Title: Small RNAs as positional signal for pattern formation

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Pattern formation in plant relies on intimate cell–cell communication exchanging positional information. While ligand–receptor interaction is commonly used by plants and animals as a means to transmit positional information, plant cells can directly exchange regulatory molecules such as transcription factors through a cytoplasmic continuum called the plasmodesmata. Recently endogenous small RNAs (sRNAs) of various biogenetic origins have been shown to function non-cell-autonomously. To date, non-cell-autonomous sRNAs have been shown to regulate leaf polarity, root vascular patterning, meristem formation in embryos, shoot meristem maintenance and female gametogenesis. All these developmental processes are fundamental to the life cycle and architecture of flowering plants, suggesting that sRNA-mediated cell-to-cell signaling has been adopted to achieve novel morphology in the course of plant evolution.

Introduction

Morphogenesis of multicellular organisms relies on tightly coordinated cell division and differentiation sequences, the process known as pattern formation. Due to the presence of rigid cell walls, virtually no cell migration and rotation occur in plants. Therefore, pattern formation in plants depends primarily on positional information in order to ensure ‘on-site’ differentiation of newly formed cells. Such patterning mechanisms suggest a major contribution of intimate cell–cell communication in plants. Indeed, research conducted for the past two decades using the model plant species *Arabidopsis thaliana* have successfully identified numerous molecules mediating cell–cell communication, including small peptides, phytohormones as well as transcription factors (see [1–3] for recent reviews).

Recently, small non-coding RNA (sRNA) has emerged as a novel class of signaling molecules that potentially move cell-to-cell and act as positional cues. Endogenous sRNAs in plants are classified into several categories based on their biogenesis and mode of action (see [4] for a recent review). MicroRNA (miRNA) is the most intensively studied class of sRNAs and suppresses target gene expression by mRNA cleavage, translational inhibition and transcriptional silencing [5–7,8]. Small interfering RNA (siRNA) is another well studied class of sRNAs [4]. siRNAs are generated from double-stranded RNAs of various origins. Similar to miRNA, some sRNAs, such as trans-acting siRNAs (ta-siRNAs), suppress gene expression by mRNA cleavage [9], while other siRNAs act directly at the transcriptional level by DNA methylation [10]. The latter pathway is essential for transposon inactivation and hence for genome maintenance [11]. Both miRNA and siRNA require DICER-LIKE (DCL) enzymes and ARGONAUTE (AGO) proteins for biogenesis and suppressing functions, respectively. sRNAs of distinct classes require different sets of DCL and AGO proteins, though they share some degree of functional redundancy

[12–14].

A role of mobile sRNA in plant pattern formation was initially described for tasiR-ARF, a *trans*-acting siRNA (ta-siRNA) that targets *AUXIN RESPONSE FACTOR3* (*ARF3*) and *ARF4* mRNAs in *Arabidopsis* (Figure 1A) [15]. Produced in the adaxial (top) layers of leaf primordia, tasiR-ARFs distribute in a gradient decreasing toward the abaxial (bottom) side. tasiR-ARF restricts *ARF3* expression to the abaxial side of young leaf primordia and thereby contributes to leaf polarity. Since then, other classes of sRNAs have also been shown to act non-cell-autonomously and play critical roles in various aspects of plant development. We here review recent studies identifying sRNA-mediated cell-to-cell signaling in plant pattern formation and provide further insights as to why sRNA-mediated signaling has been adopted as a means to transmit positional cues.

miR165/166-mediated signaling controls root tissue patterning and embryo polarity

Members of the *CLASS III HOMEODOMAIN LEUCINE ZIPPER* (*HD-ZIP III*) transcription factor family are key regulators of plant development. The *Arabidopsis* genome contains five *HD-ZIP III* transcription factor genes, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*)/*INTERFASCICULAR FIBERLESS1* (*IFL1*), *CORONA* (*CNA*) and *ATHB8*, and they are post-transcriptionally regulated by miR165 and miR166 (miR165/166). While loss-of-function mutants of a single *HD-ZIP III* gene exhibit no or subtle developmental defects, their miRNA-resistant dominant mutants exhibit strong polarity-related defects in embryos, leaf primordia and stem vascular patterns [16].

Carlsbecker *et al.* reported that in the *Arabidopsis* root meristem, miR165/166 are produced specifically in the endodermis layer, but they spread into other cell layers to suppress *HD-ZIP III* expression (Figure 1B) [17]. The endodermis-derived miR165/166 thus confer distinct

HD-ZIP III dosage to the stele cell files, which in turn specifies two distinct xylem vessel cell fates, protoxylem and metaxylem [17]. More recently, Miyashima *et al.* expressed a mutated version of miR165 (miR165mu) in the endodermis by a steroid hormone-inducible manner, and monitored spatial expression patterns of *PHBmu-GFP*, a functional *PHB* reporter to be suppressed by the miR165mu. This experimental setup allowed Miyashima *et al.* to reduce complex relationships between endogenous *MIR165/166* and *HD-ZIP III* loci into a simple one-to-one relationship between the transgenes. By using this system, Miyashima *et al.* demonstrated that miR165 produced in the endodermis dose-dependently determines *PHB* expression levels in the stele and thereby controls differentiation status of the xylem cell types [18]. The action of mobile miR165 in the specification of two xylem cell types suggests a role similar to animal morphogens, which form a concentration gradient and define multiple cell fates depending on their dosage [19]. Indeed, a mathematical simulation indicated that graded distribution of miR165/166 would result in sharp expression boundary of target *PHB* mRNA, as opposed to a graded expression pattern, if mutual degradation between miRNA and target mRNA was incorporated into the simulation [20••], providing a novel mechanism by which a graded distribution of signaling molecules is translated into a discontinuous array of cell fates.

miRNA-dependent gene expression control is also critical to embryo patterning. One such evidence is that the *serrate* (*se*) mutants of *Arabidopsis* that is compromised with miRNA biogenesis exhibit severe patterning defects in embryos [21]. Although SE protein is required for maturation of a broad range of miRNAs [22,23], the embryonic defect is largely explained by the loss of miR165/166-dependent suppression of *HD-ZIP III*, in particular those of *PHB* and *PHV* [21]. Among the five *HD-ZIP III* genes in *Arabidopsis*, all but *ATHB-8* are specifically expressed in the apical-central region of globular stage embryos, the position where shoot apical meristem (SAM) primordia are specified [24]. Moreover ectopic expression of miR-resistant versions of *HD-*

ZIP III at the root pole resulted in double-shoot embryos [24]. These data indicate that *HD-ZIP III* mRNAs specifically accumulate at the central-apical domain of globular stage embryos, not only to promote SAM specification, but also to ensure cells at the basal pole to be correctly destined to the root meristem. Because miR-resistant versions of *HD-ZIP III* transcripts accumulate in entire embryos, the region-specific accumulation of wild-type *HD-ZIP III* transcripts is largely defined by the miR165/166-dependent suppression [24].

It is known that *AGO10* (also known as *PINHEAD* or *ZWILLE*) gene is required for SAM maintenance. Loss-of-function *ago10* mutants in *Ler* background are unable to maintain stem cell population in SAM and end up forming a pin-like structure at the SAM position [25]. Levels of *HD-ZIP III* transcripts are reduced in *ago10*, a phenotype opposite to *ago1* [26]. These observations suggested that AGO10 functions differently from AGO1. By using both molecular and genetic techniques, Zhu *et al.* discovered a previously unpredicted function of AGO10; that is, sequestration of miR165/166 to protect *HD-ZIP III* mRNAs (Figure 1C) [27]. Since spatiotemporal expression pattern of *AGO10* nearly overlaps with those of *HD-ZIP III* mRNAs in embryos [24,25], it is theoretically possible that patterned expression of AGO10 defines *HD-ZIP III* expression patterns, if miR165/166 activities occur ubiquitously. Detailed expression analysis of all nine *MIR165/166* gene loci in *Arabidopsis*, however, revealed that spatial expression patterns of miR165/166 were tightly regulated in embryos [28*]. Up to the heart stage embryos, production of miR165/166 is limited to the basal-peripheral region of embryos, and in the subsequent stages, site of miR165/166 production switches to the endodermis precursor. Importantly, the site of miR165/166 production does not spatially overlap with that of *HD-ZIP III* mRNA accumulation. These observations strongly suggest that miR165/166 act non-cell-autonomously from the basal part of embryos, and eventually promote the apical fate (Figure 1C) [28*]. These observations indicate that *HD-ZIP III* expression domain is defined by the distribution of two

antagonizing activities, one mediated by non-cell-autonomous miR165/166 emanating from the basal-peripheral part of embryos, and the other mediated by AGO10-dependent sequestration of miR165/166 at the central-apical domain (Figure 1C) [28•].

miR394-mediated signaling positions stem cell pool in the shoot meristem

In *Arabidopsis*, the SAM consists of three layers, designated L1, L2 and L3. The L1 and L2 are each composed of a single cell layer, and differentiate into the epidermal and subepidermal tissues of aerial organs, respectively. The L3 is less layered in structure and differentiates into inner corpus tissues. Research conducted for the last two decades has revealed multiple modes of cell–cell communication mechanisms operating in SAM, which are mediated by a mobile transcription factor, ligand-receptor interaction, and hormonal signaling (see [29–31] for review). Briefly, a homeodomain transcription factor WUSCHEL (WUS) is expressed in the organizing center (OC), a small number of cells at the center of L3. WUS proteins move from the OC to the distal stem cell region where they activate the production of CLAVATA3 (CLV3) peptides. Secreted from the stem cells, CLV3 peptides are perceived at the OC periphery, and eventually attenuate the WUS expression in OC. While this feedback-loop well explains how the size of stem cell pool is kept constant in SAM, this mechanism alone cannot explain how cellular organization of SAM can be kept constant, because cells in OC are continuously replenished by the stem cell division. It is plausible, therefore, to assume that cells in the SAM can sense their relative position to the apical end of SAM. The plant hormone cytokinin has an important role in SAM maintenance [32–35]. Several studies suggested a crosstalk between WUS function and cytokinin signaling [36,37]. Chickarmane *et al.* developed a computational model and suggested that apically derived cytokinin together with CLV3 peptide act as a positional cue to fix the position of WUS-expressing domain [38].

Recently, Knauer *et al.* reported that L1-derived miR394 acts as a positional cue to

pattern SAM in *Arabidopsis* (Figure 1D) [39••]. They found that repression of *LEAF CURLING RESPONSIVENESS (LCR)* by miR394 played an important role in stem cell maintenance. In the course of a forward genetic screening to identify enhancers of a weak *ago10* mutant allele, they isolated a loss-of-function mutant of *MIR394B*, one of the two *MIR394* loci producing miR394 precursors. In this mutant, stem cell activity is prematurely terminated with the loss of CLV3 expression. In heart stage embryos where the layered structure of the SAM is established, *MIR394B* is transcribed specifically in the L1 layer in addition to the adaxial side of cotyledon primordia. Interestingly, *in situ* hybridization and a sensor analysis revealed the existence of both mature *miR394* and its suppressive activities not only in the L1 layer but also in the L2 and L3 layers, suggesting that miR394 can move a short distance from L1 to the inner layers. Furthermore, the expression of *miR394*-resistant *LCR* in the stem cell region is sufficient to recapitulate the stem cell termination phenotype. Altogether, these findings indicate that intercellular signaling mediated by the movement of miR394 from the L1 to inner stem cell layers plays an important role in the maintenance of SAM.

LCR encodes an F-box protein, suggesting its role in protein degradation process. Therefore it is possible that *LCR* targets as yet unidentified proteins that modify WUS function or transport. This possibility could be addressed by observing GFP-tagged WUS localization in the background with modified miR394 activity. It is also noteworthy that effects of reduced miR394 activity are largely masked by the presence of wild-type *AGO10*. Therefore, *AGO10* seems to promote SAM maintenance independently of the miR394/*LCR* module. Since the miRNA sequestration by *AGO10* are so far limited to miR165/166, the two miRNA-dependent pathways (miR165/166/HD-ZIP III and miR394/*LCR*) are likely to be independent from each other.

sRNA pathway in somatic tissue controls female gametogenesis

In most flowering species, megagametogenesis or formation of female gametes in developing ovules begins with the specification of a single subepidermal cell as the megaspore mother cell (MMC). The MMC then undergoes meiosis to produce four haploid megaspores, among which one cell can survive and differentiate as the functional megaspore, while the other three undergo cell death. This patterning process ensures only one female gametophyte (the embryo sac) resides in each ovule [40,41]. How is this evolutionarily conserved reproductive system controlled? Two independent studies suggested the involvement of sRNA-dependent suppression and communication between gametophyte and surrounding somatic tissues.

Earlier, Olmedo-Monfi *et al.* reported that in *Arabidopsis* ovules, AGO9 proteins accumulate in the cytoplasm of ovule L1 layer, but not in subepidermal cells that give rise to the MMC (Figure 1E) [42]. In T-DNA insertion mutants of *ago9*, more than one subepidermal cell is specified as the MMC in a manner dependent on the dosage of *ago9* insertion alleles. In later stages, the extra MMCs differentiate into a megaspore without meiosis, resulting in an ovule having multiple megaspores. These observations suggest that AGO9 in the ovule L1 layer non-cell-autonomously restricts the number of MMCs and megaspores [42]. In ovules, AGO9 preferentially binds to 24-nucleotide sRNAs derived from transposable elements (TEs), and indeed TEs are activated in the female gametophytes of *ago9* mutant ovules [42]. These data indicated that an AGO9-dependent sRNA pathway maintains an inactive state of TEs in female gametophytes and this regulation is essential to establish a single megaspore per ovule.

More recently, Tucker *et al.* reported that an additional sRNA-dependent process in somatic cells non-cell-autonomously promotes gametogenesis (Figure 1F) [43[•]]. They found that AGO5, an AGO protein binding to 24-nt sRNA, is specifically expressed in the somatic cells surrounding the MMC. A semidominant *ago5-4* mutant allele, which likely expresses a modified AGO5 protein, antagonizes endogenous AGO5-dependent and/or related silencing activity. In

ago5-4, MMC goes through a meiotic cell cycle to form megaspores, but subsequent megagametogenesis does not take place, resulting in a female gametophyte with only one nucleus [43•]. Expression of *ago5-4* mutant gene or viral sRNA suppressor Hc-Pro can recapitulate the same phenotype when driven by the *AGO5* promoter, but not by MMC- or megaspore-specific promoters in ovules [43•]. Therefore, in addition to the AGO9-dependent restrictive pathway, another sRNA-dependent process in the somatic cells non-cell-autonomously promotes female gametogenesis. It should be noted, however, that in both mechanisms the involvement of sRNA movement is not experimentally demonstrated. Protodermis-specific occurrence of silencing component AGO9 rather suggests that the sRNA pathway acts upstream of unknown signaling mechanisms. In the case of AGO5, endogenous silencing components inhibited by the *ago5-4* allele are not identified. Nevertheless, TE activation observed in the *ago9* female gametes suggests a relay mechanism in which sRNA effects in somatic cells are somehow transmitted to another sRNA-dependent process in gametophytes.

Conclusion

The past five years has seen a paradigm shift in the role of sRNAs in plant development. Since the first description of the essential function of mobile ta-siRNA in leaf development [15], Various classes of sRNAs have been shown to function non-cell-autonomously [10,17,18,39•,44]. It would not be surprising that more and more mobile sRNA species would be identified and shown to play essential roles in plant development. sRNA-mediated cell-to-cell signaling is a simple yet highly specific mechanism. It does not require specific receptor nor components for signal transduction pathway from signal perception to gene expression responses. Instead, it utilizes highly-specific nucleotide base pairing for direct suppression of target mRNA expression. If sRNA

mobility has been wide-spread in the plant kingdom, it is conceivable that sRNAs have been easily adopted as a means to transmit positional information during the course of plant evolution. The mechanism of passing sRNAs across the cell walls, however, is far from being understood. While plasmodesmata (PD) is the only possible route of sRNA movement across the cell walls and the observations so far support this hypothesis [45,46], no molecular components have been identified to regulate sRNA trafficking by PD, except for those affecting PD permeability in general [45]. While recent advances in the proteome analysis on PD-enriched cell wall fractions have presented a list of candidate proteins [47], neither forward nor reverse genetic analyses have so far provided clear mechanistic insights [46]. This could be either due to genetic redundancy or simply reflecting essential functions of PDs in plant morphogenesis. Future challenges include sophisticated forward genetic screening designed to specifically detect sRNA trafficking defects while avoiding lethality. Use of basal plant species with lower genetic redundancy and simpler tissue patterns than *Arabidopsis* will also help our understanding of evolutionary origin of sRNA-mediated cell-to-cell signaling.

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Figure 1

Patterning events known to be regulated by non-cell-autonomous sRNAs. **(a)** tasiR-ARF produced in the adaxial side of leaf primordia restricts *ARF3* expression to the abaxial side. **(b)** Endodermis-derived miR165/166 restrict *PHB* expression to the central vascular cell files in the root meristem region. **(c)** miR165/166 produced in the basal-peripheral domain of globular embryos restrict *PHB* expression to the central-apical domain. AGO10 antagonizes miR165/166 action to maintain *PHB* expression in the shoot meristem progenitor. **(d)** miR394 produced in the L1 layer of shoot meristem region suppresses *LCR* expression in a few inner cell layers. **(e)** TE-derived siRNAs are loaded into AGO9 in the L1 layer of ovule precursors and act to restrict MMC cell fate as well as to inactivate TEs in germ cell lineages. **(f)** Unknown siRNA pathway (AGOx) inhibited by the *ago5-4* allele promotes mitotic division of megaspores. Note that in (e) and (f), targets of the sRNA-mediated silencing are not identified and hence sRNAs may not mediate cell-cell communication itself.