SHORT COMMUNICATION

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SHORT-ROOT regulates vascular patterning, but not apical meristematic activity in the Arabidopsis root through cytokinin homeostasis

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Abbreviations: BA, 6-benzylaminopurine; miR, microRNA; XP, xylem-associated pericycle; PP, phloem-associated pericycle; ChIP, chromatin immunoprecipitation

SHORT-ROOT (SHR) is a key regulator of radial patterning and stem-cell renewal in the Arabidopsis root. Although SHR is expressed in the stele, its function in the vascular tissue was not recognized until recently. In *shr*, the protoxylem is missing due to the loss of expression of microRNA165A (miR165A) and microRNA166B (miR165B). *shr* is also defective in lateral root formation, but the mechanism remains unclear. To dissect the SHR developmental pathway, we recently have identified its direct targets at the genome scale by chromatin immunoprecipitation followed by microarray analysis (ChIP-chip). In further studies, we have shown that SHR regulates cytokinin homeostasis through cytokinin oxidase 3 and that this role of SHR is critical to vascular patterning in the root. In this communication we report that SHR also regulates miR165A and miR166B indirectly through its effect on cytokinin homeostasis. Although cytokinin is inhibitory to root growth, the root-apical-meristem defect in *shr* was not alleviated by reduction of endogenous cytokinin. These results together suggest that SHR regulates vascular patterning, but not root apical meristematic activity, through cytokinin homeostasis.

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As the major organ for water and inorganic-nutrient uptake, root is critically important for land-plant growth and development. The root increases its surface area by growing lengthwise through the mitotic activity of the root apical meristem and by branching through lateral or adventitious root formation. Because different cell types in the root have different functions, their cell-fate specification must be precisely regulated and proper ratios among them must be maintained.

A typical dicot primary root has more than seven cell types, which are arranged in two distinct patterns (**Fig. 1A**). The outer cell types, such as the epidermis, cortex, endodermis, and pericycle, form concentric rings. In the center of the root is the vascular tissue, which is composed of several cell types that are organized into separate domains of phloem and xylem. The pericycle surrounds the vascular tissue and, in most plant species, is the site of lateral root formation. The pericycle has long been regarded as a single cell type, but recent studies showed that it is composed of two populations of cells that differ both anatomically and functionally—one (XP) is associated with the xylem and the other (PP) adjoins the phloem.[1] The XP cells have dense cytoplasm and can produce lateral roots.[1] Cell division in the XP is induced by auxin but repressed by cytokinin.[2] In contrast, the PP cells have large vacuoles, indicating a greater degree of differentiation, do not divide in the presence of auxin, and are not involved in lateral root formation.[1]

Auxin and cytokinin regulate not only lateral root formation but also root growth—auxin promotes, but cytokinin inhibits, root apical meristematic activity, thereby increasing and reducing the meristem size, respectively.[3] Auxin and cytokinin also have opposite roles in vascular differentiation and patterning. On one hand, auxin induces xylem differentiation, whereas cytokinin promotes phloem specification. The ratio between their concentrations also determines the relative abundance of xylem and phloem.[4,5] On the other hand, the high levels of auxin and cytokinin in the xylem and phloem respectively in turn instruct procambium cells to differentiate according to their position, thereby maintaining the continuity of the vascular tissue.[4,5]

Although the antagonistic roles of auxin and cytokinin in vascular tissue differentiation and patterning are well documented, only recently has light been shed on the molecular basis of this

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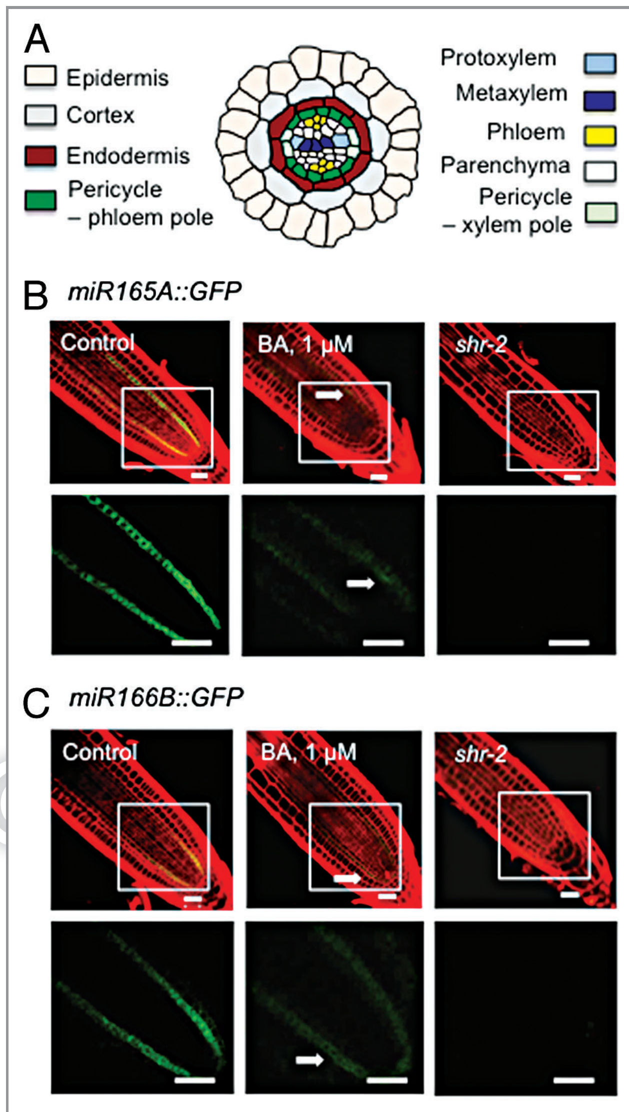

Figure 1. Cytokinin represses *miR165A* and *miR166B* expression. (A) Diagram of the cell pattern in the primary root of *Arabidopsis thaliana*. (B–C) Confocal microscopy image of *miR165A::GFP* and *miR166B::GFP* expression in wild-type root grown in normal medium or medium containing 1 μM 6-benzylaminopurine (a synthetic cytokinin) and in *shr-2* mutant. The GFP signal in the framed area is shown at a higher magnification in the lower panel. Arrow marks residual expression. One-week-seedling roots were stained for 1 min with propidium iodide (10 μg/mL dissolved in water), and images were taken with a Zeiss LSM510 confocal microscope. Bars = 20 μm.

regulation. Bishopp et al.[6] showed that cytokinin induces the expression of *PIN-FORMED7 (PIN7)* in procambium cells that are connected with phloem. PIN7 is an auxin transporter, and by exporting auxin to neighboring cells, it generates a region with a relatively high level of cytokinin (the PIN7 domain), which would assume phloem identity. In cells outside of the PIN7 domain, where the auxin level is relatively high, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN6 (AHP6)* is induced. Because AHP6 is a pseudophosphotransfer protein that blocks cytokinin signal transduction, *PIN7* is not expressed in the APH6 domain, which will maintain its relative high level of auxin and eventually differentiate into protoxylem. Although this model offers a molecular basis for the maintenance of vascular pattern, however, it does not explain how the vascular pattern is established de novo; nor does it account for the inhibitory effect of cytokinin on lateral root formation and xylem specification, because, according to this model, the AHP6 domain and the protoxylem would be maintained regardless of the level of cytokinin. Our recent study showing that SHR controls vascular patterning by regulating cytokinin homeostasis provides a critical piece to this puzzle.[7]

SHR was initially identified as a regulator of stem-cell renewal and ground-tissue patterning in the Arabidopsis root.[8] It was subsequently found also to play a role in vascular tissue patterning and lateral root formation.[9-11] Although much has been learned about the mechanisms by which SHR regulates ground tissue patterning,[8,9,12-14] how SHR controls other aspects of root development is unclear. To dissect the mechanisms by which SHR controls root morphogenesis, we have determined the genomewide location of its direct targets by ChIP-chip.[7] Through clustering analysis, we found that a large fraction of SHR targets are preferentially expressed in the pericycle and xylem. Using cell-type-specific GFP marker lines, we demonstrated that, in *shr*, the phloem and PP domains are expanded, whereas the xylem and XP domains are reduced in size. Despite the observation that auxin-signaling factors are among the list of SHR direct targets and a recent report that *shr* has an elevated level of auxin, the vascular patterning defect in *shr* is reproduced by cytokinin but not by auxin. The vascular patterning defect in *shr* is alleviated by overexpression of CKX1, a cytokinin-degradation enzyme.[15] We found that, consistent with this result, cytokinin content is elevated in *shr* and SHR directly regulates *CKX3*. Remarkably, according to the RootMap,[16] *CKX3* is preferentially expressed in the protoxylem. These results suggest that, by activating *CKX3*, SHR produces a zone with a low level of cytokinin, therefore promoting xylem and XP specification. In *shr* or when exogenous cytokinin is present, this cytokinin minimum is disrupted, and the resulting high cytokinin would repress protoxylem and XP cell fate, consequently causing a defect in lateral-root formation. Because SHR is expressed from the globular stage of embryogenesis, SHR probably plays a key role in the initial set-up of cytokinin minimum and eventually the phloem and xylem domains.

Two microRNA genes, *miR165A* and *miR166B*, were recently shown to play a pivotal role in protoxylem and pericycle specification.[12,17] It was also shown that SHR controls vascular patterning by direct activation of these genes in the endodermis, which moves into the stele to promote protoxylem differentiation.[12] Because cytokinin level is elevated in *shr* and exogenous cytokinin causes a *shr*-like vascular patterning phenotype in wild-type root, we hypothesized that SHR also regulates *miR165A* and *miR166B* indirectly through cytokinin. To investigate this possibility, we examined roots that express the GFP reporter gene under the promoters of *miR165A* and *miR166B*, in the absence or presence of the synthetic cytokinin 6-benzylaminopurine (BA). As shown in **Figure 1**, in medium

containing 1 μM BA, GFP fluorescence from the *miR165A::GFP* and *miR166B::GFP* reporter constructs was barely detectable, similar to that observed in *shr* roots. These results support the notion that SHR regulates *miR165A* and *miR166B* through its effect on cytokinin homeostasis.

SHR is essential not only for radial patterning but also for the maintenance of the root apical meristem. In *shr,* the quiescent center (QC) cells are exhausted early during postembryonic development and, as a consequence, the apical meristem is depleted and root growth ceases. Because cytokinin is inhibitory to root meristematic activity, the root growth defect in *shr* could be due to its high level of cytokinin. We first tested this hypothesis by overexpressing CKX1, a cytokinin oxidase, under the CaMV 35S promoter in *shr*. *CKX1* was used in this study, because the *35S::CKX1* transgene has been shown to be able to reduce cytokinin content effectively in wildtype root.[15] Although the vascular patterning phenotype in *shr* is rescued by the *35S::CKX1* transgene, as reported previously,[7] root growth was not improved (**Fig. 2A**). We also tested the effect of *pCRE1::CKX2*, which expresses the *CKX2* gene specifically in the stele, but again the root growth defect in *shr* was not rescued (not shown). Because root growth is determined by both mitotic activity in the apical meristem and longitudinal cell extension in the elongation zone, in order to assess the effect of cytokinin reduction on root meristematic activity in *shr*, we next compared the size of the root apical meristem in *shr* and *shr* overexpressing CKX1. As shown in **Figure 2B**, these plants had no difference in the size of their apical meristem, as indicated by the similar number of cortex cells along the longitudinal axis of the apical meristem (p = 0.82, t-test, n = 14). Although these results do not exclude a role for cytokinin in root growth in *shr*, they strongly suggest that other factors acting downstream of SHR play a major role in regulating root meristematic activity.

On the basis of all these observations, we suggest that SHR regulates vascular patterning, but not apical meristematic activity, in the Arabidopsis root through cytokinin homeostasis.

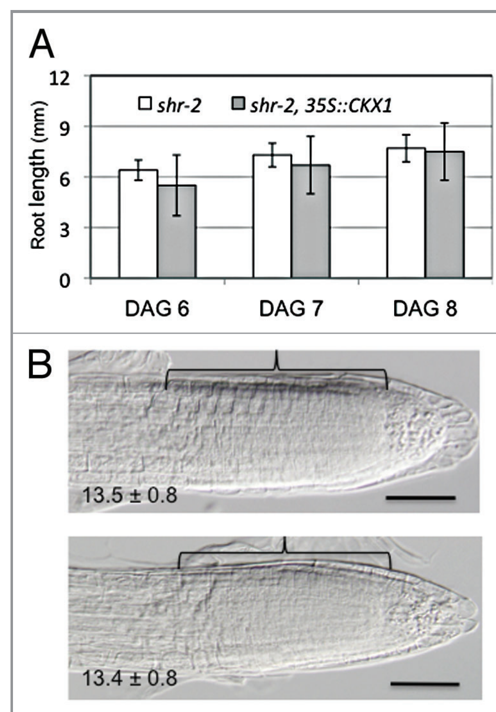

Figure 2. The root meristematic defect in *shr* is not rescued by reduction of cytokinin. (A) Root length of *shr-2* and *shr-2* expressing cytokinin oxidase 1 (*35S::CKX1*) at different time after seed germination. The error bars are standard deviations from measurements of 20 roots. p = 0.06, 0.2 and 0.8 respectively (t-test). DAG, days after germination. (B) Representative microscopic images of *shr-2* (upper) and *shr-2 35S::CKX1* (lower) roots, 7 d after germination. The value at the lower left corner is the number of ground tissue cells along the longitudinal axis of the meristem (bracket) and the standard deviations. p = 0.8 (*t*-test, n = 14). Bars = 50 μm.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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