PCP
PLANT & CELL PHYSIOLOGY

A Comprehensive Expression Analysis of the Arabidopsis *MICRORNA165/6* Gene Family during Embryogenesis Reveals a Conserved Role in Meristem Specification and a Non-Cell-Autonomous Function

Shunsuke Miyashima[1,6], Minami Honda[1], Kayo Hashimoto[1,2], Kiyoshi Tatematsu[3], Takashi Hashimoto[1], Kumi Sato-Nara[4], Kiyotaka Okada[3] and Keiji Nakajima[1,5,*]

[1]Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara, 630-0192 Japan
[2]Graduate School of Humanities and Sciences, Nara Women's University, Kitauoyanishi-machi, Nara, 630-8506 Japan
[3]National Institute for Basic Biology, 38 Nishigonaka, Myodaiji, Okazaki, Aichi, 444-8585 Japan
[4]Faculty of Science, Nara Women's University, Kitauoyanishi-machi, Nara, 630-8506 Japan
[5]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012 Japan
[6]Present address: Institute of Biotechnology, Department of Bio and Environmental Sciences, University of Helsinki, FIN-00014, Finland.
*Corresponding author: E-mail, k-nakaji@bs.naist.jp; Fax, +81-743-72-5529.
(Received October 18, 2012; Accepted December 20, 2012)

One of the most fundamental events in plant ontogeny is the specification of the shoot and root apical meristem (SAM and RAM) in embryogenesis. In Arabidopsis, the restricted expression of class III homeodomain leucine zipper (HD-ZIP III) transcription factors (TFs) at the central–apical domain of early embryos is required for the correct specification of the SAM and RAM. Because the expression of HD-ZIP III TFs is suppressed by microRNA165/166 (miR165/6), elucidation of the sites of miR165/6 production and their activity range is a key to understanding the molecular basis of SAM and RAM specification in embryogenesis. Here, we present a comprehensive reporter analysis of all nine Arabidopsis *MICRORNA165/166* (*MIR165/6*) genes during embryogenesis. We show that five *MIR165/6* genes are transcribed in a largely conserved pattern in embryos, with their expression being preferentially focused at the basal–peripheral region of embryos. Our analysis also indicated that *MIR165/6* transcription does not depend on SCARECROW (SCR) function in early embryos, in contrast to its requirement in post-embryonic roots. Furthermore, by observing the expression pattern of the miR-resistant *PHBmu-GFP* (green fluorescent protein) reporter, in either the presence or absence of the *MIR165Amu* transgene, which targets *PHBmu-GFP*, we obtained data that indicate a non-cell-autonomous function for miR165 in early embryos. These results suggest that miR165, and possibly miR166 as well, has the capacity to act as a positional cue from the basal–peripheral region of early embryos, and remotely controls SAM and RAM specification with their non-cell-autonomous function.

Keywords: *Arabidopsis thaliana* • Embryogenesis • HD-ZIP III • Meristem • MicroRNA • Pattern formation.

Abbreviations: GFP, green fluorescent protein; HD-ZIP III, class III homeodomain leucine zipper; *lt*, lower tier; miR, microRNA; PD, plasmodesmata; RAM, root apical meristem; SAM, shoot apical meristem; SEL, size-exclusion limit; TF, transcription factor; *ut*, upper tier.

Introduction

Plant pattern formation relies on intimate cell–cell communication for the continuous exchange of positional cues (Van Norman et al. 2011). Recently, the molecular identities of positional cues and their transmission mechanisms have begun to be uncovered for some aspects of plant pattern formation, including stem cell maintenance in the shoot apical meristem (SAM), pattern formation in the root, vascular differentiation, dorsoventral patterning in leaf primordia and spacing of leaf stomata (Kurata et al. 2005, Chitwood et al. 2009, Yadav et al. 2009, Carlsbecker et al. 2010, Katsir et al. 2011, Miyashima et al. 2011). While some of these patterning processes adopt a mode of exchanging positional cues shared by plants and animals, i.e. ligand–receptor interaction (Katsir et al. 2011), others use a plant-specific mechanism, i.e. direct exchange of signaling molecules through the cytoplasmic continuity at plasmodesmata (PDs) (Van Norman et al. 2011).

PDs allow passage of molecules that are smaller than their size-exclusion limit (SEL) (Maule et al. 2011). However, some proteins larger than the SEL, in many cases transcription factors (TFs), can also selectively traffic from cell to cell through PDs (Kurata et al. 2005). One of the most extensively studied examples of this is a GRAS-type TF, SHORT-ROOT (SHR). In the root meristem, SHR is produced specifically in the stele and

Plant Cell Physiol. 54(3): 375–384 (2013) doi:10.1093/pcp/pcs188, available online at www.pcp.oxfordjournals.org

moves to the adjacent cell layer (Helariutta et al. 2000, Nakajima et al. 2001). In the adjacent cell layer, SHR activates the expression of another GRAS-type TF, SCARECOW (SCR) (Nakajima et al. 2001, Levesque et al. 2006). The SCR proteins thus produced interact with SHR to form a regulatory complex that activates the transcription of genes required for endodermis differentiation (Levesque et al. 2006, Welch et al. 2007). Because SCR captures SHR in the recipient cells, and is required for the activation of downstream genes, only a single cell layer adjacent to the stele differentiates into the endodermis (Cui et al. 2007). Thus, the intercellular trafficking of SHR acts as a positional cue from the stele to the endodermis.

Recently, we and others revealed that a subset of *MICRO RNA165/166* (*MIR165/6*) genes is transcribed in the root endodermis in a manner dependent on SHR and SCR (Carlsbecker et al. 2010, Miyashima et al. 2011). The Arabidopsis *MIR165/6* gene family consists of nine members, two *MIR165* (*165A* and *165B*) and seven *MIR166* (*166A*–*166G*), which produce the 21 nucleotide microRNA165 (miR165) and microR166 (miR166), respectively. These two miR species differ only at a single nucleotide position, and target the same transcripts encoding the members of the class III homeodomain leucine zipper (HD-ZIP III) TF family (Mallory et al. 2004). In the post-embryonic root meristem, the products of *MIR165A*, *MIR166A* and *MIR166B*, most probably mature forms of microRNA165/166 (miR165/6), non-cell-autonomously suppress the expression of a HD-ZIP III TF, *PHABULOSA* (*PHB*), and possibly also other members of this TF family (Carlsbecker et al. 2010, Miyashima et al. 2011). Furthermore, by examining the *PHB* expression pattern and cell differentiation defects in response to the manipulation of miR165 levels in the endodermis, we propose a model in which endodermis-derived miR165 acts as a morphogen-like signal, forming an activity gradient across the radially organized root tissue layers and thereby controlling the multiple differentiation statuses of root tissues (Miyashima et al. 2011). Therefore, non-cell-autonomous TFs and miR play important roles in the transmission of positional cues during root pattern formation.

The early stages of Arabidopsis embryogenesis provide another unique opportunity to study plant pattern formation. In contrast to pattern formation in the post-embryonic root meristem, which is essentially a process of maintaining a pre-formed tissue pattern, embryonic pattern formation creates new patterns from a single-celled zygote. Therefore, embryonic pattern formation requires the coordinated production of new cells at specific positions and the continuous assignment of new cell fates. In the early stages of Arabidopsis embryogenesis, pattern formation is mainly controlled by the patterned expression of several TF genes (Jeong et al. 2012). For example, members of the WUSCHEL-RELATED HOMEOBOX (WOX) TF family are expressed in specific cells in early embryos. Loss-of-function *wox* mutants are perturbed in embryonic pattern formation, as well as in the expression of the other *WOX* genes (Breuninger et al. 2008). Similarly, expression of the AUXIN RESPONSE FACTOR (ARF) TFs is patterned in early embryos (Rademacher et al. 2011). ARF TFs regulate transcriptional output at the end of the TIR1/AFB-dependent auxin signal transduction pathway (Hayashi 2012). Because ARF TFs are functionally diversified, the ARF pre-pattern seems to play an important role in providing distinct developmental output from a certain auxin level (Rademacher et al. 2012).

Members of the HD-ZIP III TF family also play important roles in early embryogenesis. In globular stage embryos, three HD-ZIP III TFs, PHB, PHAVOLUTA (PHV) and REVOLUTA (REV), are expressed in the central–apical domain of the embryo proper and specify the apical fate (Grigg et al. 2009, Liu et al. 2009, Smith and Long 2010). Their ectopic expression in the basal part of embryos resulted in the formation of a SAM in place of a root apical meristem (RAM; Smith and Long 2010). HD-ZIP III expression is also regulated by miR165/6 in embryos. Loss of a zinc-finger protein *SERRATE* (*SE*), which functions as a scaffold in an miR biogenesis complex (Fujioka et al. 2007, Machida et al. 2011), leads to embryo lethality due to uniform expression of *PHB* and *PHV* in early embryos (Grigg et al. 2009). These observations indicate a key role for the miR165/6-dependent suppression of *PHB* and *PHV* in early embryos, and hence the correct specification of the SAM and RAM in embryogenesis. However, it has not been investigated in detail at which stage and at which position the miR165/6-dependent suppression operates in early embryos. While expression patterns of *MIR165/6* have been previously examined by in situ hybridization of mature miR165/6, as well as reporter analyses of *MIR165A* and *MIR166A* (Williams et al. 2005, Liu et al. 2009, Yao et al. 2009), these available data do not have an adequate signal-to-noise ratio and spatial resolution to address the mechanism by which miR165/6 control spatiotemporal expression patterns of HD-ZIP III TFs. In this study, we analyzed the spatiotemporal expression patterns of the *MIR165/6* gene family in embryogenesis, and their non-cell-autonomous functions in controlling *PHB* expression patterns. The results indicated a key role for miR165/6 production in the basal–peripheral region of early embryos. These miRs restrict *PHB* expression to the central–apical domain of embryos, which in turn is required for the correct specification of the SAM and RAM.

Results

A subset of *MICRORNA165/6* genes are expressed in largely conserved patterns during embryogenesis

We previously generated reporter lines of all nine Arabidopsis *MIR165/6* genes, by fusing the 5′ upstream region of each *MIR165/6* gene to a cell-autonomous GFPer reporter that encodes an endoplasmic reticulum-targeted green fluorescent protein (GFP) (Miyashima et al. 2011). This reporter collection allowed us to demonstrate that three *MIR165/6* genes, *MIR165A*, *MIR166A* and *MIR166B*, are expressed specifically in the endodermis and quiescent center of the post-embryonic root meristem (Miyashima et al. 2011). In the present study, we

utilized this reporter collection to examine *MIR165/6* expression patterns in the course of embryogenesis.

For each *MIR165/6* gene, embryos were isolated from 3–5 independent reporter lines, and observed by confocal laser scanning microscopy. Cellular patterns were visualized by counterstaining the embryos with propidium iodide or FM4-64, which both emit red fluorescence. Four *MIR165/6* genes, namely *MIR165A*, *MIR166A*, *MIR166B* and *MIR166G*, showed GFP fluorescence in embryos (**Fig. 1**). The expression patterns were shared by the majority of independent lines for each gene, confirming that the observed expression patterns were controlled by the inserted promoter regions. In our initial observation, the *MIR165B* reporter lines did not show GFP fluorescence in either the embryos or the post-embryonic roots (Miyashima et al. 2011). We suspected that the 2.6 kb *MIR165B* promoter region used in the reporter lines had been insufficient, because it did not include the entire 4.0 kb intergenic region (**Supplementary Fig. S1**). We therefore generated an additional reporter construct of *MIR165B* using the entire intergenic region (**Supplementary Fig. S1**). Observation of three independent lines produced with this construct indicated that *MIR165B* is indeed expressed in a pattern very similar to those of the four *MIR165/6* genes mentioned above (**Fig. 1B1–6**). As shown in **Supplementary Fig. S2**, the new *MIR165B* reporter lines also exhibited strong GFP fluorescence in the root endodermis, similarly to the *MIR165A*, *MIR166A* and *MIR166B* reporter lines (Miyashima et al. 2011). Expression of the other four genes (*MIR166C*, *MIR166D*, *MIR166E* and *MIR166F*) was not detected at any stage of embryogenesis. Considering that the entire intergenic region was used as a promoter for each of these four reporter constructs, as well as our observation that some of these reporter lines showed GFP expression in other aspects of Arabidopsis development (our unpublished results), it is likely that these four *MIR166* genes do not function in embryogenesis.

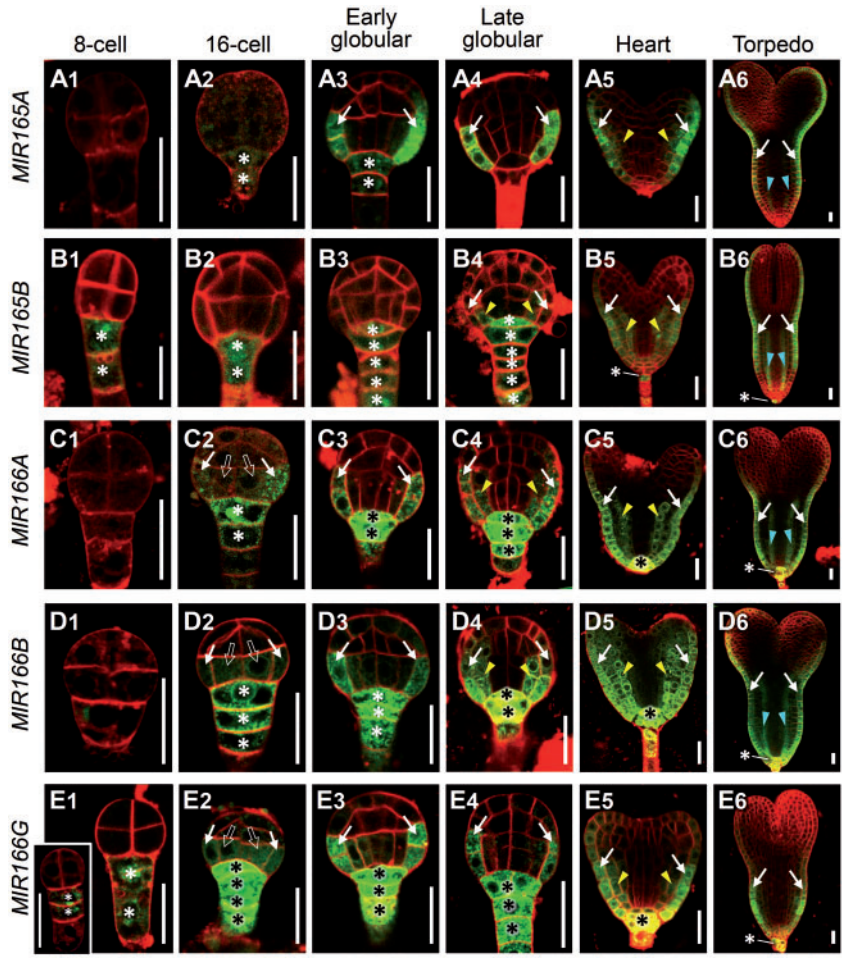

Fig. 1 *MIR165/6* genes are transcribed in a largely conserved pattern in embryogenesis. Confocal images showing the GFP reporter expression patterns (green) of *MIR165A* (A1–6), *MIR165B* (B1–6), *MIR166A* (C1–6), *MIR166B* (D1–6) and *MIR166G* (E1–6), at the stages indicated above. The inset in E1 is an image of a two-cell stage embryo of an *MIR166G* reporter line. White filled arrows, expression in the outermost cell layer; open arrows, weak expression in the inner layer of 16-cell stage embryos; yellow arrowheads, expression in the embryonic ground tissue; blue arrowheads, focused expression in the embryonic endodermis of torpedo stage embryos; asterisks (white or black), expression in the cells of the basal lineage. Bar, 20 μm.

The five *MIR165/6* genes exhibited largely conserved expression patterns (**Fig. 1**). The expression of all five *MIR165/6* genes was detected from the early embryo stages onward, although the stage in which expression was first detected varied slightly amongst the genes. The earliest expression was detected for *MIR166G*, whose expression became visible at the uppermost suspensor cell of the two-cell stage embryo (inset in **Fig. 1E1**). Expression of *MIR165B* was first detected in the uppermost suspensor of the eight-cell stage embryo (**Fig. 1B1**). Expression of *MIR165A*, *MIR166A* and *MIR166B* became detectable from the 16-cell stage onwards (**Fig. 1A2, C2, D2**). At this stage, expression was detected in the uppermost suspensor cells (asterisks in **Fig. 1A2, B2, C2, D2, E2**), though expression was weak for *MIR165A*. For the lines other than *MIR165A* and *MIR165B*, cells at the basal half domain of the embryo proper (lower tier = *lt*; Scheres et al. 1994) also expressed GFP (filled and open arrows in **Fig. 1C2, D2, E2**). In the early and late globular stages, all five *MIR165/6* genes were expressed in a similar pattern; strong GFP signal was found at the outermost *lt* layer and the 2–3 cells located at the upper end (at the side of the embryo proper) of the suspensor (arrows and asterisks in **Fig. 1A3, 4, B3, 4, C3, 4, D3, 4, E3, 4**). We will refer to this expression domain as the 'basal–peripheral' region. For all five *MIR165/6* genes, expression in the outermost *lt* layer persisted throughout the later stages of embryogenesis. Expression in this layer gradually extended toward the tip of cotyledon primordia, except for *MIR166G* (**Fig. 1A5, 6, B5, 6, C5, 6, D5, 6, E5, 6**). Expression in the upper suspensor cells disappeared in *MIR165A* reporter lines at the late globular stage (**Fig. 1A4**), while it persisted in *MIR165B*, *MIR166A*, *MIR166B* and *MIR166G* (**Fig. 1B4–6, C4–6, D4–6, E4-6**).

In addition to the expression in the outermost *lt* layer and upper suspensor cells described above, the expression of all five *MIR165/6* reporters became detectable in the inner *lt* layers in late globular to heart stage embryos (yellow arrowheads in **Fig. 1A5, B4, C4, D4, E5**). These inner *lt* layers, termed the embryonic ground tissue, are the progenitor of the cortex and endodermis layers of post-embryonic roots (Wysocka-Diller et al. 2000). For *MIR165A*, *MIR165B*, *MIR166A* and *MIR166B*, expression in the embryonic ground tissue became stronger in a single cell layer corresponding to the post-embryonic endodermis at the torpedo stage (blue arrowheads in **Fig. 1A6, B6, C6, D6**). In contrast, *MIR166G* was not expressed in the embryonic endodermis (**Fig. 1E6**).

Multiple upstream pathways regulate *MICRORNA165/6* expression in embryogenesis

We previously reported that three *MIR165/6* genes, *MIR165A*, *MIR166A* and *MIR166B*, are expressed in the root endodermis in a manner dependent on SCR (Miyashima et al. 2011). Carlsbecker et al. (2010) also reported the same results for *MIR165A* and *MIR166B*, and demonstrated the direct binding of SHR to the *MIR165A* and *MIR166B* promoters. Because SHR and SCR are known to form a TF complex that regulates a number of endodermis-specific genes in roots (Welch et al. 2007), we suspected that expression of *MIR165A*, *MIR165B*, *MIR166A* and *MIR166B* in the embryonic ground tissue is also controlled by the SHR–SCR TF complex. In support of this view, *SHR* and *SCR* are expressed in the embryonic stele and the ground tissue from the globular and triangular stages onwards, respectively (**Supplementary Fig. S3**; Helariutta et al. 2000, Wysocka-Diller et al. 2000).

To test this hypothesis, we observed the expression of *MIR165A*, *MIR166A* and *MIR166B* reporters in the loss-of-function *scr-3* mutant (Fukaki et al. 1998). In the wild-type torpedo stage embryo, *MIR165A* expression was stably maintained in the endodermis layer of late torpedo stage embryos (blue arrowheads in **Fig. 2G, H**). In contrast, the expression of the *MIR165A* reporter was not focused in the corresponding cell layer in *scr-3* embryos at the comparable stage (**Fig. 2E, F**), indicating that the SHR/SCR-dependent expression of *MIR165A* in the endodermis starts in late torpedo stage embryos and continues to the post-embryonic root. The expression of *MIR166A* and *MIR166B* also became less focused in the ground tissue layer of *scr-3* torpedo stage embryos (**Supplementary Fig. S4D, H**). In contrast, the strong GFP expression in the outermost *lt* layer of early embryos was not altered in *scr-3* embryos for all three *MIR165/6* genes tested (**Fig. 2A–C**; **Supplementary Fig. S4B, F**). Somewhat unexpectedly, a weak GFP signal in the ground tissue of heart- and early torpedo stage embryos was also maintained in the *scr-3* embryos (yellow arrowheads in **Fig. 2B, C**; **Supplementary Figs. S4C, D, G, H**). These observations indicate that while the expression of *MIR165A*, *MIR166A* and *MIR166B* in the embryonic endodermis depends on the SHR/SCR pathway after the late torpedo stage as in the root, expression in the outermost *lt* layer and in the embryonic ground tissue before the torpedo stage is controlled by unknown upstream factors.

miR165/6 non-cell-autonomously control the *PHB* expression pattern in early embryos

We have previously demonstrated that, in the post-embryonic root, endodermis-derived miR165/6 non-cell-autonomously restrict *PHB* expression to the central stele, and thereby function as a dose-dependent positional cue to pattern root tissue organization (Miyashima et al. 2011). While the involvement of HD-ZIP III TFs in axis formation and organogenesis during embryogenesis has been well documented, the miR-mediated spatiotemporal regulation of HD-ZIP III expression patterns has not been analyzed at the resolution of embryonic cellular patterns. It is also not known whether or not miR165/6 act in a non-cell-autonomous manner in embryos, as has been demonstrated in the post-embryonic root meristem (Carlsbecker et al. 2010, Miyashima et al. 2011).

To address these questions, we utilized the *PHB-GFP*, *PHBmu-GFP* and *PHBmu-GFP/MIR165Amu* transgenic plants generated in our laboratory (**Fig. 3A**; Miyashima et al. 2011). Briefly, *PHB-GFP* plants contain a transgenic copy of the *PHB*

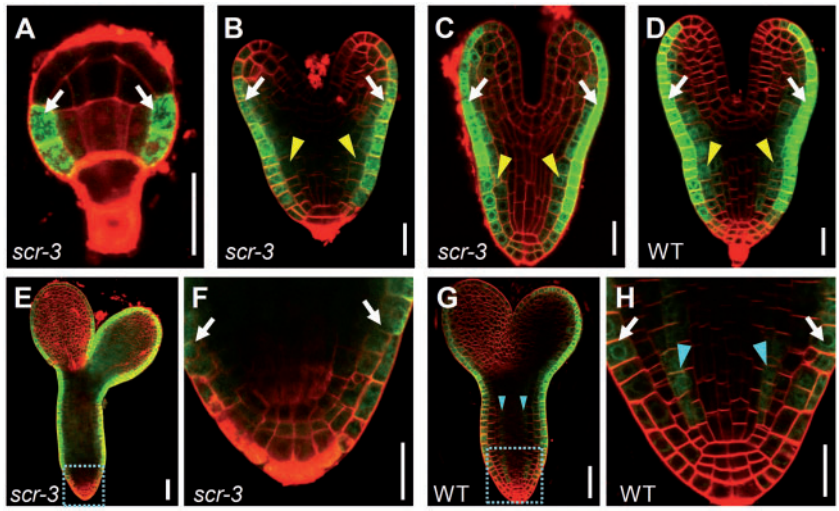

Fig. 2 The SHR/SCR pathway does not control *MIR165A* expression in early embryos. Confocal images showing the GFP reporter expression patterns (green) of *MIR165A* in *scr-3* (A–C, E, F) and wild-type (D, G, H) embryos. F and H are magnifications of the boxed regions in E and G, respectively. White filled arrows, expression in the outermost cell layer; yellow arrowheads, expression in the embryonic ground tissue; blue arrowheads, focused expression in the embryonic endodermis; Bar, 20 μm (A–D, F, H); 50 μm (E, G).

genomic fragment fused in-frame with a GFP-coding sequence at the C-terminal end. GFP fluorescence in this line closely mimics the endogenous distribution pattern of *PHB* transcripts that are suppressed by miR165/6. The second line, *PHBmu-GFP*, contains a transgene that is identical to *PHB-GFP* except for the presence of two point mutations that abolish miR165/6-dependent suppression. Because the protein products from *PHB-GFP* and *PHBmu-GFP* transgenes are identical in amino acid sequence and possess PHB protein functions, *PHBmu-GFP* plants phenocopy miR-resistant *phb-D* mutants. Moreover, by comparing the GFP expression patterns of *PHB-GFP* and *PHBmu-GFP* plants, one can investigate the extent of miR165/6-dependent post-transcriptional regulation of *PHB* expression (Miyashima et al. 2011). The third line, *PHBmu-GFP/MIR165Amu*, contains another transgene, *MIR165Amu*, in addition to *PHBmu-GFP*. The *MIR165Amu* transgene harbors two point mutations that restore complementarity to *PHBmu-GFP*. *MIR165Amu* can completely suppress the *phb-D*-like phenotypes of *PHBmu-GFP* plants (Miyashima et al. 2011).

PHB-GFP embryos exhibited no morphological defects and their GFP expression faithfully recapitulated the expression pattern of endogenous *PHB* transcripts in the course of wild-type embryogenesis (**Fig. 3B–G**; Smith and Long 2010). In *PHB-GFP* embryos, the GFP signal was first detected in the upper four cells in the eight-cell stage embryo (arrows in **Fig. 3C**; upper tier = *ut*; Scheres et al. 1994). Subsequently, the GFP signal became restricted to the apical domain of the embryo proper (**Fig. 3D**) and then to the central–apical region (**Fig. 3E, F**). In torpedo stage embryos, the GFP signal was restricted to the region comprising the central vascular cylinder, presumptive SAM and the adaxial side of cotyledon primordia (**Fig. 3G**).

We next observed embryos derived from *PHBmu-GFP/MIR165Amu* double homozygous parents. GFP expression patterns in the embryos of this genotype were almost identical to those of the *PHB-GFP* embryos described above (compare **Fig. 3H–M** with **3B–G**), with the exception that the eight-cell stage *PHBmu-GFP/MIR165Amu* embryos expressed GFP in all eight cells in the embryo proper (compare **Fig. 3C** and **I**, arrows). These observations indicate that miR165 derived from *MIR165A* is sufficient to pattern wild-type *PHB* expression in the course of embryogenesis, except in eight-cell stage embryos. Ectopic expression of *PHBmu-GFP* in the basal region of eight-cell stage *PHBmu-GFP/MIR165Amu* embryos suggests that *MIR165/6* genes other than *MIR165A*, i.e. *MIR165B*, *MIR166A*, *MIR166B* or *MIR166G*, are required for the suppression of *PHB* in the basal part of eight-cell stage embryos. However, at the eight-cell stage or earlier, the only detectable *MIR165/6* expression is that of *MIR165B* and *MIR166G* in the uppermost suspensor cells (**Fig. 1B1, E1**), and not in the basal embryo proper cells per se. These results suggest that the miR165/6 produced from the *MIR165B* and *MIR166G* loci in the upper suspensor cells non-cell-autonomously suppresses *PHB* expression in the basal region of the embryo proper (**Fig. 4**).

We then observed GFP fluorescence in the *PHBmu-GFP* embryos, which reflects the *PHB* expression patterns in the absence of miR-dependent suppression. Because the *PHBmu-GFP* line is not maintainable on its own, we observed embryos from the parental plants that were homozygous for *PHBmu-GFP* and hemizygous for *MIR165Amu*. As expected, about a quarter of the embryos showed GFP expression in nearly every cell in the embryo proper up to the early torpedo stage (**Fig. 3N–Q**). We regarded these embryos as segregants without *MIR165Amu*. After the heart stage, the morphology of *PHBmu-GFP* embryos deviated from those of the wild type (**Fig. 3P, Q**). A comparison of the GFP expression patterns between *PHBmu-GFP* (**Fig. 3N–Q**) and *PHBmu-GFP/MIR165Amu* (**Fig. 3J–M**) embryos suggested that miR165 had additional

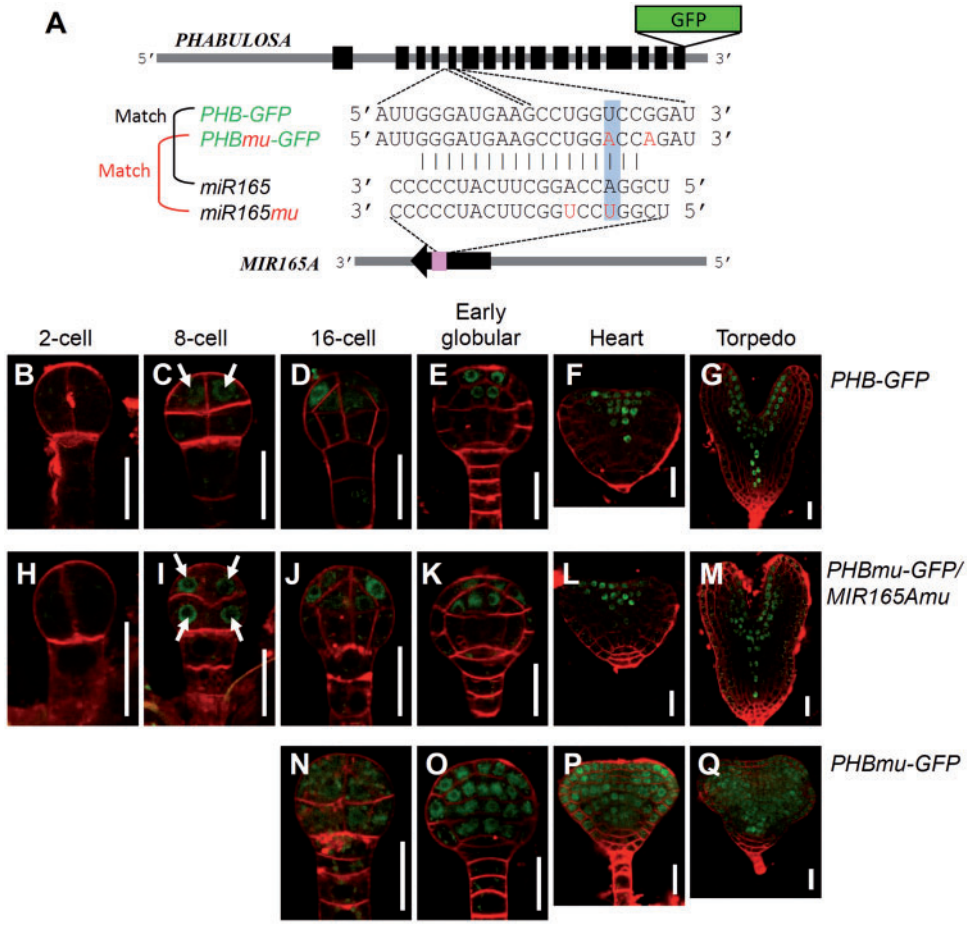

Fig. 3 The expression patterns of *PHB* in the presence or absence of miR165/6-dependent suppression. (A) A diagram showing the configurations of *PHB-GFP* reporters and *MIR165A* transgene fragments. Nucleotide sequences of miR165 and their target site in *PHB*, as well as mutations introduced into these constructs (in red font) are shown. Base pairing at the position shown in a blue background is critical to the miR-dependent cleavage (Mallory et al. 2004). This diagram is modified from Miyashima et al. (2011). (B–Q) GFP expression patterns (green) in embryos transformed with *PHB-GFP* (B–G), *PHBmu-GFP/MIR165Amu* (H–M) and *PHBmu-GFP* (N–Q). Arrows indicate GFP expression in eight-cell stage embryos. Bar, 20 μm.

non-cell-autonomous functions. In the globular stage embryos, for example, *MIR165Amu* restricted the expression of *PHBmu-GFP* to a few cells at the central–apical domain of the embryo proper (compare **Fig. 3K and O**). At this stage, however, *MIR165A* is expressed only in the outermost *lt* layer (**Fig. 1A4**). These observations suggest that the miR165 produced in the outermost *lt* layer non-cell-autonomously restricts *PHB* expression to the central–apical domain. In heart and torpedo stage embryos, *PHBmu-GFP* expression is restricted to a narrow domain along the central embryo axis (**Fig. 3L, M**). At these stages, *MIR165A* expression is found in the outer layers away from the GFP-expressing domain (**Fig. 1A5–6**), suggesting that miR165 produced in the outer embryo layers non-cell-autonomously restricts *PHB* expression to the central domain.

Discussion

Our comprehensive reporter analysis revealed the expression patterns of *MIR165/6* genes in the course of embryogenesis in much higher resolution than previous reports. The data indicated that five *MIR165/6* genes, namely *MIR165A*, *MIR165B*, *MIR166A*, *MIR166B* and *MIR166G*, are expressed in an overall similar pattern, i.e. in the 'basal–peripheral' region of embryos (**Fig. 4A**, cells shown in green). The conserved expression patterns across the members of the gene family suggest that this expression pattern arose before the duplication of *MIR165/6* genes. Maher et al. (2006) analyzed the phylogenetic relationship between Arabidopsis *MIR166* genes. They found that *MIR166A* and *MIR166B* reside in the duplicated genomic segments. Similarly, the tandemly arranged *MIR166C*/*MIR166D* pair and *MIR166G* are located in the duplicated segments. While a conserved expression pattern of *MIR166A* and *MIR166B* in the post-embryonic root endodermis is in good agreement with their close phylogenetic relationship, the conserved expression across the phylogenetically distant *MIR166A/B* and *MIR166G* in early embryos suggests that this expression pattern reflects an ancient role for the miR166/HD-ZIP III module in embryogenesis. Considering the evolutionarily

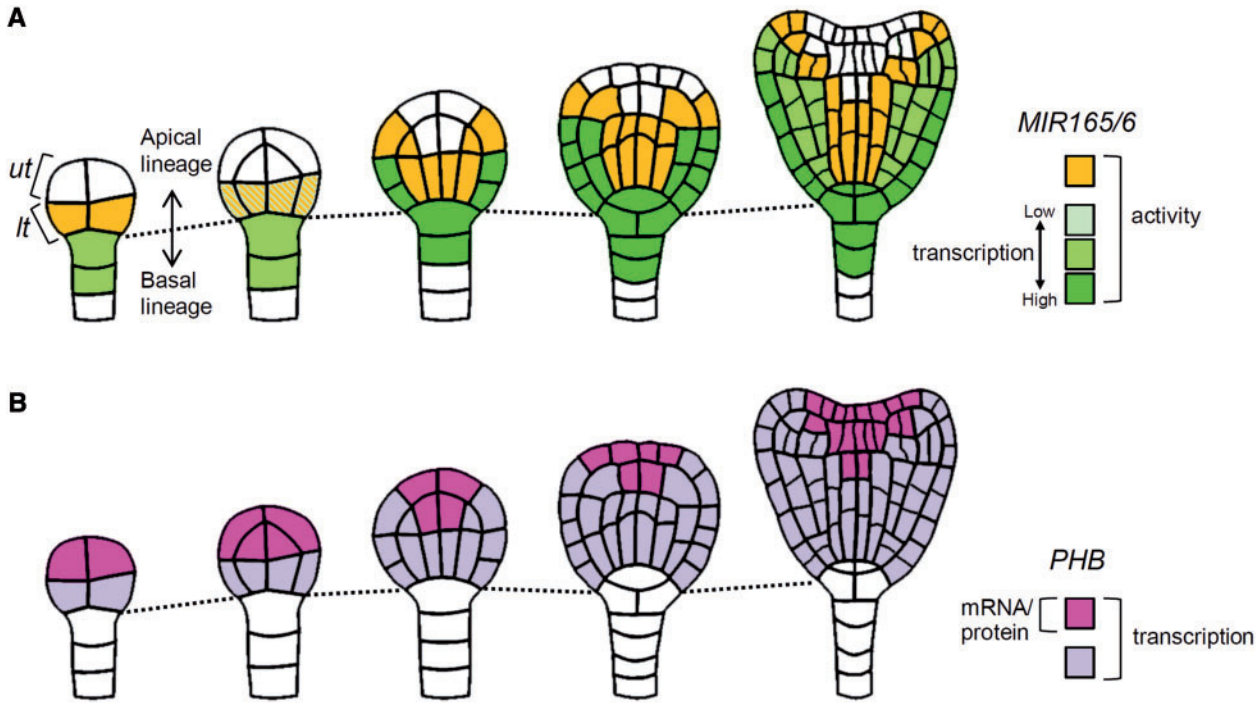

Fig. 4 Diagrams showing the spatial control of the PHB expression domain by miR165/6 in embryogenesis. (A) The site of miR165/6 production (green) and their predicted activity range (green and orange). In the 16-cell stage embryo, expression in the basal embryo proper (hatched area) was very weak. The strong PHB suppression observed in these cells suggests that the miR produced in the basal suspensor cells has non-cell-autonomous effects. (B) The site of *PHB* transcription (pink and lilac) and the distribution patterns of *PHB* mRNA and proteins in embryogenesis (pink).

conserved roles of HD-ZIP III TFs and the major contribution of miR165/6-dependent restriction to the spatial control of HD-ZIP III expression in land plants (Floyd and Bowman 2004, Floyd et al. 2006, Prigge and Clark 2006), it is conceivable that the acquisition of the 'basal–peripheral' expression pattern could date back to the older node of land plant evolution. While the miR166 expression patterns have been investigated in some stages of rice embryos (Nagasaki et al. 2007), a detailed expression analysis of embryos from more plant species is necessary to define the evolutionary origin of the miR165/6 expression patterns in embryos.

Our data indicated that the expression of *MIR165A*, *MIR166A* and *MIR166B* does not depend on SCR function before the late torpedo stage. This is even the case for globular and heart stage embryos (**Fig. 2**; **Supplementary Fig. S4**), in which SHR and SCR are already expressed and regulating the periclinal divisions of embryonic ground tissue (**Supplementary Fig. S3**) (Helariutta et al. 2000, Wysocka-Diller et al. 2000). These observations suggest that the SHR/SCR-dependent expression of *MIR165/6* requires an as yet unknown factor(s) present in the torpedo stage embryos, and that this regulation is carried over to pattern formation in the post-embryonic root meristem (Carlsbecker et al. 2010, Miyashima et al. 2011). The SHR/SCR-independent expression of the *MIR165/6* genes in early embryos raised another question as to what defines the observed expression patterns of *MIR165/6* in early embryogenesis. The basal–peripheral region-specific expression of *MIR165/6* suggests that expression is regulated by the combinatorial action of region-specific TFs (Jeong et al. 2012). In addition, the conserved expression pattern of the five *MIR165/6* genes suggests the existence of shared *cis*-regulatory elements in their promoters. However, our sequence comparison did not provide promising candidates for further analysis. We are currently performing promoter deletion analysis of the *MIR165/6* genes. A comprehensive interpretation of such experimental data and in silico analysis will shed light on the molecular mechanism that enables the production of miR165/6 in the 'basal–peripheral' region of early embryos, which appears to make a key contribution to the shoot and root meristem specification.

We have previously shown that the *PHB-GFP* marker used in this study faithfully recapitulates the expression pattern of endogenous *PHB* in post-embryonic roots (Miyashima et al. 2011). The present study extends this notion to embryogenesis, since the *PHB-GFP* expression pattern was almost identical to that determined previously by in situ hybridization, i.e. *PHB* transcripts accumulated specifically at the central–apical domain in globular stage embryos onward (**Fig. 4B**, pink; Smith and Long 2010). Our observation further indicated that *PHB* expression starts as early as in eight-cell stage embryos, where the expression is already confined to the apical half region of the embryo proper (**Fig. 3C**). In contrast, expression of the miR-resistant

PHBmu-GFP marker was expanded over the entire embryo proper in eight-cell to torpedo stage embryos (**Fig. 3I, N–Q**; **Fig. 4B**, pink and lilac). Co-expression of *MIR165Amu* and *PHBmu-GFP* resulted in GFP expression that was identical to that of wild-type *PHB-GFP* after the 16-cell stage. Since *MIR165Amu* is constructed in the *MIR165A* genomic fragment, the mutated miR165 is expected to be produced in the region marked by *MIR165A* reporter expression (**Fig. 1A1–6**). The discrepancy between the site of miR165mu production (**Fig 4A**, green) and the region of miR165mu activity as predicted by the comparison of *PHBmu-GFP* and *PHB-GFP* expression (**Fig. 4A**, orange) suggests that miR165mu can act non-cell-autonomously in early embryos, as has been demonstrated in the post-embryonic root meristem (Carlsbecker et al. 2010, Miyashima et al. 2011). In eight-cell stage embryos, *PHBmu-GFP* expression was still expanded to the basal cells even in the presence of *MIR165Amu*. Since *MIR165A* expression has not yet been initiated by this stage, the miR165/6 produced from the other loci are responsible for the suppression of *PHB* in the wild-type eight-cell stage embryos. Based on our reporter analysis, we suspect that *MIR165B* and *MIR166G* adopt this role, though this possibility has to be tested by modifying *MIR165B* and *MIR166G* to target *PHBmu-GFP*, and introducing them into the *PHBmu-GFP* background. It is notable that the observed non-cell-autonomous function of miR165/6 in early embryos is consistent with the previous observation that early Arabidopsis embryos consist of a single symplastic domain (Kim and Zambryski 2005). It should also be noted that Zhu et al. (2011) recently reported that the ARGONAUTE10 (AGO10) protein inhibits miR-dependent suppression of HD-ZIP III by sequestering miR165/6. Since AGO10 is expressed in the central region of early embryos (Lynn et al. 1999, Tucker et al. 2008), the range of miR165 activity revealed in this study could have been even broader in the absence of AGO10 action.

The involvement of non-cell-autonomous miR165 (and possibly miR166) in embryogenesis led us to ponder the developmental significance of such non-cell-autonomous miR in embryonic pattern formation. An interesting possibility is that a small embryonic region at the outermost *lt* position acts as a signaling center that emits an miR-mediated positional cue, and thereby remotely defines the position of the SAM. Region-specific disruption of miR165/6 function is necessary to test this possibility, by, for example, the targeted expression of miRNA-sequestering molecules in these cells (Yan et al. 2012).

Materials and Methods

Plant materials

The *MIR165/6* reporter lines were generated previously (Miyashima et al. 2011). Additional GFPer reporter lines for *MIR165B* were generated by sequentially inserting a GFPer-coding region derived from G3GFP (Kawakami and Watanabe 1997) and the entire 4.0 kb intergenic region upstream of *MIR165B* into the pGWB-NB1 vector (Toyokura et al. 2011) using Gateway® technology (Invitrogen). This construct was introduced into wild-type Col-0 plants to yield the reporter lines for *MIR165B*. *PHB-GFP*, *PHBmu-GFP*, *PHBmu-GFP/MIR165Amu* and *pSCR-GFP-SCR* transgenic plants have been described previously (Gallagher et al. 2004, Miyashima et al. 2011). The *pSHR-GFPer* line was generated by transforming Col-0 plants with the *UAS-GFPer-pSHR-GV* construct, which was prepared by inserting a 2.5 kb *SHR* promoter fragment (Helariutta et al. 2000) upstream of the GAL4:VP16 (GV)-coding region of *pBIN-UAS-GFPer-hssb-GV* (Waki et al. 2011).

Microscopy

Ovules were isolated from developing siliques and soaked in a drop of a solution containing 0.4 M glucose and either 7 $\mu g\,ml^{-1}$ propidium iodide (Sigma-Aldrich) or 10 $\mu g\,ml^{-1}$ FM4-64 (Invitrogen) on a slide glass. Ovules were opened using the tips of dental injection needles (DENTSPLY SANKIN) to release embryos into the solution. After about 5 min, a coverslip was placed on the sample solution and the embryos were observed using a C2 confocal laser scanning microscope (Nikon).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan [Grant-in-Aid for Scientific Research on Priority Areas (23012030 and 80273853 to K.N., and 19060004 to K.O.)]; the Japan Society for the Promotion of Science (JSPS) [a Grant-in-Aid for Creative Scientific Research (19GS0315 to K.O.) and Research Fellowships for Young Scientists (to S.M.)].

Acknowledgments

We are grateful to Yuko Ono, Emi Murata, Nagisa Sugimoto and the Model Plant Research Facility at the NIBB Bioresource Center for technical assistance.

References

Breuninger, H., Rikirsch, E., Hermann, M., Ueda, M. and Laux, T. (2008) Differential expression of WOX genes mediates apical–basal axis formation in the Arabidopsis embryo. *Dev. Cell* 14: 867–876.

Carlsbecker, A., Lee, J.Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J. et al. (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* 465: 316–321.

Chitwood, D.H., Nogueira, F.T., Howell, M.D., Montgomery, T.A., Carrington, J.C. and Timmermans, M.C. (2009) Pattern formation via small RNA mobility. *Genes Dev.* 23: 549–554.

Cui, H., Levesque, M.P., Vernoux, T., Jung, J.W., Paquette, A.J., Gallagher, K.L. et al. (2007) An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. *Science* 316: 421–425.

Floyd, S.K. and Bowman, J.L. (2004) Gene regulation: ancient microRNA target sequences in plants. *Nature* 428: 485–486.

Floyd, S.K., Zalewski, C.S. and Bowman, J.L. (2006) Evolution of class III homeodomain-leucine zipper genes in streptophytes. *Genetics* 173: 373–388.

Fujioka, Y., Utsumi, M., Ohba, Y. and Watanabe, Y. (2007) Location of a possible miRNA processing site in SmD3/SmB nuclear bodies in Arabidopsis. *Plant Cell Physiol.* 48: 1243–1253.

Fukaki, H., Wysocka-Diller, J., Kato, T., Fujisawa, H., Benfey, P.N. and Tasaka, M. (1998) Genetic evidence that the endodermis is essential for shoot gravitropism in Arabidopsis thaliana. *Plant J.* 14: 425–430.

Gallagher, K.L., Paquette, A.J., Nakajima, K. and Benfey, P.N. (2004) Mechanisms regulating SHORT-ROOT intercellular movement. *Curr. Biol.* 14: 1847–1851.

Grigg, S.P., Galinha, C., Kornet, N., Canales, C., Scheres, B. and Tsiantis, M. (2009) Repression of apical homeobox genes is required for embryonic root development in Arabidopsis. *Curr. Biol.* 19: 1485–1490.

Hayashi, K. (2012) The interaction and integration of auxin signaling components. *Plant Cell Physiol.* 53: 965–975.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G. et al. (2000) The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. *Cell* 101: 555–567.

Jeong, S., Volny, M. and Lukowitz, W. (2012) Axis formation in Arabidopsis—transcription factors tell their side of the story. *Curr. Opin. Plant Biol.* 15: 4–9.

Katsir, L., Davies, K.A., Bergmann, D.C. and Laux, T. (2011) Peptide signaling in plant development. *Curr. Biol.* 21: R356–R364.

Kawakami, S. and Watanabe, Y. (1997) Use of green fluorescent protein as a molecular marker tag of protein movement in vitro. *Plant Biotechnol.* 14: 127–130.

Kim, I. and Zambryski, P.C. (2005) Cell-to-cell communication via plasmodesmata during Arabidopsis embryogenesis. *Curr. Opin. Plant Biol.* 8: 593–599.

Kurata, T., Okada, K. and Wada, T. (2005) Intercellular movement of transcription factors. *Curr. Opin. Plant Biol.* 8: 600–605.

Levesque, M.P., Vernoux, T., Busch, W., Cui, H., Wang, J.Y., Blilou, Y. et al. (2006) Whole-genome analysis of the SHORT-ROOT developmental pathway in Arabidopsis. *PLoS Biol.* 4: e143.

Liu, Q., Yao, X., Pi, L., Wang, H., Cui, X. and Huang, H. (2009) The ARGONAUTE10 gene modulates shoot apical meristem maintenance and establishment of leaf polarity by repressing miR165/166 in Arabidopsis. *Plant J.* 58: 27–40.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P. et al. (1999) The PINHEAD/ZWILLE gene acts pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene. *Development* 126: 469–481.

Machida, S., Chen, H.Y. and Adam Yuan, Y. (2011) Molecular insights into miRNA processing by Arabidopsis thaliana SERRATE. *Nucleic Acids Res.* 39: 7828–7836.

Maher, C., Stein, L. and Ware, D. (2006) Evolution of Arabidopsis microRNA families through duplication events. *Genome Res.* 16: 510–519.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K. et al. (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. *EMBO J.* 23: 3356–3364.

Maule, A.J., Benitez-Alfonso, Y. and Faulkner, C. (2011) Plasmodesmata—membrane tunnels with attitude. *Curr. Opin. Plant Biol.* 14: 683–690.

Miyashima, S., Koi, S., Hashimoto, T. and Nakajima, K. (2011) Non-cell-autonomous microRNA165 acts in a dose-dependent manner to regulate multiple differentiation status in the Arabidopsis root. *Development* 138: 2303–2313.

Nagasaki, H., Itoh, J., Hayashi, K., Hibara, K., Satoh-Nagasawa, N., Nosaka, M. et al. (2007) The small interfering RNA production pathway is required for shoot meristem initiation in rice. *Proc. Natl Acad. Sci. USA* 104: 14867–14871.

Nakajima, K., Sena, G., Nawy, T. and Benfey, P.N. (2001) Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* 413: 307–311.

Prigge, M.J. and Clark, S.E. (2006) Evolution of the class III HD-Zip gene family in land plants. *Evol. Dev.* 8: 350–361.

Rademacher, E.H., Lokerse, A.S., Schlereth, A., Llavata-Peris, C.I., Bayer, M., Kientz, M. et al. (2012) Different auxin response machineries control distinct cell fates in the early plant embryo. *Dev. Cell* 22: 211–222.

Rademacher, E.H., Moller, B., Lokerse, A.S., Llavata-Peris, C.I., van den Berg, W. and Weijers, D. (2011) A cellular expression map of the Arabidopsis AUXIN RESPONSE FACTOR gene family. *Plant J.* 68: 597–606.

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. et al. (1994) Embryonic origin of the Arabidopsis primary root and root meristem initials. *Development* 120: 2475–2487.

Smith, Z.R. and Long, J.A. (2010) Control of Arabidopsis apical–basal embryo polarity by antagonistic transcription factors. *Nature* 464: 423–426.

Tucker, M.R., Hinze, A., Tucker, E.J., Takada, S., Jürgens, G. and Laux, T. (2008) Vascular signalling mediated by ZWILLE potentiates WUSCHEL function during shoot meristem stem cell development in the Arabidopsis embryo. *Development* 135: 2839–2843.

Toyokura, K., Watanabe, K., Oiwaka, A., Kusano, M., Tameshige, T., Tatematsu, K. et al. (2011) Succinic semialdehyde dehydrogenase is involved in the robust patterning of Arabidopsis leaves along the adaxial–abaxial axis. *Plant Cell Physiol.* 52: 1340–1353.

Van Norman, J.M., Breakfield, N.W. and Benfey, P.N. (2011) Intercellular communication during plant development. *Plant Cell* 23: 855–864.

Waki, T., Hiki, T., Watanabe, R., Hashimoto, T. and Nakajima, K. (2011) The Arabidopsis RWP-RK protein RKD4 triggers gene expression and pattern formation in early embryogenesis. *Curr. Biol.* 21: 1277–1281.

Welch, D., Hassan, H., Blilou, I., Immink, R., Heidstra, R. and Scheres, B. (2007) Arabidopsis JACKDAW and MAGPIE zinc finger proteins delimit asymmetric cell division and stabilize tissue boundaries by restricting SHORT-ROOT action. *Genes Dev.* 21: 2196–2204.

Williams, L., Grigg, S.P., Xie, M., Christensen, S. and Fletcher, J.C. (2005) Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. *Development* 132: 3657–3668.

Wysocka-Diller, J.W., Helariutta, Y., Fukaki, H., Malamy, J.E. and Benfey, P.N. (2000) Molecular analysis of SCARECROW function

reveals a radial patterning mechanism common to root and shoot. *Development* 127: 595–603.

Yadav, R.K., Girke, T., Pasala, S., Xie, M. and Reddy, G.V. (2009) Gene expression map of the Arabidopsis shoot apical meristem stem cell niche. *Proc. Natl Acad. Sci. USA* 106: 4941–4946.

Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X. et al. (2012) Effective small RNA destruction by the expression of a short tandem target mimic in Arabidopsis. *Plant Cell* 24: 415–427.

Yao, X., Wang, H., Li, H., Yuan, Z., Li, F., Yang, L. et al. (2009) Two types of cis-acting elements control the abaxial epidermis-specific transcription of the MIR165a and MIR166a genes. *FEBS Lett.* 583: 3711–3717.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.H., Liou, L.W. et al. (2011) Arabidopsis Argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. *Cell* 145: 242–256.