“And yet it moves”: Cell-to-cell and long-distance signaling by plant microRNAs

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Abstract

MicroRNAs (miRNAs) are key regulators of numerous genes in many eukaryotes. Some plant miRNAs are involved in developmental and physiological processes that require intercellular or inter-organ signaling. Movement of other small RNAs within plants has been established. Recent findings also demonstrate intercellular signaling by miRNAs and strongly support that a subset of these regulatory molecules move from one cell to another or over long distances. Phloem exudates contain diverse miRNAs and at least two of them, involved in responses to nutrient availability, are transmitted through grafts, indicating long-distance movement. Two miRNAs that regulate developmental processes are present in cells outside their domains of expression. Several results strongly support that one of them moves from cell to cell. Research on a mutant affected in plasmodesmata trafficking indicates that these intercellular channels are required for transmission of miRNA activity to adjacent cells. Moreover, ARGONAUTE proteins might be involved in the regulation of miRNA trafficking. Hypothesis on the features and mechanisms that may determine miRNA mobility are presented. Future challenges include identifying other mobile miRNAs; demonstrating that miRNA movement is required for non-cell autonomous action; and characterizing the mechanisms of translocation and genetic pathways that regulate miRNA movement.

Keywords: microRNA, miRNA movement, long-distance signaling, cell-to-cell signaling, phloem transport

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1. Introduction

Since the first reports of microRNA (miRNA) from the nematode *Caenorhabditis elegans* in 1993 [1,2], our knowledge of the biogenesis, functions and mechanisms of action of small non-coding RNAs has rapidly advanced. Small RNAs are produced from double-stranded RNA (dsRNA) precursors or from single-stranded RNA (ssRNA) molecules that fold into a structure that is partially double stranded. In plants, these precursors are cleaved by DICER-LIKE (DCL) ribonucleases, generating 21–24 nt RNAs. Small RNAs are loaded into RNA-induced silencing complexes (RISCs), which contain a member of the ARGONAUTE (AGO) protein family and direct the small RNAs to complementary RNAs or DNAs. RISCs silence gene activity by at least three mechanisms: mRNA cleavage, inhibition of translation and chromatin modification through DNA methylation [3–6]. Small RNAs are involved in virtually all aspects of plant biology, including development, responses to biotic and abiotic stresses, nutrient allocation and maintenance of genome integrity [7–9].

There are two main classes of small RNAs in plants: small interfering RNAs (siRNAs) and microRNAs (miRNAs) [3–6]. siRNAs are produced from dsRNAs derived from endogenous repeated sequences, convergently transcribed RNAs or transposons, or from exogenous transgenes and viral sequences. Most of these dsRNAs are diced by DCL2, DCL3 or DCL4, producing 21, 22 or 24-nt siRNAs, which are sorted into AGO1, AGO4, AGO6 or AGO9 RISCs that induce transcriptional or post-transcriptional silencing of complementary sequences [5,10]. Long (30–40 nt) siRNAs

also exist [11] and binding of these or other siRNAs to additional AGOs has been shown [11–13].

MIRNA genes are transcribed by RNA polymerase II as primary transcripts (pri-miRNAs), which contain a hairpin structure and are usually cleaved by DCL1, yielding pre-miRNAs. These are cleaved again by DCL1 producing a duplex comprising the mature miRNA imperfectly base-paired to a miRNA* strand. Mature miRNAs, which are approximately 21-nt long, are usually incorporated into AGO1-containing RISCs that silence endogenous mRNAs [3,6]. Longer miRNAs processed by DCL3 and sorted into AGO4 complexes have also been reported [14]. A few miRNAs bind to transcripts produced from endogenous non-coding genes, known as TAS genes, which are also transcribed by RNA pol II. Upon binding of a miRNA, TAS transcripts are cleaved by an AGO1 or AGO7-containing RISC. The cleaved RNA is used as a template by an RNA-dependent RNA polymerase, RDR6, producing a dsRNA, which is in turn cleaved by DCL4 giving rise to several phased 21 nt siRNAs, called trans-acting siRNAs (tasiRNAs). TasiRNAs target transcripts unrelated to their precursors through binding to AGO1 [4,6].

The intricate miRNA world includes: (a) families of very closely related miRNAs; (b) identical mature miRNAs encoded by different genes; (c) transcriptional regulation of MIRNA genes; (d) differential processing of precursors; (e) targeting of multiple genes; (f) sequential action of miRNAs; (g) production of tasiRNAs; (h) target mimics, which are non-coding RNAs that sequester miRNAs through partial complementarity; (i) alternative splicing of target mRNAs that can delete or create miRNA binding sites; and

(j) several modes of RNA silencing [6,15–17]. More richness has been added to this landscape with the recent finding that miRNAs can act in a non-cell autonomous manner, that is, in cells in which they are not produced (reviewed in [7,18]).

Intercellular communication, mediated by diverse molecules, is essential for coordinated development and responses to environmental challenges [19–23]. Much evidence has accumulated on short- and long-range movement of siRNAs [5,10], but in the case of miRNAs there were reports suggesting that they could act non-cell autonomously within a short range [24–27], and others indicating that they act only in the places where they are produced [28–30]. This has led to the general assumption that miRNAs act mostly in a cell autonomous manner [6,30,31].

This review focuses on recent reports that question this view by providing strong evidence of short and long-distance signaling by plant miRNAs, and indicating movement of these small RNAs [24,32–39].

2. Cell-to-cell signaling by plant miRNAs

Plasmodesmata, the channels that connect the cytoplasm of adjacent cells, allow the passive diffusion of water, small solutes and the selective intercellular movement of macromolecules and viruses [40]. Plasmodesmal aperture, or size exclusion limit, varies among tissues, developmental stages and environmental conditions and can be modified by actin filaments, myosin, regulatory proteins and callose deposition in the surrounding cell wall [40,41]. Both mRNA and siRNA move from cell to cell and data suggest that

this movement occurs through plasmodesmata [5,10,42]. The first proposals that a miRNA can translocate to adjacent cells came from studies of miR165/166 in maize and *Arabidopsis thaliana* [25,26]. In addition, the effects of some artificial miRNAs are evident outside their expression domains [24,27]. Recent results strongly support the notion that endogenous miRNAs move intercellularly.

2.1. miR390 acts non-cell autonomously

Several developmental events involve the action of miR390, which can be derived from one to four loci (MIR390a, MIR390b, MIR390c and MIR390d) in different plants. The mature miR390 species produced from these genes are identical. miR390-guided cleavage of non-coding TAS3 transcripts depends on a specific RISC that contains AGO7 [4,6]. This cleavage triggers the production of tasiRNAs, a subset of which, named tasiR-ARFs, target three members of the transcription factor family AUXIN RESPONSE FACTOR (ARF), ARF2, ARF3 and ARF4. The miR390/TAS3/tasiR-ARF/ARF pathway plays roles in dorso-ventral leaf patterning, juvenile-to-adult phase transition, floral organ development and lateral root development [35,43–45]. Although single *Arabidopsis* ago7 mutations do not alter adaxial/abaxial (upper/lower) leaf patterning [46], they cause leaf polarity phenotypes in combination with mutations weakly affecting leaf morphology [44,47]. Maize mutants defective in tasiR-ARF biogenesis also exhibit radialized leaves [48,49].

To determine whether mobility of small RNAs contributes to specification of the adaxial/abaxial polarity in leaves, the places of expression and activity of tasiR-ARFs

were compared with those of the factors required for tasiR-ARF production in *Arabidopsis* [33]. The miR390 precursor loci, MIR390a and MIR390b, are transcribed in the vascular system and pith region below the shoot apical meristem, but not in the meristem itself or very young leaf primordia. In developing leaves they are expressed preferentially on the adaxial side. Both transcripts also accumulate on the abaxial epidermis of leaves, a place where their transcription is not detected. This may suggest movement of the precursors, although the authors propose differential processing between the epidermis and the underlying cells. Mature miR390 is present where its precursors accumulate, but also in the shoot apical meristem and throughout very young leaf primordia, strongly suggesting that this miRNA is a mobile molecule [33]. In addition, a lack of agreement between the accumulation of miR390 precursors and mature miR390 in the maize shoot apex suggests that this miRNA might also move in maize [37].

The mobility of miR390 has also been explored in roots. Lateral root formation is initiated from pericycle cells in response to the local accumulation of auxin [50]. miR390 is initially expressed before the initiation of lateral root primordia and then extends and overlaps with auxin accumulation. As the lateral root primordium develops, miR390 expression is restricted to the base and flanks of the primordium and is excluded from the centre. However, specific miR390 activity is detected in the whole primordium, indicating that miR390 acts beyond its domain of expression. This suggests that miR390 moves across a few cells in the root [35]. Other research, however, indicates that miR390 might be expressed in the whole primordium [51] and, therefore, additional work is required to test whether miR390 moves in roots.

Results indicating that tasiR-ARFs probably move in leaf primordia and the shoot apical meristem [31,33], and that they act in root cells in which TAS3 is not transcribed [35] have been reported. Since non-cell autonomous activity of tasiRNAs is also supported by other works [24,30] and TAS3 transcripts do not move in leaves [33], it seems plausible that tasiR-ARFs, rather than TAS3 transcripts, are transmitted from cell to cell.

Although intercellular traffic of tasiRNAs is more widely accepted than that of miRNAs [6,7,30,31], the experimental support for cell-to-cell transfer of miR390 and tasiR-ARFs in shoot apical meristem and leaf primordia is similar [31,33,37]. Both accumulate and act in places in which their precursors are not detected. Therefore we do not find any objective reason to give more credibility to the movement of tasiR-ARFs than to that of miR390 in the shoot apex. The use of sensor constructs that carry miR390 or tasiR-ARF binding sites in the context of a reporter sequence completely unrelated to the endogenous target transcripts [31,35] is a good strategy to exclude the possibility of indirect effects on their targets. Further experiments, however, are required to fully demonstrate that miR390 and/or tasiR-ARFs are transmitted and to test the relevance of this movement.

2.2. Intercellular signaling by miR165/166

Several Arabidopsis genes encode miR165 and miR166, which differ by a single nucleotide. In other species, like maize, several MIR166 genes also exist, but miR165 has not been found. These miRNAs are involved in meristem formation, establishment

of adaxial/abaxial leaf polarity, radial root patterning and vascular specification through down-regulation of class III homeodomain-leucine zipper (HD-ZIP III) transcription factors [21,52]. At very early stages of maize leaf development, miR166 accumulates mainly on the abaxial side and, as leaves develop, it extends to a broader domain, forming a gradient from the abaxial to the adaxial surface [25]. This gradient, together with the presence of miR165/166 in phloem cells and the effect of ago1 mutations on the spatial distribution of miR165/166, led to the proposal that this miRNA might move from its site of expression [25,26]. This hypothesis has received very strong support from several papers that study the function of miR165/166 in root patterning [32,36,39].

Reciprocal signaling between the vascular tissue and the surrounding cell layers in *Arabidopsis* roots specifies the patterning of root tissues. This signaling is achieved through the movement of a transcription factor in one direction and a miRNA in the opposite direction [32]. Approaches similar to those employed for miR390 and tasiR-ARFs (see above) were elegantly combined with genetic analyses of miR165/166 targets and genes regulating transcription of MIR165/166 precursors [32]. MIR165a and MIR166b are transcribed mainly in the endodermis and there is no detectable transcription in the pericycle. Mature miR166 accumulates and is active not only in the endodermis, but also in the surrounding cell layers. This strongly suggests that miR165/166 moves from the endodermis to adjacent cell layers. Thus, miR165/166 suppresses HD-ZIP III mRNAs in the pericycle and endodermis and restricts the activity of these transcription factors to the vascular cylinder. This restriction is required for proper xylem patterning, such that a high dose of HD-ZIP III specifies metaxylem at the centre of the vascular cylinder and a low dose specifies protoxylem in the periphery

[32] (Fig. 1). Expression of the remaining MIR165/166 genes was not detected by one group in roots [32], but other reports showed that MIR166a and MIR166g are expressed there [36,53]. MIR166a is specifically expressed in the endodermis and quiescent centre [36]. A detailed analysis of the expression pattern of MIR166g in root tissues would be required to rule out that mature miR166 present outside the endodermis derives from expression of this precursor.

Experiments using a mutated form of a MIR165 gene (MIR165mu) and a mutated version of the miR165/166 target gene PHABULOSA (PHB) bound by miR165mu indicate that mobile MIR165 gene products generated in the endodermis exert their function in recipient pericycle and xylem cells [36]. This is corroborated by the fact that ectopic expression of miR165 in the stele is sufficient to down-regulate PHB and cause protoxylem development in the place of metaxylem, a phenotype observed in double or triple *hd-zip III* mutants [32,36]. The effect of a mutated MIRNA gene beyond its domain of expression is a demonstration that MIRNA gene products move, although this does not demonstrate translocation of the mature miRNA. It can also be argued that the use of a mutated MIRNA gene does not necessarily reflect what the endogenous gene does. However, together with all the other evidence pointing to movement of mature miRNAs, the transmission of the effect of miR165mu from cell to cell makes a strong case for the movement of mature miRNA.

2.3. *The miR390 pathway regulates miR165/166*

MiR390, together with TAS3/tasiR-ARFs, and mature miR166 localize in essentially complementary domains in young leaf primordia of maize [37,48]. Analysis of the accumulation of MIR166 primary transcripts and mature miR166 in a mutant defective in tasiRNA production indicates that the miR390/tasiR-ARF pathway is involved in the regulation of miR166 expression [48]. The following model has been proposed (Fig. 2): the distribution of miR390, which might result in part from intercellular movement, together with the localization of factors involved in tasiRNA biogenesis, would determine the production of tasiR-ARFs on the adaxial side of the leaf. These small RNAs move from the adaxial to the abaxial leaf domain, establishing a gradient along the adaxial/abaxial axis. This gradient accurately defines the adaxial/abaxial boundary and limits the spatial localization of ARF proteins and miR166 to the abaxial side. ARFs specify abaxial fate and miR166 restricts the HD-ZIP III transcription factors, which specify adaxial fate, to the adaxial domain [33,37,48]. Thus, the difference between the adaxial and abaxial regions of the leaf would be, at least in part, a consequence of the movement of small RNAs. It will be interesting to test whether miR165/166 moves in leaves, as had been suggested [25,26], although, in principle, the tasiR-ARF gradient might establish an opposite miR166 distribution pattern. Expression of a viral protein that affects cell-to-cell trafficking causes severe defects in leaf polarity [54], which is consistent with the notion that small RNAs move in leaves.

By moving from cell to cell, a small RNA may act as a morphogenetic signal that establishes patterns of cell specification by creating boundaries that limit the activity of target genes [33,36,37]. In some cases, a threshold level below which the miRNA is not able to silence its targets may exist. Computational pre-dictions indicate that miRNA

gradients can help define sharp boundaries for miRNA targets [55]. This seems to be the case for miR390, tasiR-ARFs and their target ARF3 [33]. The situation seems more complex for miR165/166. The regulation of PHB mRNA levels by this miRNA is dose dependent, but the boundaries for the accumulation of the five miR165/166 target genes are different [32,36]. Several possibilities might explain this: different cleavage efficiencies for miRNA-target gene combinations due to different degrees of complementarity; different expression levels of the target genes; or differential mobility of miRNA family members. Future research should address all these questions.

Altogether these results indicate that members of at least two miRNA families, miR165/166 and miR390, together with miR390-dependent tasiRNAs, act non-cell autonomously and probably move [31–33,35–37,39]. However, the techniques used have some limitations that must be taken into account. Firstly, most methods used to detect mature miRNAs do not distinguish between very similar mature miRNAs derived from different genes. Second, it is not yet clear how faithfully the reporter and sensor constructs show the real pattern of miRNA expression or activity. There are inconsistencies between laboratories in the patterns of MIR390 expression and response to auxin [35,51] and in the expression of several MIR166 genes in the root [32,36,53]. Third, most of these approaches are not quantitative and are limited by the sensitivity and resolution of detection systems, so that low levels of a miRNA precursor might remain unnoticed. Also, factors other than small RNAs may affect the accumulation of the target or reporter constructs [33]. This highlights the need to use more than one method to confirm the results obtained and to perform additional experiments to test mobility.

3. Long-distance movement of plant miRNAs

Plant survival requires an accurate transport of nutrients, water and signaling molecules over long distances. Long-range signaling has a major role in the regulation and coordination of different biological processes, including flowering, leaf and vascular development, resource allocation, pathogen defense and silencing of RNAs.

Metabolites, hormones, proteins and nucleic acids, including mRNAs and small RNA species, act as cell-to-cell or systemic signals that provide an efficient exchange of information between tissues [5,10,19,22,56–59]. In this section we discuss the long-range movement of miRNAs. The importance of this transport is related to the function of miRNAs as negative regulators of genes involved in processes that require long-distance signaling [21,23,57,60,61].

3.1. miRNAs are present in phloem exudates

The vascular system, in particular the phloem, provides the pathway for the systemic translocation of macromolecules [19,22]. Although long-distance movement of mRNAs through the phloem is well documented in diverse species [22,56], less is known about miRNA movement. Given that phloem sieve elements have lost their capability to transcribe and translate, the RNAs present in these cells should either be remnants from developing precursor cells or derive from adjacent cells, probably phloem companion cells.

Detailed biochemical, proteomic and transcriptomic analyses have brought advances in the understanding of phloem sap composition [19,22]. The first hint that miRNAs might be systemic molecules in plants was the finding of endogenous populations of these RNAs in phloem exudates of pumpkin, cucumber, castor bean and yucca [62]. The presence of miRNAs in phloem exudates was later confirmed in oilseed rape (*Brassica napus*) [63] and white lupin (*Lupinus albus*) [64]. Phloem exudates were collected by bleeding from incisions made to the vasculature. The species analyzed present spontaneous exudation upon wounding, facilitating collection of large volumes of exudate. As this method is restricted to species that naturally exude, other methods have been used to analyze phloem composition. Chelating agents, such as EDTA, prevent callose formation and occlusion of wounds, providing a quick and easy method for collecting exudates. EDTA-facilitated exudation has been used in analyses of proteomic phloem composition, but there are no data about whether miRNAs have been found by this method. Furthermore, practical problems associated with this method include the impossibility of measuring component concentrations and the induction of artefacts and changes in phloem composition [65].

Another technique, considered the most elegant and least invasive method, is stylectomy, the analysis of phloem exudates from cut stylets of sap sucking insects [66]. It has been successfully employed to detect mRNA in the phloem sap of rice [67] and barley [68,69] and, more recently, to detect miRNAs in apple phloem sap [70]. Due to the selectivity of aphids to feed from sieve elements, solute contamination from adjacent cells is reduced, yielding relatively pure phloem sap. Nevertheless, stylectomy

is restricted to plant species on which insects feed and the volume of the exuding drop is very limited [66,69].

All these techniques also have some common disadvantages. They can promote changes in phloem gene expression, especially in genes involved in wound or defense responses. Moreover, contamination from adjacent cells can be a serious problem. In cucurbits, much of the exudate volume from cut stems or petioles derives from the xylem and cut cells and only a small proportion represents real phloem sap [71]. To limit and monitor possible contaminations, it is necessary to exclude the initial drop and check for molecules, such as hexoses and Rubisco, known to be absent in sieve elements. These controls, however, also have limitations [71].

These combined approaches have revealed more than one hundred miRNAs, belonging to tens of families, in exudates of different plants [60,62–64,70]. Although the first drops were discarded in samples collected from incisions or cuts [60,62–64,70] and no small or large RNAs have been detected in xylem exudates [63], it is still possible that some of these miRNAs may derive from contaminating tissues. MiRNAs that are detected in both bleeding and stylectomy samples are more likely to represent phloem sap miRNAs. Exudate miRNAs range in sizes from 16 to 22 nt, although 24-nt miRNAs might also be present [62–64]. The inventory includes some well characterized miRNAs involved in different biological processes, such as development (e.g. miR156, miR159, miR166, miR172, miR390) or responses to nutrient stresses (miR169, miR395, miR398 and miR399) (Table 1). MiR171 has been used as a negative control to rule out contamination from adjacent cells [60]. However, the detection of low levels of miR171

in phloem exudates in two reports [63,70] casts doubts on the convenience of using this miRNA as a control, or suggests that there might have been contamination.

Phloem exudates are enriched in specific miRNAs, e.g. miR156, miR168, miR169, miR390, miR395 and miR399, when compared with other tissues, such as inflorescence stems, leaves, roots, pods or vascular bundles, while miR167 and miR171 have been consistently found under-represented in exudates and other miRNAs have not been detected [60,63,64,70].

The highly conserved repertoire of miRNAs in phloem exudates of distantly related species, including herbaceous and woody plants [60,62–64,70], suggests a functional role for these miRNAs. However, some differences in exudate composition have been found between plant species. Because miRNAs are strongly influenced by environmental conditions as well as by developmental stage [72], it might be interesting to obtain data from a wide range of species, growth conditions and developmental phases to obtain a complete picture of the miRNA landscape of the phloem sap.

Many phloem exudate miRNAs have been detected in phloem tissue or vascular bundles using other methods [25,33,37,70,73,74]. It has been suggested that miRNAs in sieve tubes may simply represent leakage molecules from adjacent companion cells [60]. However, the lack of clear correlations between miRNA levels in phloem tissues and phloem sap [70] argues against this hypothesis. In addition, not all miRNAs detected in phloem tissue have been found in phloem sap [70], and exudates from different parts of the plant show a different relative composition of miRNAs [64],

indicating that entry of miRNAs into the phloem stream must be somehow regulated. The accumulation of specific miRNAs in the phloem exudate suggests that they are there to perform a function, possibly as mobile signals, although this is not necessarily true for all exudate miRNAs. Mobility and systemic signaling have to be examined case by case.

3.2. miR399 is a long-distance signal for phosphate homeostasis

Communication from root to shoot, and vice versa, is essential to orchestrate responses to fluctuations in the availability of mineral nutrients. Hormones and nutrients themselves are candidates for long-distance signals controlling mineral macronutrient homeostasis [57]. MiR399 has emerged as an important long-range regulator of inorganic phosphate (Pi) homeostasis. Mature miR399 accumulates to high levels under Pi deficiency, consistent with the suppression of the accumulation of its target mRNA PHO2 in the roots and the promotion of Pi uptake and translocation [75]. Whereas levels of mature miR399 are similar in Pi-starved shoots and roots [73,76], MIR399 primary transcripts show much stronger up-regulation in shoots than roots [77]. Furthermore, MIR399 genes are expressed in the vasculature [73] and, as indicated above, mature miR399 is present in phloem exudates, which show higher miR399 levels in Pi-starved plants [38]. These findings suggested that the pool of mature miR399 in the root might derive in part from the shoot via phloem transport.

To test this hypothesis, Arabidopsis miR399-over-expressing shoots were grafted onto wild-type roots (miR399-OX/WT) and mature miR399 massively accumulated in the

rootstocks. Low levels of pri-miR399 and miR399* in roots indicated that the mature miRNA did not derive from de novo synthesis of miR399 in roots [34,38]. This indicates that miR399 molecules from the over-expressing scion moved across the graft junction to the wild-type rootstock and cleaved PHO2 mRNA directly in roots, but also in shoots. However, reciprocal grafts (WT/miR399-OX) did not show evidence of miR399 transport from root to shoot [38]. Two miR399 species, miR399b and miR399f, which differ in two nucleotides, move systemically with similar efficiencies [34].

Arabidopsis miR399 also moves in *Nicotiana benthamiana* plants [34]. In addition, accumulation of pri-miR399 and mature miR399 was much faster in shoots (hours) than in roots (days) in Pi-starved wild-type plants. This suggests that movement of miR399 from shoots to roots could suppress PHO2 at an early stage, a crucial signal for a rapid response to Pi deficiency. However, after prolonged Pi-starvation, roots expressed sufficient endogenous miR399 to suppress PHO2 [34].

Despite these results, it remained unknown whether movement also takes place in wild-type plants, in the absence of miR399 over-expression. To address this question, *hen1-1* mutants, which are affected in a methyltransferase required to stabilize miRNAs [6], were used. *hen1-1* mutants show reduced growth, diverse developmental defects and dramatically decreased levels of mature miRNAs [78,79], with no detectable levels of mature miR399 [60]. Grafting of wild-type scions on *hen1-1* mutant rootstocks confirmed the translocation of miR399 through graft unions from wild-type shoots to roots under Pi deficiency, although the effect of the translocated miRNA on its target genes was not tested in this case [60].

These studies suggest that miR399 acts as a long-distance signal that suppresses target gene expression in distal organs under Pi deficiency in several species (Fig. 3).

Moreover, the identification of miR399 and PHO2 orthologs in other plants [77], and the detection of miR399 in phloem exudates of several distant species [38,63,64], suggest that the movement and regulatory network of miR399 could be conserved. However, in order to better prove that the translocation of miR399 is biologically relevant, it should be shown that restricting its movement prevents PHO2 mRNA degradation in roots and has a consequence in the early response to Pi deficiency.

3.3. Other stress-induced miRNAs

Phloem exudate contains, in addition to miR399, a specific set of small RNAs that respond to the lack of essential nutrients, such as sulfate, copper or iron [60]. Similar to miR399, levels of miR395 show a drastic increase in plants grown under S starvation [80], with a higher increase in phloem exudate than in roots, stems or leaves [60], suggesting that its presence in the phloem exudate might be functionally relevant. Several MIR395 loci are expressed in the companion cells of the root and leaf phloem [81]. MiR395 post-transcriptionally controls the expression of the low-affinity sulfate transporter (SULTR2;1) and two ATP sulfurylases (APS1 and APS4) [80,82].

It was examined whether miR395 was able to cross graft junctions in response to S starvation, using WT/hen1-1 grafts, in an effort to clarify the capacity of miR395 to move. Signals of miR395 translocation between wild-type scions and hen1-1 mutant rootstocks were observed, consistent with down-regulation of APS4, but not of other

miR395 targets, in *hen1-1* rootstocks [60]. Despite these results, it is unclear whether transport of miR395 from shoots to roots is necessary for sulfate homeostasis, given that MIR395 promoter activity in wild-type roots is almost as high as in leaves in S-starved plants [60]. Two possible alternatives were proposed: roots might be unable to produce sufficient amounts of this miRNA; or some organs might respond earlier than others to stress [60]. Furthermore, SULTR2;1 mRNA is up-regulated in the root vascular system under S deficiency [60,81], contradicting the expected down-regulation of the target gene. Based on the observation that miR395 is expressed in root companion cells, while SULTR2;1 is mainly expressed in xylem parenchyma cells, the authors suggested a possible repression of the target gene in the companion cells, where SULTR2;1 expression would be low, restricting its accumulation to the xylem under S starvation. These and other questions have to be investigated in detail in future studies.

MiR398 mediates the response to Cu deficiency and other stresses by directing degradation of Cu/Zn superoxide dismutase mRNA in *Arabidopsis* [83,84]. MiR398 was not found by sequencing of phloem exudate samples of oilseed rape [63], yet it was detected in these samples by RNA gel blots and microarray analysis [60,63] and transcription of one precursor had been previously observed in the *Arabidopsis* vasculature [83]. Oilseed rape showed up-regulation of miR398 under Cu deprivation in leaf, stem and also in phloem exudate [60,63]. Recent studies have suggested that SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 7 (SPL7) might be a central regulator of Cu-deficiency responses, being able to induce the accumulation of miR398, among other miRNAs [85]. Nevertheless, transport of miR398 has not been demonstrated so far.

That miR395 and miR399 can be transmitted through grafts and down-regulate their target genes in recipient rootstocks [34,38,60] supports long-distance movement. It is still unclear whether all miRNAs present in the phloem sap or only some of them may move. In this regard, miR171 has been detected in phloem exudates of oilseed rape and pumpkin [63,70], but it does not seem to move through grafts in *Arabidopsis* [60]. In addition, the lack of graft transmission of the effects of artificial miRNAs expressed using the ubiquitous 35S promoter suggests that expression in the phloem is not sufficient for long-range activity [28]. Taken together, all these results suggest that some miRNAs are systemic regulatory signals, although this has to be further elucidated.

4. Hypothetical movement of other miRNAs

Inconsistencies between the accumulation pattern of a miRNA and the transcription of its precursors may suggest movement from cell to cell, as is the case with miR391 [33]. This miRNA is up-regulated by Pi starvation and, as explained above, Pi homeostasis requires both local and systemic signaling [75,86], but no reports on miR391 movement have been published so far.

MiRNAs present in vascular bundles and, in particular, in the phloem exudate are potentially mobile. A substantial proportion of the miRNAs detected in phloem exudates target genes involved in processes that require systemic signals, such as flowering (e.g. miR156, miR159 and miR172) [87–89], nutrient homeostasis (e.g. miR169, miR395, miR398 and miR399) [57,90] and nodulation (e.g. miR169) [91],

pointing to these miRNAs as possible candidates for long-distance regulators of these processes.

Systemic signals for flowering and tuber induction can substitute for each other [92,93], indicating that they are very similar. These signals move from the leaves to the shoot apical meristem for flowering and to underground stolons for tuberization [59,94]. Several FLOWERING LOCUS T (FT) proteins and the plant growth regulators gibberellins (GAs) have been identified as mobile flowering signals [95–98].

In potato, over-expression of miR172 promotes tuberization and this effect is transmitted through grafts. Changes of miR172 levels in stolons, rather than in leaves, correlate with tuberization [99]. These results have led to the proposal that miR172 moves from leaves to stolons or regulates long-distance signals affecting tuber induction. That over-expression of miR172 in stocks is not sufficient to induce tuber formation [99] argues against a role of miR172 as a mobile signal, although it does not rule it out completely. It is possible that repressive signals produced in wild-type scions counteract the effect of over-expressing miR172 in stocks. Alternatively, there might be factors required for processing of the miRNA precursor that act only in leaves.

Differential processing of a pre-miRNA in certain cell types has been described in mammals [16], making this hypothesis plausible. MiR172 expressed from a viral promoter seems to move through graft junctions in *N. benthamiana* [100]. However, the results are not conclusive given that only a small fraction of grafted plants showed a slight increase in miR172 levels and, in addition, the miR172 transgene did not have

any effect on target genes. Given that miR172 regulates FT in Arabidopsis [101], the systemic effect of this miRNA might be mediated by FT.

It has also been speculated that miR172 might be involved in cell-to-cell signaling from mesophyll to phloem companion cells [94], on the basis that the photoreceptor phytochrome B (PHYB) regulates miR172 [99] and is required in mesophyll cells to inhibit FT expression in companion cells [102]. In addition, two miR156 target genes, SPL9 and SPL10, regulate the levels of miR172 [17] and the effect of SPL9 on vegetative development is non-cell autonomous [103], suggesting that either SPL proteins or down-stream factors, e.g. miR172 or FT, might be transmissible.

The phloem exudate also contains miR159, which targets genes involved in GA signaling and affects flowering [87]. In addition, GA regulates miR159 levels indirectly [87]. Although the mechanism by which miR159 affects flowering is not clear [104], it is intriguing that a putatively mobile miRNA affects signaling by a molecule, GA, that acts as a long-distance flowering regulator. This finding deserves more attention.

MiR399 and its target gene PHO2 are involved in the response of flowering to ambient temperature [105]. As miR399 is a phloem-mobile signal for Pi homeostasis [34,38], it is tempting to speculate that this miRNA might also act as a systemic flowering signal. However, it has not been ruled out that the flowering-time phenotype of miR399 over-expressors or pho2 mutants is an indirect result of the stress caused by Pi over-accumulation [105].

Two miRNAs present in phloem exudates, miR162 and miR168, target genes involved in miRNA biogenesis, DCL1 and AGO1, respectively [106,107], and another one, miR390, triggers formation of tasiRNAs [6], suggesting that the production of small RNAs might itself be modulated by long-distance signals. The detection of miR390 in vascular bundles [33,35,37] and the findings that suggest cell-to-cell trafficking of this miRNA give credibility to the hypothesis that miR390 can reach the sieve tubes. However, no evidence that this miRNA acts as a long-range signal has been reported so far.

In addition to miR390, another miRNA in phloem exudates, miR165/166, is involved in cell-to-cell signaling and has been detected by other methods in the vasculature of several organs [25,32,70]. This suggests that complex mechanisms of short and long-distance regulation may exist, as is the case for Pi homeostasis [75]. However, it is also possible that miRNAs that are both able to move from cell to cell and expressed in phloem companion cells simply leak into sieve elements and do not play a role as systemic signals.

Therefore, a number of miRNAs are putatively transmissible, but further research is needed to determine whether they move.

5. How do miRNAs move?

Many questions will have to be answered after establishing that miRNAs can move. In this section, we examine the factors that may affect mobility and the possible mechanisms of transfer.

5.1. Features of miRNAs that may affect movement

It has been proposed that differences in miRNA size might influence the capacity to move [7]. Most miRNAs are 21-nt long and all size classes of endogenous and transgene-derived siRNAs move long distances [108,109], with preferential movement of 24-nt over 21-nt siRNAs [109]. Both 21 and 24-nt siRNAs also move from cell to cell [108,110]. Therefore, size is probably not a factor that determines, per se, whether a small RNA moves or not, although it is possible that the mobility range is affected by size, perhaps as a consequence of differential interactions with other molecules.

Other features of the MIRNA gene, the pri- or pre-miRNA or the mature miRNA itself, such as sequence, secondary structure or stability of the miRNA* may also have an effect [7]. The 5 nt of small RNAs very often determines into which RISC they will be loaded [6]. It has been suggested that this may affect mobility [6]. However, all possible nucleotides exist at the 5 position in the miRNAs found in phloem exudates (Table 2). Also, the 5 nt of miR165/166 is different from that of miR390 (Table 2). It is very unlikely, then, that the 5 nt establishes whether a miRNA can reach the phloem conduit or move from cell to cell. Results using precursors with different sequences that contain the same mature artificial miRNA indicate that local transmission of miRNA activity from phloem companion cells is independent of precursor sequence [24]. Whether this is also true for other miRNAs and, more importantly, for endogenous miRNAs, remains to be resolved. However, other characteristics of miRNAs may determine preferential interaction with certain AGO proteins [111] and perhaps influence mobility (see below).

Place, time and level of expression can contribute to determine transmissibility. For example, expression in phloem companion cells probably helps miRNAs reach the sieve tubes and commence long-distance movement. The degree of plasmodesmal connectivity between different cell types is not uniform and varies through development and in response to exogenous factors [40,112]. In addition, there is differential processing of precursors in different cell types or tissues at least in animals [16], and this may affect mobility. It is expected that cell-to-cell trafficking of miRNAs is regulated by interactions with other cell components. These interactions may vary among cells or tissues and may be influenced by the time and level of miRNA expression.

The abundance of endogenous and artificial miRNAs may determine the range of transmission, probably due to dilution effects [24,36]. This may explain the discrepancies between reports that examined the mobility of small RNAs [24,28,30]. For example, a two-construct approach was used to indirectly drive expression of artificial or endogenous miRNAs in specific cell types and the effect of the miRNAs analyzed was not transmitted to neighboring cells [28,30]. However, indirect control of miRNA expression may have led to lower levels of miRNAs than direct control by a cell-specific promoter [24].

Additional work is needed to understand which features make some miRNAs mobile.

5.2. Other factors that may affect movement

Given that the composition of the processing machinery is different for different classes of small RNAs and that this determines into which type of RISC they are loaded, it has been hypothesized that the biogenesis pathway and/or the RISC may determine the mobility of silencing signals [6,7]. Work with artificial small RNAs indicates that the ability to act non-cell autonomously does not depend on the biogenesis pathway, although the extent of the non-cell autonomy can be influenced by biogenesis. The miRNAs and siRNAs act within a similar range, whereas the effect of tasiRNAs can spread much further [24]. Conversely, in another report, the mobilities of tasiRNA and siRNA silencing signals were similar, whereas miRNAs acted cell autonomously [30]. Perhaps the use of artificial miRNAs or the expression of natural miRNAs using heterologous backbones or in places where they are normally absent has inadvertently overlooked some of the features affecting movement. This might explain the contrasting results obtained by different laboratories.

Initial movement of primary siRNAs over 10–15 cells from the site in which they are produced can be amplified through the production of secondary 21-nt siRNAs, which move further away [113]. It has been proposed that tasiRNAs could help spread over longer distances the effect of miRNAs with limited or no mobility [6,24,30,31], but clear evidence showing this for endogenous small RNAs has not been obtained so far. As discussed previously, it is not clear, for example, that tasiR-ARFs move more extensively than miR390 [33]. Further research with additional endogenous small RNAs will be required to elucidate whether biogenesis pathways influence the mobility of these RNAs.

Another question that must be addressed is whether miRNAs move as naked RNAs or in complexes with other molecules. On the basis that these small RNAs act in RISCs, bound to proteins, that RNAs are very sensitive to degradation by nucleases, and that RNA binding proteins are involved in cell-to-cell transmission of mRNAs [22,42], it has been assumed that miRNAs may move within ribonucleoprotein complexes, which would protect miRNAs from degradation. However, so far there is no conclusive demonstration that this is the case for any small RNA. AGO1, despite being required for mobile silencing [5], acts cell-autonomously and, therefore, it is improbable that 21-nt siRNAs and miRNAs move bound to AGO1 [110].

A phloem protein from pumpkin, PSRP1, binds single and double-stranded small RNAs and facilitates cell-to-cell movement of 25-nt small RNAs in microinjection experiments [62]. Whether PSRP1 binds 21-nt miRNAs is not known. This protein is not evolutionarily conserved, what makes improbable that it plays a general role in small RNA movement. Furthermore, no biological function has been reported for PSRP1. The pri-miRNAs of one mobile (miR399) and two putatively mobile miRNAs (miR398 and miR172) interact with FCA, an RNA-binding protein involved in flowering-time control that promotes pri-miR172 processing [114]. Whether this affects miRNA movement has not been tested yet.

A hypothesis on how miRNA movement may be regulated derives from work on the effect of AGO10 on miR165/166 [111]. AGO10 sequesters miR165/166 and prevents interaction of this miRNA with AGO1, precluding miR165/166 function in the shoot apical meristem. Furthermore, ago10 mutants ectopically accumulate this miRNA in the

shoot apical meristem [115]. Therefore, it is possible that regulation of miR165/166 trafficking is achieved, at least in part, through the activity of AGO10. Exploring this hypothesis will undoubtedly help to understand how miRNAs move. Whether this mode of miRNA regulation is specific for miR165/166 or controls other miRNAs that interact with AGO10 [111] is still unknown. Combining ago10 mutations with reporters of miRNA activity and comparing the expression pattern of AGO10 with the localization of these miRNAs will help answer the question about whether AGO10 spatially restricts the function of these miRNAs. Structural features of the miRNA:miRNA* duplex, rather than miRNA sequence, are required for the interaction of miR165/166 with AGO10 [111], pointing to a miRNA trait that might affect mobility. AGO1 is required to confine miR165/166 localization [26] and is associated to peripheral membranes [116]. Given that AGO10 belongs to a protein family present in all higher eukaryotes, perhaps other AGO proteins may play a similar role to AGO10 in restricting miRNA activity.

IPS1, the target mimic of miR399, limits the activity of this miRNA by sequestering it [117]. It will be interesting to determine whether the spatial localization of miR399 is regulated by IPS1. Perhaps target mimics may restrict movement of miRNAs in a similar way as that proposed for AGO10 [111].

Not only are plant miRNAs mobile, human miRNAs are present in blood plasma, serum and other body fluids in an extracellular form and these circulating RNAs can be used as disease biomarkers [118]. Many plasma and serum miRNAs are present in protein complexes that contain AGO2, and others are found in vesicles [119,120]. In addition,

human miRNAs are transferred from one cell to another through extracellular vesicles, probably exosomes, and are functional in recipient cells [121]. The delivery to recipient cells is mediated by lipoproteins and scavenger receptors, which are plasma membrane proteins able to bind lipoproteins and polynucleotides, among other ligands [122,123]. It will be interesting to study whether similar mechanisms operate in plants. Vesicle-mediated transport of molecules has been shown in plants [124] and a role for endo-lysosomal membranes and exosomes in cell-to-cell and long-distance transmission of miRNAs in plants has been hypothesized [125]. In this regard, the association of AGO1 and AGO7 to membranes is intriguing [116,126].

The most likely scenario is that multiple factors determine whether a miRNA is mobile or not as well as the range of translocation. Mobility is probably regulated in space and time and responds to environmental conditions. In addition, the extent of the influence of different factors on mobility might vary for different miRNAs.

5.3. Which is the mobile species?

Two important issues that deserve further attention are whether mature miRNAs or their precursors move and whether miRNAs traffic between cells as single or double-stranded species. In section 2 we have discussed that it is likely that mature miR165/166, miR390 and tasiR-ARFs, rather than their precursors, move from cell to cell [32,33,35–37], but this is not fully demonstrated yet. As for long-distance movement, these issues are relevant for three steps: entry into the sieve elements, translocation in the phloem stream and exit from the phloem. Which miRNA form

enters the sieve tubes is not yet known. The finding of mature miRNAs in phloem exudates of several species [62–64,70] and the results of grafting experiments [34,38,60] suggest that mature miRNAs are transported in the vasculature. It has not been ruled out that pri-miRNAs or pre-miRNAs can move in the phloem, although it seems unlikely because the miRNA processing machinery has not been detected in sieve elements [63].

To identify the mobile silencing signal, small RNAs have been sequenced in stocks of *Arabidopsis* dcl2 dcl3 dcl4 triple mutants, which do not produce 22, 23 or 24-nt mature siRNAs, grafted with wild-type scions. Endogenous 23 and 24-nt siRNAs have been detected in the mutant stocks [109], leading to the conclusion that siRNAs, rather than their precursors, are the mobile species [109]. In *C. elegans*, both long and small RNAs act as mobile silencing signals (reviewed in [5]), leaving open the possibility that small RNA precursors can move at least in some organisms.

Regarding the question of whether miRNAs move as ssRNAs or as miRNA:miRNA* duplexes, the miRNA population of pump-kin phloem exudates is mainly single stranded [62]. However, the presence of miRNA* strands in the phloem is puzzling [63]. Sequencing results suggest that the abundance of most miRNAs* is lower than that of the corresponding miRNAs. In addition, RNase digestion experiments indicate that exudate miRNAs are predominantly in a single-stranded form [63]. Although the authors of this work admit that double-stranded molecules might separate during the isolation procedure, the observation that only mature miRNAs and not miRNAs* increase during stress treatments suggested that single strands, and not

miRNA:miRNA* duplexes are the physiologically relevant molecular species. Yet, graft-transmission of miR399* from miR399-over-expressing scions has been observed [127]. This may be due to excessive expression of miR399 precursors in the over-expressing plants. Alternatively, it is possible that both miR399 and miR399* move in wild-type plants, either as ssRNAs or forming a miRNA:miRNA* duplex.

Using a viral silencing suppressor that specifically sequesters 21-nt siRNA duplexes, movement of silencing signals was inhibited [110], indicating that duplexes are mobile. Bombardment of fluorescently labeled 21 or 24-nt siRNA duplexes confirmed cell-to-cell and systemic movement of these small RNAs, whereas movement of single-stranded siRNAs was not observed [108,110]. By contrast, single-stranded but not double-stranded siRNAs moved from cell to cell when co-injected with an RNA-binding protein [62]. Experiments performed with exogenously supplied small RNAs have to be interpreted with caution, because they can be affected by artefacts and may not necessarily represent what happens with endogenous small RNAs. Therefore, whether single-stranded siRNAs move or not is not clearly established. Also, it is not known to which extent the conclusions on movement of siRNAs can be extrapolated to miRNAs.

5.4. Movement pathways and mechanisms

Intercellular communication can occur via symplastic (through plasmodesmata) or apoplastic pathways (through plasma membranes and cell walls). Restricting movement of molecules through plasmodesmata by increasing callose deposition limits the non-

cell-autonomous action of miR165, indicating that plasmodesmata are required for cell-to-cell movement of at least one miRNA [39]. Phloem loading and unloading of small RNAs may, in principle, use pathways similar to those for cell-to-cell movement. The most widely accepted hypothesis is that miRNAs are loaded symplastically through plasmodesmata [7,18,42] (Fig. 4). Plasmodesmata connecting sieve elements with companion cells differ from plasmodesmata that connect other cell types [128]. Therefore, it is possible that symplastic phloem loading and unloading may be different from movement between non-phloem cells.

Given the important roles that miRNAs play in plant biology, and the fact that very few of them seem to move, it is expected that miRNA trafficking is tightly regulated, but this might be achieved either by actively facilitating transport or by restricting free diffusion. It is not known whether miRNAs use passive or active mechanisms to move through plasmodesmata or enter the phloem stream. siRNAs probably move in a passive manner (reviewed in [6]). The size exclusion limit of plasmodesmata is variable and the size of single-stranded miRNAs, around 7 kDa, would theoretically allow free diffusion between many cell types [129]. Nevertheless, shape may also affect the ability of molecules to freely pass through these channels [41] and the size of miRNAs themselves would be irrelevant if they move in complexes with other molecules rather than as naked RNAs.

As miRNA expression patterns, regulatory mechanisms and modes of action are diverse, it would not be too daring to hypothesize that miRNAs may use more than one translocation pathway. Whether miRNAs can enter the sieve elements through

apoplastic loading has not been discarded. In fact, apoplastic movement of small RNAs has been suggested. Results indicate non-cell autonomous effects of endogenous endosperm-derived siRNAs on the developing embryo and movement of transposon-derived siRNAs from the vegetative cell to sperm cells in pollen (reviewed in [7,61]). Given that the embryo is symplastically isolated and sperm cells probably do not have plasmodesmata, it has been proposed that these cases might involve apoplastic movement of siRNAs (reviewed in [5]). Thus, it might be worth testing whether miRNAs can be transmitted apoplastically.

It is assumed that miRNAs reach the sieve elements from adjacent companion cells, but this has not been demonstrated so far. It is not known if some phloem exudate miRNAs can originate in more distant cells. Also, the places in which these miRNAs are processed have not been determined. Results indicate that miRNAs, as well as siRNAs, move with the phloem mass flow, preferentially from sources to sinks [34,38,60,108,109] (Fig. 4). That two miRNAs move through grafts and lead to down-regulation of target genes in recipient stocks [34,38,60] suggests that these miRNAs can exit the vasculature and perform their functions in recipient tissues. However, since these analyses did not distinguish between phloem and other stock tissues, a proof that miRNAs can leave the phloem is not available yet.

Viral movement proteins, which increase the size exclusion limit of plasmodesmata [42], and viral suppressors of silencing, which affect miRNA activity by inhibiting different steps of the RNA silencing process [130–135], could be used as tools to test the mechanisms of miRNA movement.

6. Conclusions and future prospects

Evidence supporting the hypothesis that certain miRNAs move has been obtained in several tissues and in several species with a range of experimental approaches. We can almost say with certainty, in relation to miRNA, “and yet, it moves” (*“eppur si muove”*), the sentence that the legend attributes to Galileo Galilei, referring to the movement of the Earth around the Sun, after being forced to deny this theory before the infamous Inquisition. Local transmission only occurs within a few cells, but the fact that miRNAs can also be transported in the phloem suggests that these small RNAs are more mobile than was previously thought. It is probable that more miRNAs will join the list of putative cell-to-cell and long-distance signals. Still, mobility seems to be a specific feature, not a general attribute, of a limited subset of miRNAs.

Additional experiments are required to undoubtedly demonstrate that endogenous miRNAs move and that movement is required for acting at a distance. Methods for the dynamic visualization of small RNAs and their activity will be essential to provide a final proof of miRNA movement. Grafting experiments to test movement of a mutated miRNA from a donor plant to a receptor carrying a complementarily mutated target gene will also be helpful. Mutants affected in intercellular trafficking of macromolecules [39] could be useful tools to test movement of different miRNAs. The research done so far opens new questions and novel ways to understand the function of miRNAs in plants. The detection of uncharacterized miRNAs in the phloem exudate can help to address the role of these miRNAs in future studies. More work is needed to identify the mobile species and the features that determine whether a miRNA is mobile

or not. Understanding the origins of the discrepancies between results obtained in similar studies will help advance in this field. To fully comprehend the roles of mobile miRNAs, it will be necessary to determine the expression patterns, localization and functions of different family members, as well as to elucidate if different family members show differential mobility. Some work in this direction has already been started in several laboratories (e.g. [32,34,48]).

The mechanisms, as well as the regulators, of intercellular and interorgan signaling by miRNAs remain largely unknown. Taking together all the information presented here, we propose that miRNAs can passively diffuse through plasmodesmata, but movement can be restricted between certain cell types, at certain developmental stages or in response to external signals by retention mechanisms, e.g. by binding to proteins or other cellular components. Nevertheless, other pathways for translocation might also exist.

Macromolecules can be transmitted between different organisms. Examples of this are the delivery of bacterial and fungal effector proteins to plants [136,137] and the translocation of RNAs, proteins and RNA silencing signals from host plants to parasitic weeds [138]. Testing whether miRNAs can also move between species could open a new area of research. The rapid progress in the field of small RNA investigation in recent years promises novel and significant discoveries in the near future.

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Fig. 1. Cell-to-cell transmission of miR165/166. MIR165/166 genes are transcribed in the root endodermis. Their products, probably mature miR165/166, move to the adjacent cell layers, becoming progressively less concentrated as they move further away. High levels of miR165/166 suppress HD-ZIP III mRNA accumulation in the endodermis and pericycle. Moderate abundance of miR165/166 allows the accumulation of low levels of HD-ZIP III and lead to specification of protoxylem in the periphery of the stele. In the centre of the stele there are very low levels of miR165/166 and therefore high levels of HD-ZIP III, which specify metaxylem. Arrows indicate movement and a line ending in a bar indicates down-regulation.

Fig. 2. Model for the regulation of adaxial/abaxial determination of leaves.

Transcription of MIR390 loci in the epidermis and adaxial part of the developing leaf produces mature miR390, which is probably transmitted to almost the whole primordium. MiR390 binds to TAS3 transcripts, triggering the formation of tasiR-ARFs in the adaxial domain. TasiR-ARFs down-regulate ARF transcription factors on the adaxial side and move to the abaxial domain, creating a gradient distribution that establishes the adaxial/abaxial boundary and limits ARF accumulation to the abaxial domain. Down-regulation of miR165/166 on the adaxial side by tasiR-ARFs, presumably through the repression of ARFs, which would act as inducers of miR165/166, allows the accumulation of HD-ZIP III transcription factors, which determine adaxial fate. TasiR-ARFs are not produced in the abaxial domain, due to the absence of TAS3 and AGO7 in this part of the leaf primordium. Very low levels or absence of tasiR-ARFs in the abaxial part enable accumulation of ARFs and miR165/166, which down-regulates HD-ZIP III. ARFs specify abaxial fate. Wavy arrows indicate movement, dashed arrows indicate hypothetical positive regulation and lines ending in a bar indicate down-regulation.

Fig. 3. Long-distance movement of miR399. Under a shortage of phosphate (Pi) miR399 is expressed in the shoot and is transmitted through the phloem to the root, where it down-regulates its target gene PHO2. Wavy arrows indicate movement and a line ending in a bar indicates down-regulation.

Fig. 4. Model for long-distance movement of miRNAs. MiRNAs present in phloem companion cells may use three different mechanisms to move to sieve elements: (A) Passive diffusion through plasmodesmata; (B) active symplastic movement in complexes with other cellular components, e.g. protein or lipids; and (C) apoplastic transmission. Once in the sieve elements, miRNAs move from source to sink tissues with the phloem flow. Exit of miRNAs from the phloem conduit to target tissues has not been shown yet, but in principle it could use similar mechanisms to those for entry into the sieve elements. Arrows indicate movement

Table 1. miRNAs found in phloem exudates of different plant species.

Table 2. miRNAs found in phloem exudates contain all possible 5 nucleotides.