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Plant microRNAs: key regulators of root architecture and biotic interactions

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Summary

Plants have evolved a remarkable faculty of adaptation to deal with various and changing environmental conditions. In this context, the roots have taken over nutritional aspects and the root system architecture can be modulated in response to nutrient availability or biotic interactions with soil microorganisms. This adaptability requires a fine tuning of gene expression. Indeed, root specification and development are highly complex processes requiring gene regulatory networks involved in hormonal regulations and cell identity. Among the different molecular partners governing root development, microRNAs (miRNAs) are key players for the fast regulation of gene expression. miRNAs are small RNAs involved in most developmental processes and are required for the normal growth of organisms, by the negative regulation of key genes, such as transcription factors and hormone receptors. Here, we review the known roles of miRNAs in root specification and development, from the embryonic roots to the establishment of root symbioses, highlighting the major roles of miRNAs in these processes.

I. Introduction

One of the fascinating properties of land plants is represented by an indeterminate post-embryonic growth. This feature notably relies on particular cellular microenvironments, called the primary meristems, located at the shoot and root apices (the shoot apical meristems and root apical meristems, SAM and RAM, respectively; for reviews, see Scheres, 2007; Stahl \& Simon, 2010). The stem cell niches (SCNs) of SAM and RAM fulfill two functions. First, they are involved in their own self-renewal via the integration of various signals for meristem maintenance (for reviews, see Petricka \textit{et al.}, 2012; Drisch \& Stahl, 2015). Second, they provide all the cells that are necessary for organogenesis through division, elongation and differentiation activities. In Arabidopsis roots, the SCN is...
composed of a central and rarely dividing quiescent center (QC) surrounded by the stem cells or initials that will divide to produce the different cell types (Fig. 1). Rootward to the SCN, a protective root cap is produced, and shootward, three main regions can be distinguished: the meristematic, elongation and differentiation zones (Fig. 1). Radially, Arabidopsis root cells are organized in concentric cylinders of epidermis, cortex, endodermis, pericycle and a central vasculature with xylem and phloem poles.

Being sessile organisms, plants are also characterized by a high degree of plasticity depending on their environment (for a review, see Gaillochet & Lohmann, 2015). For example, the root system architecture (RSA) can be modulated to cope with the limiting availability (or excess) of minerals and water, depending on their biotic environment (for reviews, see Lugtenberg & Kamilova, 2009; Dastidar et al., 2012; Gutjahr & Paszkowski, 2013; Kazan, 2013). The modulation of RSA notably relies on the regulation of primary root growth through division and elongation activities, and also on the regulation of lateral or adventitious root branching. Understanding how plants integrate external signals to modulate RSA is also particularly important for breeding as it participates in plant fitness (for a review, see Rogers & Benfey, 2015). Another strategy used by plants relies on the interactions with their biotic environment at the root level. It is generally assumed that the capability of land plant ancestors to interact with arbuscular mycorrhizal fungi (AMF) was an important factor for successful terrestrial colonization in terms of stress tolerance, limiting water and mineral nutrients in this newly colonized environment (Smith & Read, 2008). In this symbiosis, roots cells are colonized intracellularly by fungi, characterized by a high degree of invagination in colonized cells. This feature obviously increases the exchange interface between the roots and the fungus. These fungal arbuscular cells are connected to an extensive extraradical hyphal network that also considerably increases the availability of nutrients for the plant (reviewed in Gutjahr & Parniske, 2013 and Gobbato, 2015). This intimate association is widespread and estimated to be found in 80% of land plant species (Smith & Read, 2008). Some plants, mainly from the Legume family, also acquire the ability to host intracellularly soil bacteria collectively known as rhizobia in specialized root-derived organs, symbiotic nodules. In this interaction, the plants host symbiotic rhizobia in a favorable ecological niche with a carbohydrate supply to the bacteria. Conversely, bacterial nitrogenase allows the conversion of atmospheric dinitrogen into ammonium, which is a form of nitrogen (N) that can be assimilated by the plant (for a review, see Suzuki et al., 2015). Given the huge amount of atmospheric dinitrogen (78% of atmospheric gas), this represents an inexhaustible potential of N fertilizer for legume plants. Interestingly, the mycorrhization and nodulation, which favor phosphorus (P) uptake and N fixation, respectively, are each inhibited by a high concentration of the respective compound (Thornton, 1936; Gibson & Pagan, 1977; Graham et al., 1981; Balzergue et al., 2011).

Beyond the linear and binary scheme in which a gene contains the coding information to produce a protein through an intermediary messenger RNA (mRNA), several processes for the fine tuning of this multi-step and compartmentalized biological reaction have arisen in recent decades. Among them, the small regulatory non-coding RNAs are of particular importance and have considerably increased the number of non-coding genetic loci. Two main classes of small non-coding RNAs can be established based on their origin and biogenesis (Axtell, 2013). Among them, the microRNAs (miRNAs; Box 1) are small regulatory RNAs (20–24 nucleotides) that are encoded by genes and participate in numerous

Fig. 1 Schematic representation of the root system and meristem in Arabidopsis thaliana. LR, lateral root; SCN, stem cell niche. The stem cells and quiescent center are surrounded by white lines.
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Box 1 Main features and properties of microRNAs
- Short regulatory RNAs (mainly 21–22 nucleotides).
- Produced from primary transcripts after a complex biogenesis.
- Found in several multicellular eukaryotic lineages.
- Participate in most of the developmental processes and stress responses.
- May act as mobile signals.
- Negatively regulate gene expression.
- Trigger transcript cleavage or translational repression.

The number of plant miRNAs deposited in public databases, such as miRBase and PNRD (for Plant Non-coding RNA Database), has increased considerably in the last two decades (Yi et al., 2015; Zhang & Wang, 2015). Many have been in silico annotated from the increasing number of available genomic sequences, but also identified from small RNA profiling studies using next-generation sequencing technologies. Overall, the latter studies have highlighted many miRNA families responsive to abiotic and biotic stresses or specific to a developmental process. Notably, they have allowed the discovery of new miRNAs, but also provide a powerful framework at the spatiotemporal level for further functional studies. Although, in most cases, the up-regulation or, conversely, down-regulation of miRNAs might be of functional relevance in a given context, their modified expression might also represent a secondary side effect. For this reason, the experimental validation of miRNAs is still a necessary approach. Ideally, the use of a null mutant in a given miRNA represents a classical approach, but has been little used in plants. Conversely, miRNA overexpression, mainly using the Cauliflower Mosaic Virus (CaMV) 35S strong promoter, has been extensively used, but has the main disadvantage that it may lead to ectopic silencing of target genes. Finally, the use of target genes mutated in their miRNA binding site, and thus insensitive or less sensitive to miRNA inhibition (miR-resistant), represents a very powerful approach to unravel the contribution of miRNAs in the regulation of their targets. In a complementary approach, target mimicry allows the sequestration of miRNAs from the same family and the efficient study of their overall contribution to target inhibition (Franco-Zorrilla et al., 2007).

In this review, we propose to emphasize miRNAs validated mainly by the latter approaches in the context of root development, the modulation of root architecture and root biotic interactions (Box 3), mainly using Arabidopsis and legume plants as models, because numerous studies presenting an exhaustive list of miRNA profiles in these biological contexts have been discussed elsewhere.

II. The roles of miRNAs in the specification of embryonic roots

Plants differ from animals by their continuous post-embryonic growth. Likewise, in plants, the embryo is very simple and does not contain adult organs, such as leaves, branches and lateral roots, but also lacks the reproductive lineages. Most studies have focused on Arabidopsis, in particular because embryo patterning from a single zygotic cell is easily accessible, and because of the very regular,

Box 2 Main features and properties of microRNAs (miRNAs) in plants
- They often target transcription factors.
- They are mostly found in multicellular families.
- They only exert a negative action on their target genes (mainly via transcript cleavage).
- Some may be sequestered by long non-coding RNAs (lncRNAs) or ARGONAUTE (AGO) proteins.
- The primary transcripts of miRNAs may also encode for small regulatory peptides (miPEPs).

Box 3 Main features and properties of microRNAs in roots

They participate in:
- Embryo and organ polarity.
- Meristem establishment and maintenance.
- Vasculature differentiation.
- Lateral and adventitious root formation.
- The regulation of symbioses (nodulation and mycorrhization).
predictable and reproducible cell divisions (for a review, see ten Hove et al., 2015). Root specification in the Arabidopsis embryo starts at the globular stage, where the uppermost suspensor cell, called the hypophysis, divides to furnish QC and columella precursor cells. The importance of miRNAs for correct embryogenesis has been highlighted using mutants impaired in miRNA accumulation, strong alleles in such genes often resulting in embryo lethality. Accordingly, c. 400 miRNAs were detected in the Arabidopsis embryo (Willmann et al., 2011), suggesting an important role for post-transcriptional regulation during this developmental stage.

The C2H2 zinc finger protein SERRATE (SE) is involved in miRNA processing and various se alleles have been associated with embryo patterning defect or lethality (Prigge & Wagner, 2001; Lobbes et al., 2006; Yang et al., 2006). Among the embryo defects observed using a se null mutant, aberrant cell division occurs in the hypophysis and, subsequently, QC precursor cells are not established and embryonic root development is impaired. At the molecular level, the accumulation of mature miRNAs is largely affected in se, in particular for miR165/166 (Grigg et al., 2005; Lobbes et al., 2006; Yang et al., 2006). The miR165/166 family targets members of the class III homeodomain leucine zipper (HD-ZIP): PHAVULOTA (PHV), PABULOSA (PHB), CORONA (CNA), REVOLUTA (REV) and ARABIDOPSIS THALIANA HOMEBOX-8 (ATHB8) (Emery et al., 2003; Mallory et al., 2004b). They are involved in numerous developmental processes, such as leaf polarity, vascular differentiation, and RAM and SAM functioning (reviewed in Byrne, 2006). During embryogenesis, PHV and PHB are expressed in the central apical domain at the globular stage (Grigg et al., 2009; Smith & Long, 2010), whereas miR165/166 are expressed more basally (Miyashima et al., 2013). Interestingly, the basal expression of PHV and PHB in the embryo results in the conversion of RAM into SAM, showing that the exclusion of PHV/PHB from this domain is necessary for the correct specification of root identity (Smith & Long, 2010). Accordingly, miR165/166 have been shown to act non-cell autonomously to restrict PHV and PHB from the base of the embryo (Miyashima et al., 2013). Another level of complexity has arisen from recent studies in which AGO10 sequesters miR165/166 and thus represses their activity in the expression domain of PHB and REV, thus competing with AGO1 which is necessary for class III HD-ZIP inhibition by miR165/166 (Zhu et al., 2011; Zhou et al., 2015a).

dcl1 mutants have also been associated with defective or lethal embryo formation depending on the mutation (Schwartz et al., 1994; McElver et al., 2001; Nodine & Bartel, 2010; Seefried et al., 2014). Among them, dcl1 null alleles failed to establish correct cell division at the base of the embryo, a defect resulting in an abnormal hypophysis (Schwartz et al., 1994; Nodine & Bartel, 2010). The loss of root identity is attested by the absence of the QC marker WUSCHEL HOMEOBOX5 (WOX5) in dcl1-5 globular embryos (Nodine & Bartel, 2010). Numerous miRNA targeted genes are upregulated in dcl1-5 embryos and mis-regulation of SQUAMOSA PROMOTER-LIKE 10 (SPL10) and SPL11 by miR156 could explain part of the dcl1-5 defects (Nodine & Bartel, 2010); it has also been proposed that another possible miRNA-target couple might also be involved in embryo patterning defects of dcl1 (Seefried et al., 2014).

III. The roles of miRNAs at the post-embryonic root meristem (Fig. 2)

Distally to the root SCN in the rootward orientation, the root cap fulfilling two main functions: the protection of the root meristem and SCN when foraging the soil, and the perception of gravitropism. The redundant AUXIN RESPONSE FACTORS 10 (ARF10), ARF16 and ARF17 are targeted by miR160 (Mallory et al., 2005; Wang et al., 2005). Simultaneous loss of function of ARF10 and ARF16 or miR160 overexpression leads to a significant decrease in root elongation and defective root cap formation, thus resulting in agravitropic roots. Interestingly, miR160 overexpression phenotypes are suppressed when expressing miR-resistant versions of

**Fig. 2** MicroRNAs (miRNAs) involved in root apical meristem functioning. Quiescent center (red) is surrounded by mitotically active initials (green). ARF, AUXIN RESPONSE FACTOR; bHLH, basic helix-loop-helix; GRF, GROWTH-REGULATING FACTOR; HAMs, Hairy MERISTEMS; HD-ZIP, homeodomain leucine zipper; NFYA, nuclear factor-YA; SCN, stem cell niche.
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2012; Heyman et al., 2014). After the first division of root initials in the shootward orientation, their progenitor cells enter into rapid and transit-amplifying cell divisions (Scheres, 2007). In Medicago and Arabidopsis, miR396 overexpression has been shown to reduce root elongation with possible contradictory effects when monitoring the root meristem size of plants either overexpressing or sequestering miR396 (Bazin et al., 2013; Rodriguez et al., 2015). However, in the last two studies, an increased miR396 abundance resulted in a reduced expression of cell cycle marker genes. The GROWTH-REGULATING FACTORS (GRFs) are conserved targets of the miR396 family in plants (Debeernardi et al., 2012), and have been shown to promote leaf growth and to be expressed in transit-amplifying cells at the root meristem where they repress PLT gene expression (Debeernardi et al., 2014; Rodriguez et al., 2015). Using a combination of elegant experiments, Rodriguez et al. (2015) deciphered an miR396/GRFs-PLT regulatory module to balance the division activities in the SCN and transit-amplifying cells. In this model, PLT activates miR396 in the SCN, which, in turn, downregulates GRF in this territory. Stabilized expression of GRFs (miR-resistant and miR396 mimicry) in the SCN will lead to distorted QC and columella cells. Accordingly, the exclusion of GRFs is proposed to be essential for formative periclinal cell divisions to occur in the SCN, as is observed in transit-amplifying cells when GRFs are downregulated or, conversely, by increasing PLT levels (Galinha et al., 2007). In the transit-amplifying cells, GRFs are necessary for correct spatiotemporal regulation of this mitotically active territory, notably through the inhibition of PLT expression. In conclusion, the mutual repression between GRF and PLT, with miR396 as an intermediate partner, is crucial for the correct positioning of SCN and transit-amplifying cells, mainly by balancing cell division activity (Rodriguez et al., 2015).

A recent study nicely highlighted the importance of the LOST MERISTEMS (LOMs)/HAIRY MERISTEM (HAM) in the control of plant SCNs, notably via interaction with the QC marker WOX5, but presumably also in a WOX5-independent manner (Zhou et al., 2015b). HAMs are GRAS transcription factors of the SCR-LIKE family required for both shoot and root indeterminacy (Stuurman et al., 2002; Engstrom et al., 2011). In Arabidopsis, four HAM copies exist, three of which (HAM1, HAM2 and HAM3) are cleaved by the miR171 family (Llave et al., 2002; Engstrom et al., 2011). They participate in a wide variety of developmental processes, such as the regulation of reproductive transition, meristem determinacy and trichome patterning in various species (Stuurman et al., 2002; Schulze et al., 2010; Wang et al., 2010; Engstrom et al., 2011; Curaba et al., 2013; David-Schwartz et al., 2013; Xue et al., 2014). Interestingly, miR171c overexpression reduces the primary root length, as is the case with simultaneous mutation in the HAM genes (Wang et al., 2010; Engstrom et al., 2011; Zhou et al., 2015b). Indeed, quadruple mutants for the HAM genes display aberrant QC and columella stem cells, resulting in root growth arrest, according to the detection of HAM2 transcriptional and translational fusions in the QC and root meristem (Zhou et al., 2015b). It remains to be shown how HAM restriction by the different miR171 members participates in root SCN maintenance as miR171 is expressed in Medicago and Arabidopsis roots (Wang et al., 2010; Laussery et al., 2015).

In addition to their roles during embryogenesis, miR165/166 also occupy a central role in the regulation of root development, in particular for the correct differentiation of the vascular elements and the regulation of meristem size. The SHR protein moves from the stele, its site of production, to the endodermis, where it activates the expression of miR165a and miR166b. In turn, miR165/166 diffuse in the central stele, thus establishing an opposite gradient to that of their class III HD-ZIP targets (Carlsbecker et al., 2010). Similar to the shr mutant, the miR-resistant phd-7d allele is characterized by the frequent replacement of protoxylem by metaxylem cells. Conversely, simultaneous mutations of phv, phd, can, rev and athb8 result in protoxylem instead of metaxylem cells. Interestingly, phb-shr double loss-of-function mutants display normal vascular patterning, and miR165a under the control of a ground-tissue specific promoter was able to rescue vascular defects of the shr mutant (Carlsbecker et al., 2010). Finally, using the green fluorescent protein (GFP) sensor system for miR165/166 actions, it has been confirmed that these miRNAs act non-cell autonomously in a dose-dependent manner (Carlsbecker et al., 2010; Miyashima et al., 2011). Together, the last two studies elegantly demonstrated that opposite gradients of miR165/166 and class III HD-ZIP are crucial for correct xylem differentiation (high miR165/166 and low HD-ZIP) on control of the mobile SHR diffusible signal. Collectively, this provides a case study in which mobile transcription factors, opposite gradients of miRNAs and targets participate in the establishment of sharp and robust developmental boundaries (Benkovics & Timmermans, 2014). In addition, a mathematical model showed that the integration of auxin, cytokinin (CK), SHR, PHB and miR165/166 can explain the establishment of the bisymmetric pattern in the root of Arabidopsis (Muraro et al., 2014). Recently, mi857 was shown to target LACCASE7 in Arabidopsis and, using both overexpression and mutant approaches, the authors revealed a negative role for miR857 in the regulation of lignin content and secondary xylem differentiation in Arabidopsis (Zhao Y et al., 2015).

phb-1d, another miR-resistant allele of PHB, displays a short root phenotype (Hawker & Bowman, 2004), and the root meristem size of both phb-1d and phv-1d alleles is reduced, opposite to that observed in phv-phb double mutants (Dello Ioio et al., 2012). As for the embryonic defects of se, phv-phb double mutations suppress the short root phenotype observed in se (Grigg et al., 2009), suggesting an additional and important role for the miR165/166 family in the regulation of root growth and meristem longitudinally. CK plays an important role in the control of root meristem size (Dello Ioio et al., 2007, 2008), and PHB has been shown to directly activate a CK biosynthetic gene ISOPENTYL TRANSFERASE7 (IPT7). In turn, the resulting CK inhibits...
miR165a transcription, thus building an incoherent feedback regulatory loop between CK, PHB and miR165a. This circuit has been proposed to regulate the balance between division and differentiation, on CK fluctuations in the roots, thus controlling meristem size (Dello Ioio et al., 2012). Recently, it has been proposed that IPT3, in addition to IPT7, might contribute to the activation of CK biosynthesis by the PHB–SHR circuit in this context of meristem size adjustment (Sebastian et al., 2015).

IV. The roles of miRNAs during lateral and adventitious root formation (Fig. 3)

In plants, RSA varies greatly and is highly plastic depending on the environment. Lateral and adventitious roots are distinguished based on their origin: root tissue or not, respectively (reviewed in Atkinson et al., 2014; Bellini et al., 2014). In dicots, the primary root generally arises from the embryonic radicle and branches into lateral roots of several orders and thus contributes to RSA establishment (taproots or allorhizic systems). In monocots, adventitious roots are of particular importance for root system establishment (fibrous roots or homorhizic systems). They derive from below-ground or underground shoot nodes, thus forming crown and brace roots, respectively. In Arabidopsis, adventitious roots derive from the hypocotyl or at the root–hypocotyl junction. These junction roots can be viewed as a rescue system when the primary root arrests or is damaged (Lucas et al., 2011). The formation of lateral and adventitious roots has been particularly studied in recent decades; it represents a post-embryonic root specification process from cells that have already been engaged into a differentiation route. In Arabidopsis, lateral roots form from the reactivation of pericycle cells facing the xylem poles that undergo anticlinal, periclinal and tangential cell divisions to form a primordium (reviewed in Péret et al., 2009). Despite the very similar patterning and final anatomy of lateral and adventitious roots, different molecular players contribute to their establishment in Arabidopsis (reviewed in Atkinson et al., 2014; Bellini et al., 2014). Although a series of elegant experiments have led to the formulation of two non-exclusive hypotheses to explain the mechanism of the early specification and patterning of lateral roots in Arabidopsis, their relative contributions and interplay are still a matter of debate (De Smet et al., 2007; Ditengou et al., 2008; Dubrovsky et al., 2008; Moreno-Risueno et al., 2010; Kircher & Schopfer, 2016). The availability of macro- and micro-nutrients is an important factor modulating RSA. Several miRNAs participate in the regulation of these processes from the modulation of nutrient uptake, translocation, sensing and assimilation to an adaptive response via the modulation of the root architecture. As exhaustive and recent articles have reviewed the miRNAs involved in N, P and potassium (K) homeostasis (Kulcheski et al., 2015; Nguyen et al., 2015), more attention is paid here to the miRNA-target nodes that participate in the modulation of root architecture. Many factors contribute to the regulation of lateral root formation, from preinitiation events to meristem activation (reviewed in Péret et al., 2009; Overvoorde et al., 2010; Petricka et al., 2012). Among them, the hormone auxin plays a central role (Lavenus et al., 2013) at three possible levels: homeostasis, transport and signaling.

Three ARFs are targeted by miR160: ARF10, ARF16 and ARF17 (Mallory et al., 2005; Wang et al., 2005). miR-resistant alleles of ARF16 and ARF17 exhibit reduced root branching, whereas double arf10-arf16 loss-of-function mutants and miR160c-overexpressing plants show the opposite defects (Mallory et al., 2005; Wang et al., 2005). ARF16 and ARF17 downregulation by miR160 thus exerts a positive role on lateral root development in Arabidopsis. Surprisingly, neither arf10-arf16 single loss-of-function mutants nor an miR-resistant ARF10 allele exhibits an obvious phenotype in terms of lateral root formation (Wang et al., 2005; Liu et al., 2007).

Other members of the ARF family involved in auxin homeostasis, namely ARF2, ARF3 and ARF4, are targeted by trans-acting short interfering RNAs (tasiRNAs; Allen et al., 2005; Williams et al., 2005). These tasiRNAs are produced from miRNA-mediated cleavage products of non-coding TAS transcripts. The
produced tasiRNAs will, in turn, trans-regulate target genes. For example, TAS3 is cleaved by miR390 and the resulting tasiRNAs negatively regulate ARF2, ARF3 and ARF4 in the context of polarity establishment of aerial organs and developmental timing (Fahlgren et al., 2006; Garcia et al., 2006; Hunter et al., 2006). A role of TAS3/miR390/ARF modules has been revealed in the context of lateral root formation (Marin et al., 2010; Yoon et al., 2010). miR390 expression is induced by auxin and a complex interplay between ARF2, ARF3 and ARF4 (Marin et al., 2010; Yoon et al., 2010). During lateral root initiation, miR390 is expressed in pericycle cells facing the xylem poles, and, in turn, will lead to tasiARF production that ultimately negatively regulates ARF2, ARF3 and ARF4 in lateral root primordia, thus allowing their correct growth (Marin et al., 2010). In summary, Marin et al. (2010) elegantly revealed a complex autoregulatory loop between miR390, tasiARFs, ARF2/3/4 and auxin to quantitatively regulate the growth of lateral roots in Arabidopsis.

The miR164 family is predicted to target five members of the NAC transcription factor family (for NAM, ATAF, CUC: NO APICAL MERISTEM, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR, CUP-SHAPED COTYLEDON, respectively; Guo et al., 2005; for a review, see Olsen et al., 2005). In addition to their roles in aerial development (Laufs et al., 2004; Mallory et al., 2004a; for a review, see Blein et al., 2010), one member of the NAC family, NAC1, positively regulates lateral root formation, notably via the transduction of the auxin signal downstream of an F-box auxin receptor, TRANSPORT INHIBITOR RESPONSE1, TIR1 (Ruegger et al., 1998; Xie et al., 2000; Guo et al., 2005). NAC1 is expressed in pericycle cells during lateral root initiation (Xie et al., 2000) and miR164 is induced by auxin, which, in turn, cleaves the NAC1 transcript (Guo et al., 2005). Using miR164a and miR164b mutants, miR-resistant NAC1 and the inducible expression system for both miR164 and NAC1, Guo et al. (2005) revealed an autoregulatory loop between auxin, miR164 and NAC1 for lateral root initiation, where miR164 acts as a negative regulator. Interestingly, a later study revealed a correlation between RSA, miR164 and NAC1 levels in maize (Li J et al., 2012), and miR164 expression is modified in response to N and P deficiencies in lupin and maize roots and leaves (Zhu et al., 2010). Together, these results might place the auxin/miR164/NAC1 loop as a conserved module participating in the adaptation of root architecture to nutrient availability.

The auxin receptors TIR1, AUXIN SIGNALING F-BOX PROTEIN 2 (AFB2) and AFB3 (also known as TAAR, for TIR1/AFB1 auxin receptors) are targeted by miR393a and miR393b in Arabidopsis (Jones-Rhoades & Bartel, 2004; Dharmasiri et al., 2005). AFB1 is partially resistant to miR393 cleavage, a property which might be a result of the mismatch in the miR393 binding site of AFB1, and is repressed by miR393 at the translational level (Navarro et al., 2006; Parry et al., 2009). Parry et al. (2009) also demonstrated that tir1-1 mutants, as miR393a- and miR393b-overexpressing lines, are less responsive to auxin in the stimulation of lateral roots. Interestingly, auxin has long been proposed to be involved in the integration of the nitrate signal into the modulation of root development (Forde, 2002; Walch-Liu et al., 2006). miR393 and AFB3 have been shown to be upregulated in Arabidopsis following nitrate treatment (Vidal et al., 2010). In particular, miR393 was induced specifically in roots 2 h following nitrate treatment. According to this and by contrast with AFB3, only reduced and assimilated (so-called) nitrate, and not nitrate treatment itself, led to miR393 induction. In addition, miR393 overexpression, as a null afb3 mutation, suppresses root modification following nitrate treatment: (1) inhibition of root elongation and (2) stimulation of lateral roots. Interestingly, none of the other miR393 targets (TIR1, AFB1 and AFB2) are necessary for the establishment of RSA modulation triggered by exogenous nitrate supply. This apparently incoherent AFB3/miR393 feed-forward loop might provide a mechanism by which miR393 can be rapidly and precisely adjusted to regulate its target AFB3. In other words, this may provide a robust mechanism for rapid adaptation of RSA depending on the fluctuating internal and external nitrate concentrations (Vidal et al., 2010). pri-miR393b is also induced following auxin treatment (Chen et al., 2011). Overexpression of a miR393-resistant form of TIR1 significantly increased lateral root number and, conversely, either tir1 null mutation or miR393a/b overexpression is associated with reduced lateral root number (Chen et al., 2011). miR393b is the predominant source of miR393 in all aerial organs studied so far (Si-Ammour et al., 2011), and miR393a and miR393b both contribute to miR393 production in roots (Windels et al., 2014). In the latter study, it was also shown that AFB2 and AFB3 degradation by miR393 led to the production of secondary short interfering RNAs (si-TARs) using tasiRNA pathways (Si-Ammour et al., 2011). The TAAR-miR393 modules have also been shown to be required for: (1) adaptive root growth inhibition promoted by osmotic stress or ABA treatment (Chen et al., 2012); (2) root acclimation to saline stress via the regulation of redox status and auxin signaling (Iglesias et al., 2014); and (3) correct establishment of auxin signaling output (Windels et al., 2014). As nitrate treatment induced the auxin synthetic responsive construct DR5:GUS in Arabidopsis (Vidal et al., 2010), the possible crosstalk and interference between miR393, its targets, auxin, and internal and external nitrate concentration still need to be deciphered in detail. Moreover, by combining computational and experimental data, it was shown that NAC4, which is also regulated by the miR164 family, acts downstream of AFB3 for the root adaptation to nitrate, at least for the modulation of lateral root formation, in an NRT1.1-dependent manner (Vidal et al., 2013, 2014), thus adding another layer of interplay between nitrate homeostasis, TIR1/miR393 and NAC1/miR164 modules in the regulation of root architecture (Xie et al., 2000, 2002).
requirement of three auxin responsive genes between these three ARFs, their miRNAs, light and adventitious genetics, overexpression and quantitative expression analyses, the authors demonstrated a complex interplay and feedback loops between these three ARFs, their miRNAs, light and adventitious root formation. Schematically, ARF6 and ARF8 stimulate adventitious root formation, whereas ARF17 does the opposite, and their mutual regulation and post-transcriptional repression by miR167 and miR160 are crucial for the correct formation of shoot-borne roots (Gutierrez et al., 2009). A subsequent study demonstrated the requirement of three auxin responsive genes GRETCHEN HAGEN 3 (GH3) and jasmonate homeostasis downstream of this complex ARFs/miR160/miR167 network (Gutierrez et al., 2012). miR167a also targets IAA-Ala RESISTANT 3 (IAR3) on osmotic stress to adapt root architecture, whereas ARF6 and ARF8 cleavage by miR167 appears to be dispensable for this plant adaptive response (Kinoshita et al., 2012). IAR3 is an enzyme responsible for the conversion of an inactive auxin form, Ala-IAA, into the bioactive auxin IAA (Davies et al., 1999). On high osmotic stress, miR167a and miR167b, and IAR3, are downregulated and upregulated, respectively. According to this, iar3 mutants have reduced IAA levels and lack the root architecture modifications triggered by osmotic stress. Conversely, overexpression of miR167-resistant IAR3 led to increased IAA accumulation and increased lateral root development. Finally, a positive role for IAR3 in drought stress tolerance was demonstrated, and Kinoshita et al. (2012) proposed a model with specific miR167 regulation depending on the tissue and stress (roots vs aerial, osmotic vs N supply). This last example illustrates the importance of the biological context in the regulation of different targets by the same miRNA family.

INDOLE-3-ACETIC ACID INDUCIBLE 28 (IAA28) is an Aux/IAA repressor initially identified in a genetic study of the iaa28-1 gain-of-function allele. IAA28 has recently been shown to be necessary for the transcriptional repression of auxin-inducible genes required for lateral root promotion (Rogg et al., 2001). IAA28 is post-transcriptionally regulated by miR847, a low abundant miRNA that is induced on auxin treatment (Wang & Guo, 2015). Accordingly, miR847 overexpression and the iaa28 null mutant exhibited more lateral roots, whereas overexpression of the IAA28-1 gain-of-function allele or miR847-resistant IAA28 had a negative impact on the lateral root number (Wang & Guo, 2015). Interestingly, post-transcriptional cleavage of IAA28 triggered by miR847 is synergistic to the proteasome degradation of Aux/IAAs, and both degradation processes are stimulated by auxin, thus providing a robust degradation mechanism. Using a somatic embryogenesis assay, the authors proposed that IAA28 degradation affects lateral organ growth via the regulation of cell proliferation.

Many other studies have suggested the potential implication of miRNAs in the regulation of lateral roots. For example, miRNA participation in the homeostasis of macronutrients (uptake, assimilation, translocation, etc.) might, in fact, modify RSA through an indirect mechanism, as the perception of nutrient concentration and their assimilates has a drastic effect on root growth. Small RNA profiling studies have also suggested the implication of some miRNAs in the regulation of lateral roots in response to nutrient starvation. For example, Liang et al. (2012) proposed that the combined action of miR160-ARF10/16/17, miR167/ARF6/8 and miR171-HAM1/2/3 modules might be important for the modulation of RSA on N starvation. The miR171 family and their HAM targets might be suspected to play a role in the regulation of lateral root formation, as miR171b is expressed in lateral root primordia and miR171b overexpression reduces the lateral root density in Medicago (Lauressergues et al., 2015). In the same line, miR396 detection in Arabidopsis and Medicago lateral root primordia and the reduced root dry weight of MIM396 plants in Medicago might favor a role for miR396 in the formation of lateral roots (Bazin et al., 2013; Bao et al., 2014). In Medicago, miR166a overexpression led to a reduced lateral root number (Boualem et al., 2008), as is the case in the triple phb-phv-rev mutant in Arabidopsis (Hawker & Bowman, 2004). According to this and the increased lateral root density in the miR165/166-resistant rev-10d allele, the miR165/6-HD-ZIP module might also be suspected to play a role in the regulation of lateral root formation, in addition to those well established in the longitudinal and radial patterning of RAM in Arabidopsis.

V. The roles of miRNAs during root endosymbioses

The mycorrhization process has been studied for decades because of its wide distribution in the plant lineage, its ecological importance and the advantages it provides in terms of nutrition and stress tolerance. This process was first studied in a plant–microbe interaction perspective, but can also be viewed as a dynamic and reversible developmental process for the plant cell. Indeed, root cortical cells that are invaded by arbuscules are already engaged in a defined differentiation stage. On P deficiency, these cells can host fungi based on molecular exchanges between the two partners that ultimately lead to cell specialization. Conversely, arbuscules can collapse within a few days, leaving cortical cells devoid of branched arbuscules (Kobae et al., 2016). For this reason, it would not be surprising to unravel an increasing number of developmental regulators participating in this particular cell differentiation program and reversible infection process. Among them, miR396, which is known to regulate GRF transcription factors, basic helix-loop-helix (bHLH) and cell proliferation activity (see above), has also been shown to regulate the mycorrhizal colonization rate in Medicago (Bazin et al., 2013). Indeed, Medicago plants overexpressing or sequestering miR396 are characterized by decreased and increased colonization rates, respectively, thus defining an inhibitory role for miR396 on the mycorrhization process. To our knowledge, it remains to be shown which of the miR396 targets participate in the regulation of fungal colonization. Low auxin levels have also been shown to stimulate mycorrhization in Medicago, tomato and rice according to DR5::GUS induction in arbuscules and pre-miR393 repression during mycorrhization (Etemadi et al., 2014). However, the targets of miR393, AFB/TIR1, auxin transport and other signaling elements involved during mycorrhization still need to be further investigated. The GRAS transcription factor, NODULATION SIGNALING PATHWAY 2 (NSP2), was first isolated on the basis of its essential role for the formation of symbiotic nodules in the Legume family (Oldroyd & Long, 2003). Later, it was shown that NSP2 is also required for mycorrhizal colonization and is regulated by miR171h.
Legume nodules are generally root-derived organs, and although they are formed on stems, their formation is tightly linked to shoot-borne root primordia. Two main classes of nodule can be distinguished based on the persistence or not of a nodule meristem: indeterminate nodules (e.g. in Medicago) and determinate round-shaped nodules (e.g. in soybean, bean and Lotus). Despite the anatomical differences between roots and nodules, the nodulation program has co-opted part of the root developmental program (Couzigou et al., 2012, 2013; Franssen et al., 2015). The nodulation signaling pathway has also been acquired through the co-option of the mycorrhizal signaling pathway. It is interesting to note here that both mycorrhizal fungi and rhizobia, as their symbiotic signals (Myc and Nod factors), trigger lateral root organogenesis (Mailet et al., 2011). As for lateral roots, nodule initiation and organogenesis are tightly controlled processes, in which miRNAs have been reported to participate. Excellent reviews have described the nodulation signaling program compared with that of the lateral roots (Desbrosses & Stougaard, 2011; Oldroyd et al., 2011). In Medicago, miR166 targets a class III HD ZIP transcription factor, and has a negative effect on both lateral root and nodule formation when it is overexpressed (Boualem et al., 2008). Overexpression of miR166 also leads to aberrant patterning of vascular bundles in roots (multiplication of xylem poles), according to the well-known functions of the miR166/HD-ZIP module in this latter process in Arabidopsis (see earlier). miR169 regulates HAP2-1, a transcription factor of the NFY-A family (Combier et al., 2006). The overexpression of miR169 as RNA interference against its target, HAP2-1, leads to delayed nodule formation and arrested meristem development, thus resulting in non-fixating round-shaped nodules. By contrast, plants expressing a miR169-resistant version of HAP2-1 develop nodules associated with a defect in nodule apical growth, thus resulting in shorter nodules, defining an important role for HAP2-1 restriction during the course of nodule organogenesis. Interestingly, the miR169/e/h-g-NFYA2/10 modules have also been shown to regulate root architecture in Arabidopsis (Sorin et al., 2014). Soybean is perhaps the legume model for which most examples of miRNAs involved in the regulation of nodulation exist. Indeed, several miRNAs affecting nodule number have been identified recently (Li et al., 2010; Turner et al., 2013; Wang et al., 2014, 2015; Yan et al., 2015). In these latter studies, overexpression of miR167c, miR172c, miR482, miR1512, miR1515 and miR2606b has a positive effect on nodule formation in soybean, whereas overexpression of miR156, miR160 and miR4416 has the opposite effect. The miR172c-AP2 module has been shown to play a similar role during nodule organogenesis in Phaseolus vulgaris (Nova-Franco et al., 2015), and recent evidence suggests a similar situation in Lotus japonicus (Holt et al., 2015). Generally, these studies have combined miRNA overexpression, miR-resistant target constructs and miRNA mimicry to unravel the miRNA-target module in the symbiotic process. It should be noted here that an miRNA affecting the indeterminate type of nodule does not necessarily affect determinate nodule development, in particular in terms of the regulation of auxin signaling (Mao et al., 2013) (Fig. 5). During infection by soil nematodes, root cells are also reprogrammed, and some studies have suggested local and systemic implications of miRNA-target modules in the establishment of this interaction (Hewezi et al., 2008; Li X et al., 2012; Zhao W et al., 2015). Finally, small RNA profiling studies have suggested the implication of miRNA post-transcriptional regulation in the context of infection by root pathogens (Radwan et al., 2011; Verma et al., 2014). However,

![miRNA Distribution](image.jpg)

**Fig. 4** MicroRNAs (miRNAs) involved in the control of mycorrhization. Photograph of a lateral root colonized by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. Red arrows indicate a negative role on the process. Note: the targets and the connection between miRNA/target modules have been omitted for clarity. The fungal structures (green) have been stained with ink (see Lauressergues et al., 2012).

![miRNA Distribution](image.jpg)

**Fig. 5** MicroRNAs (miRNAs) involved in the control of nodulation. Red arrows, negative role on the process; green arrows, positive role on the process. Note: the targets and the connection between miRNA/target modules have been omitted for clarity.
functional studies still need to be performed to investigate the roles of these miRNA-target modules in these detrimental biotic interactions.

VI. Concluding remarks and future perspectives

Since their initial discovery in the early 1990s (Lee et al., 1993; Wightman et al., 1993) and their distinction as a new class of regulatory molecules in the early 2000s (Pasquinelli et al., 2000; Reinhart et al., 2000, 2002; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001; Llave et al., 2002), miRNAs have emerged as widespread regulatory RNAs in most, if not all, biological processes in plants and animals. Nowadays, they are crucial actors in the regular patterning of embryo and adult organisms, and also in the adaptation to fluctuating biotic and abiotic environments. The simple scenario in which an miRNA represses gene expression via transcript cleavage or translation inhibition seems to be even more complex today with the emergence of additional players in this scheme: activating miRNAs, pseudo targets, miRNA sponges, target mimicry, miRNA-encoded peptides (miPEPs) and, finally, many other classes of non-coding RNAs, such as tasiRNAs and long non-coding (lnc) RNAs (for reviews see Cech & Steitz, 2014; Guil & Esteller, 2015). In addition, the regulation of miRNA biogenesis and turnover is made up of extremely complex and tightly controlled mechanisms. One breakthrough was the discovery of IPS1 IncRNA, which sequesters miR399 and thus antagonizes the cleavage of the miR399 target, PHO2 (Franco-Zorrilla et al., 2007). Since then, the proposed artificial strategy to sequester any miRNA isoform via a target mimic has been successfully and widely used. It appears that other miRNA endogenous target mimics (eTMs) may also exist in plants; most of the miRNAs discussed above might have an eTM in Arabidopsis (Wu et al., 2013). In plants, recent studies have highlighted the importance of IncRNA regulation (Ariel et al., 2015) and have shown that a significant amount of IncRNAs (as miRNAs) can be loaded into ribosomes, thus suggesting a possible role in translational regulation for these so-called non-coding transcripts (Juntawong et al., 2014). In animals, there is also increasing evidence for regulatory peptides derived from IncRNA transcripts, first thought to be non-coding (Payre & Desplan, 2016). Recently, we have shown that miRNA transcripts can also produce small regulatory peptides, called miPEPs, which, in turn, favor the accumulation of their corresponding miRNAs (Lauressergues et al., 2015; Couzigou et al., 2016). This latter finding needs to be studied in other miRNA families and species to determine the extent of this mechanism. If widespread, these new regulatory peptides might also serve as tools for the study of separate miRNAs from the same family because of their apparent specificity (Couzigou et al., 2015). A major challenge will be to decipher how this miRNA-miPEP couple might be transcriptionally regulated and how these peptides might act on their miRNA. In the context of plant development, several miRNAs have been shown to act as mobile signals, thus resembling animal morphogens (Benkovics & Timmermans, 2014). However, the regulatory and selectivity mechanism by which a miRNA moves or not is still not clear. Indeed, opposite gradients of miRNA and targets could be required for the correct determination of root vasculature, SAM and embryogenesis. This non-cell autonomous action of miRNA has been shown to be tightly controlled by the opposite action ofAGO1 and AGO10 in Arabidopsis (Zhu et al., 2011; Zhou et al., 2015a). A similar mechanism in the context of root development has not yet been elucidated. Another challenge for miRNAs with dual roles in the regulation of root meristem homeostasis and lateral roots will be to dissect their contribution at the lateral root level locally, and possibly from their role in the meristem, a region that is close to the site of lateral root priming. A common feature of miRNA-target modules is the presence of incoherent regulatory loops and the complex interplay between these modules. The existence of incoherent regulatory loops can, however, be interpreted as a possible mechanism to provide robustness and a rapid adaptability to regulatory gene networks (Li et al., 2009; Osella et al., 2011). The global study of these miRNA-target modules, their interconnection and their biological relevance in response to perturbed environments (e.g. single vs multiple nutrient deprivation) is now possible in the era of systems biology and the tools that have been developed. From an evolutionary perspective, it is not surprising to find miRNA-target nodes involved in both shoot and root regulation in the light of the ‘shoot–root dichotomy hypothesis’, where the root morphogenetic program can be thus considered as a co-opted shoot program. Moreover, the conservation of such miRNAs in basal embryophytes (Cupera et al., 2011; Jones-Rhoades, 2012) that do not have roots or even vascular elements raises the question of their functions in organisms with such different lifestyles. More extensively, it might be interesting to study the miRNA-target nodes involved in embryogenesis, developmental timing and reproductive development in species with an opposite balance of diploid and haploid phases. The development of efficient model systems in each of these clades will undoubtedly allow the study of miRNAs in an evolutionary and developmental perspective in future years. The study of the relative roles of each miRNA isoform and their associated targets present in various numbers among species also represents an interesting framework for our understanding of miRNA evolution. After almost two decades of intense research in the non-coding RNA field, it appears that many elements still need to be, and will be, discovered. In other words, and as mentioned in a recent review summarizing 50 yr of Arabidopsis research (Provart et al., 2016), Churchill’s famous sentence about World War II can also apply to the non-coding RNA field: ‘This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning’.

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