lateral root development for the more efficient extraction of nutrients from such patches. Nitrate absorption in turn activates the expression of the miR393 target, and auxin receptor, AFB3. Finally, nitrate assimilation in the amino acids glutamine and glutamate activates expression of miR393, reducing auxin perception and thus dampening the initial signal. Likewise, nitrate assimilation contributes to trigger auxin signalling increasing ARF8 activity through reduction of miR167 levels (Figure 2a) [83,85].

The miR167–ARF6/ARF8 nodes interact with the miR160–ARF17 node to support development of shoot-borne, adventitious roots in *Arabidopsis* [88]. ARF17 affects both miR167-dependent and independent regulation of ARF6 and ARF8. Conversely, ARF6 represses ARF17 by activating miR160, while ARF8 directly represses ARF17 (Figure 2b). Finally, miR160 and miR167 targets appear to have opposite roles in controlling the expression of the auxin homeostatic enzyme GH3.6 [82,89]. Thus, miR160 targets reduce active auxin and adventitious rooting, while the opposite is true for miR167 targets (Figure 2b).

Conclusions and future perspectives

One of the emerging themes in studies of the role of miRNAs in plant development is that, despite great sequence similarity between miRNAs, different members of miRNA families can make very specific contributions to the spatial and temporal control of their targets. It is also striking that despite the relatively small number of confirmed miRNA targets, these are so highly interconnected. Apart from determining how many of these interactions are indeed direct, intra- and inter-specific variation in miRNA node networks will be an important are of future research. On the other end of the spectrum, it is also important to reveal in detail the cellular and physiological contexts in which the miRNA networks operate. Finally, we believe that the central role that miRNA networks play in the control of key agronomic traits makes them appealing biotechnological targets for the production of varieties with improved performance. Of particular interest in this context is the ability to quantitatively and spatially manipulate both miRNAs and miRNA targets, e.g. by introducing mutations into target genes and specific miRNA loci that reduce, but not abolish the level of miRNA control.

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