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SCARECROW reinforces SHORT-ROOT signaling and inhibits periclinal cell divisions in the ground tissue by maintaining SHR at high levels in the endodermis

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In contrast to development in animals, much of the patterning of plants occurs post-embryonically in specialized structures called meristems. The root apical meristem of *Arabidopsis thaliana* is a readily accessible structure that has been extensively studied to uncover the factors that control root growth and cellular patterning. Recently we showed that one of the key factors in patterning the root, the mobile transcription factor SHORT-ROOT (SHR), acts in a concentration-dependent manner to initiate or suppress asymmetric divisions in the endodermis. The amount of SHR varies dynamically in the endodermis with the age of the root. Here we show that this variation is controlled in part through the activity of the transcription factor, SCARECROW (SCR), which regulates SHR movement and therefore its effective concentration and function in the endodermis.

The Arabidopsis root is composed of single layers of epidermis, cortex, endodermis and pericycle, which encircle the vascular tissues.[1] This regular pattern is established during embryogenesis, and is maintained as the root grows through the stereotypical cell divisions of the root initial cells and their immediate daughters.[2] Later in the development of the Arabidopsis root (at about day 7), asymmetric periclinal divisions occur in the endodermis to produce an additional cell layer, the middle cortex (**Fig. 1A**).[3,4] Collectively the endodermis, cortex and middle cortex comprise the ground tissue. Previous results showed that the GRAS family transcription factors, SHORT-ROOT (SHR) and SCARECROW (SCR), are required for the establishment and maintenance of separate cortical and endodermal cell layers.[5-10] The SHR protein is expressed in the stele and moves out of the stele into the endodermis and surrounding initials, where it upregulates *SCR*.[7] Together, both SHR and SCR directly turn on expression of *CYCLIN D 6;1* (*CYCD6;1*) in the cortical endodermal daughter cells, triggering the asymmetric periclinal division that produces the endodermis and cortex. Following this division, *CYCD6;1* expression is turned off in the ground tissue until middle cortex formation occurs (about day 7–14 in wild-type Columbia roots).[10,11]

SHR and SCR have different roles during the formation of middle cortex. *SCR* expression is not required for the development of middle cortex and appears to delay the expression of *CYCD6;1* and the initiation of periclinal cell divisions in the endodermis.[4,10] In contrast, SHR is required for middle cortex formation. Plants that lack SHR activity (e.g., *shr-2* null mutants) fail to activate expression of *CYCD6;1* in the endodermis and make middle cortex.[4] Recently we showed that the amount of SHR in the endodermis controls when and where middle cortex forms.[10] During the formation of middle cortex in wild-type roots, the level of SHR protein in the endodermis drops by approximately 40% just prior to initiation of the periclinal cell divisions that create the middle cortex layer. Following the asymmetric cell division, SHR levels then increase 2.4 fold in the endodermal cell that now borders the new middle cortex cell. These results suggest that when SHR is maintained at high levels in the endodermis, periclinal cell divisions are inhibited; whereas a decrease in SHR stimulates cell division. Consistent with this model, partial inhibition of SHR movement into the endodermis or a reduction in SHR activity triggers precocious activation of *CYCD6;1* and the initiation of middle cortex. Collectively these results show that SHR acts in a concentration dependent manner to regulate cell divisions in the endodermis, with high levels of SHR inhibiting expression of *CYCD6;1* and moderate levels of SHR promoting the expression of *CYCD6;1* and thus periclinal cell division.[10]

As SHR is a mobile protein that is expressed exclusively in the stele, one way that the amount of SHR in the endodermis may be controlled is through the regulation of SHR movement. A key factor in SHR movement is SCR.[9] SCR increases the expression of the SIEL (SHR INTERACTING EMBRYONIC LETHAL) protein, which promotes movement of SHR out of the stele, suggesting a positive role for SCR in the maintenance of SHR

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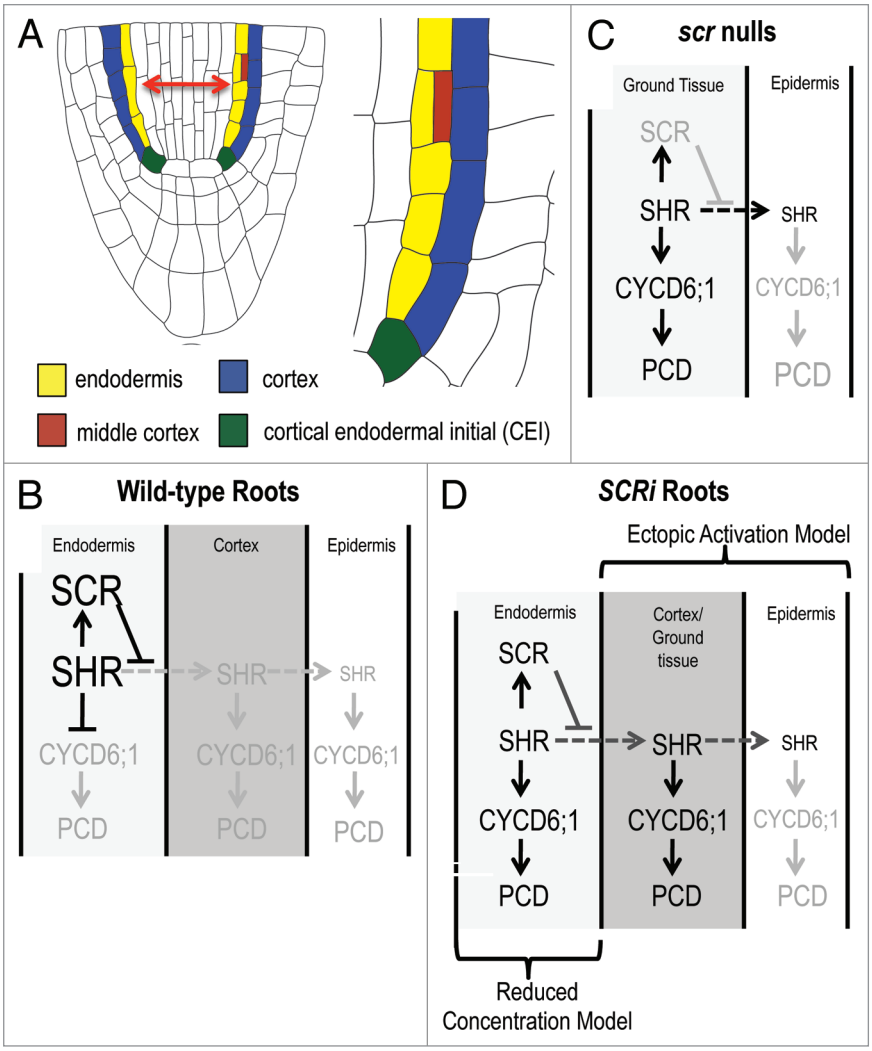

Figure 1. Formation of Middle Cortex. (A) Tracing of a longitudinal section through the root meristem. The middle cortex is formed by periclinal cell divisions in the endodermis. The double-arrow shows the stele. (B) In wild-type roots, SHR moves from the stele into the endodermis where it upregulates *SCR*. SCR in turn promotes nuclear localization of SHR and restricts movement of SHR out of the endodermis. This allows SHR to accumulate to levels sufficient to inhibit expression of *CYCD6:1*, which when expressed promotes periclinal cell division (PCD). (C) In *scr-4* roots, SHR is not restricted to the endodermis. This reduces the amount of SHR in the inner ground tissue layer to below what is sufficient to block expression of *CYD6;1* and PCDs are initiated. (D) In the *SCRi* lines, *SCR* levels are reduced leading to less efficient sequestration of SHR in the endodermis. This causes the SHR concentration in the endodermis to fall below the level required to inhibit *CYCD6;1* (Reduced Concentration Model) thus middle cortex-like divisions are activated. As SHR moves out of the endodermis, it is able to activate expression of *CYCD6;1* in the outer ground tissue layers and thus induce periclinal cell divisions in cells that would otherwise only divide anticlinally (Ectopic Activation Model). The size of the font is meant to indicate the strength of the signal. Light gray fonts are pathways that are not activated.

movement.[12] SCR has also been shown to promote the nuclear localization of SHR in endodermal cells and to prevent movement of SHR out of the endodermis.[9,13,14] SCR's role in inhibiting SHR movement is not dependent its role in promoting the nuclear localization of SHR.[13] As *scr* nulls form middle cortex precociously and the knockdown of SCR causes the formation of additional ground tissue layers, we examine here what effects a loss or a reduction in SCR function has on the amount of SHR in the ground tissue of *scr-4* and *SCR RNAi* (*SCRi*) lines respectively.[4,9] We also quantify SCR protein levels during the formation of middle cortex in wild-type roots. The results of these assays along with a reexamination of *SCRi* lines reinforce a model in which accumulation of SHR in the endodermis is enhanced and cell divisions are inhibited through SCR's ability to retain SHR in the endodermis.

At the time of germination, *scr-4* roots have a single layer of ground tissue between the epidermis and pericycle.[5] By day 4 (approximately 3–10 d earlier than wildtype), *scr-4* mutants begin to form middle cortex (**Fig. 1A** and **Fig. 2A**).[4] The formation of middle cortex in *scr-4* correlates with expression of *pCYCD6;1:GFP-GUS* in the ground tissue (**Fig. 2A** inset).[10] In wild-type roots, the formation of middle cortex and expression of *pCYCD6;1:GFP-GUS* is initiated by a reduction in SHR-GFP levels in the endodermis.[10] As *scr-4* roots form middle cortex very soon after germination, we examined SHR-GFP levels and localization in *scr-4* seedlings. In wild-type roots SHR localizes exclusively to the nuclei of endodermal cells (**Fig. 2B**), however in the ground tissue of *scr-4* roots (as previously reported), there is a reduction in the nuclear localization of SHR-GFP.[8,9] In some roots, there was no indication of nuclear accumulation of SHR-GFP in the ground tissue (**Fig. 2C**); whereas in others nuclear localization was apparent (**Fig. 2D**). In many roots, SHR-GFP signal was detected beyond the ground tissue layers (**Fig. 2E**). However ectopic divisions were never observed in these cells.

To quantify the distribution of SHR-GFP in *scr-4*, we measured SHR-GFP fluorescence in the ground tissue relative to SHR-GFP fluorescence in the stele in 4 and 6 d-old roots. In previous analyses of wild-type roots, this was done by measuring nuclear fluorescence in the endodermis of medial confocal cross-sections and comparing this value to the fluorescence in an adjacent region in the stele (**Fig. 2F** inset).[10,12] Because SHR-GFP is not restricted to the nucleus in *scr-4* ground tissue, measurements of SHR-GFP for both *scr-4* and wild-type roots were taken by measuring the average fluorescence in the inner ground tissue layer and the corresponding region of the stele (**Fig. 2F**; G:S ratio). These assays yielded a G:S ratio of 0.90 for wild-type roots and 0.46 for *scr-4*, indicating that SHR-GFP is reduced in the ground tissue of *scr-4* roots by approximately 50%. In previous results, we found that

SHR levels in the endodermis decreased by more than 40% prior to formation of middle cortex.[10] Therefore the reduction in SHR-GFP levels in ground tissue cells of *scr-4* roots compared with wildtype may explain the precocious formation of middle cortex in *scr-4* and is consistent with a model by which SCR maintains high levels of SHR in the endodermis (**Fig. 1B and C**).

In wild-type roots that are forming middle cortex, the ratio of SHR-GFP fluorescence in endodermal cells that have recently divided (**Fig. 2G** inset 1 white arrowhead) to those that are in line to divide (**Fig. 2G** inset 1 yellow arrowhead) was 2.4.[10] In the *scr-4* roots expressing SHR-GFP, there was no difference in SHR-GFP levels in ground tissue cells that had divided vs. those that had not (**Fig. 2G** ratio 1.04). These results indicate that SCR is required to maintain SHR-GFP at different levels in divided vs. undivided endodermal cells. As SCR has been shown to directly interact with SHR and to promote nuclear localization of SHR,[8,9] one mechanism by which this regulation could occur is through a change in the amount of the SCR protein. To determine whether SCR-GFP showed a similar pattern to what was observed for SHR-GFP (**Fig. 2G** inset 1), we examined wild-type roots expressing SCR-GFP that were in the process of forming middle cortex. In these roots, we saw no difference in the levels of SCR-GFP in endodermal cells that had divided relative to those that had not (**Fig. 2G** inset 2). These results suggest that a change in the amount of SCR is not what accounts for the differences in SHR-GFP levels in the endodermal cells of roots that are forming middle cortex and that factors downstream of SCR may be regulating SHR concentrations.

Previously it was shown that a reduction in *SCR* through *SCRi* resulted in an increase in the number of ground tissue layers (**Fig. 3A**). As the expression of the *SCRi* transgene also resulted in an increase in the domain of SHR movement (**Fig. 3B**), the extra ground tissue layers were ascribed to ectopic activity of SHR *i.e.* the presence of SHR in cells in which it was not normally found was able to activate divisions in these cells (ectopic activation model in **Fig. 1D**).[9] However it is also possible that the ability of SHR to move out of the endodermis into the cortex and epidermis reduces the concentration of SHR in the endodermis, which leads to the induction of middle cortex-like divisions in the endodermis (reduced concentration model **Fig. 1D**). In this case the supernumerary layers are not the direct result of increased SHR movement per se, but more accurately an inability to concentrate SHR. If this is the case, it would lend further support to our contention that SHR concentration, rather than mere presence or absence is what regulates late ground tissue patterning.[10,12] To distinguish between the above two possibilities, we examined expression of *pCYCD6;1:GFP-GUS* in the *SCRi* lines. If movement of SHR into the cortex or the epidermis is responsible for triggering the extra periclinal cell divisions in the *SCRi* lines, then *pCYCD6;1* should be induced in these cells. In contrast, as SHR is normally present in the endodermis, there is no expectation that *pCYCD6;1* should be activated in the endodermis in a 5 d-old root, unless the movement of SHR out of the endodermis allows the concentration of SHR to drop below the critical level required to inhibit expression of *CYCD6;1* in the endodermis.

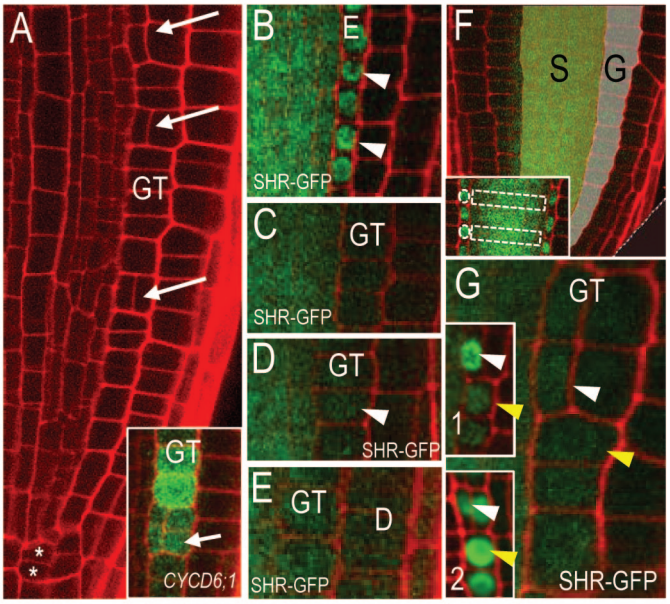

Figure 2. Loss of SCR causes a reduction in SHR levels in the ground tissue. (A) Confocal section through a 4 d-old *scr-4* root showing the formation of middle cortex (arrows). The inset in (A) shows expression of *pCYCD6;1:GFP-GUS* in 5-d old *scr-4* root. (B) Localization of SHR-GFP in a wild-type plant shows accumulation of SHR-GFP in the endodermis and nuclear localization (arrowheads). (C-E) Expression of *pSHR:SHR-GFP* in *scr-4*; arrowhead indicates the nucleus. (F) The G:S ratio was calculated by measuring in medial confocal sections of *scr-4* or wildtype roots the average pixel intensity of the SHR-GFP signal in the ground tissue layer (white shading) and the stele (yellow shading). In wild-type roots, the ground tissue was endodermis in cases where the ground tissue layer was divided in *scr-4* only the inner layer was measured. (F inset) In previous assays looking at endodermal to stele ratios of SHR-GFP in wildtype, the nuclei were specifically used to quantify SHR levels. This could not be done for these studies, as SHR was not restricted to the nuclei of the ground tissue cells in *scr-4*. (G) In contrast to wild-type roots (G inset 1), SHR-GFP *in scr-4* is not increased in the inner ground tissue layer (white arrowhead) after periclinal cell division compared with the undivided ground tissue (yellow arrowhead) nor is SCR-GFP in wildtype (G inset 2). GT and G = ground tissue, E = endodermis, S = stele, D = epidermis.

When we examined expression of *pCYCD6;1:GFP-GUS* in the *SCRi* roots, we saw expression in the cortical endodermal daughter cells and the outer ground tissue layers, which under normal circumstances would form cortex (**Fig. 3C and D**; blue arrowheads). We did not detect expression in any of the initial cells outside of the ground tissue layer or in the epidermis, indicating that ectopic cell division are not induced in these layers, although SHR-GFP is often present in these cells. These results are consistent with the original interpretation of the *SCRi* phenotype by Cui et al. (2007): movement of SHR into the outer ground tissue layer induces ectopic divisions in these cells. However, we also saw expression of *pCYCD6;1:GFP-GUS* in the endodermis of the *SCRi* roots (**Fig. 3C**), which is consistent with a reduction in the concentration of SHR in this inner ground tissue layer triggering middle cortex-like cell divisions.

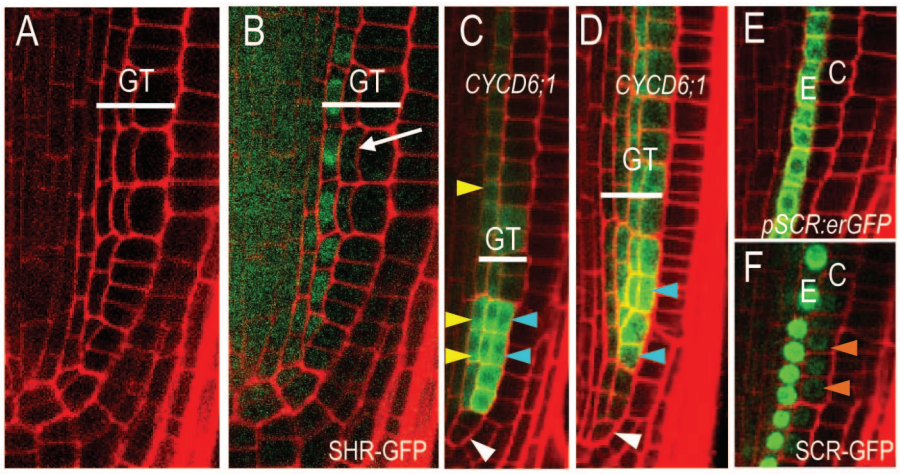

Figure 3. A reduction of *SCR* increases the extent of SHR movement and decreases the amount of SHR-GFP in the endodermis. (A and B) Confocal longitudinal section through a *SCRi* root expressing the *pSHR:SHR-GFP* transgene; the arrow points to SHR-GFP signal in one of the outer ground tissue layers. (C and D) Expression of *pCYCD6;1:GFP-GUS* in two different *SCRi* roots. White arrowheads mark the cortical endodermal initial cells, blue arrowheads mark transit-amplifying cells of the outer ground tissue layer (normal position of the cortex), yellow arrowheads mark the presumptive endodermis. (E) Expression of the *pSCR:erGFP* construct is restricted to the endodermis. Whereas in (F) *pSCR:SCR-GFP* shows signal in the nuclei of both the endodermis and cortex (orange arrowheads).

To directly assess whether there is a reduction in SHR-GFP levels in the endodermis (inner-most ground tissue layer) of *SCRi* roots, we measured the ratio of SHR-GFP fluorescence in the inner-most ground tissue layer as compared with the stele (using the same method for quantification as for **Fig. 2F**). These assays yielded a ratio of SHR-GFP fluorescence of 0.60, which represents a 34% reduction in the level of SHR-GFP in the inner ground tissue layer compared with wildtype. Our results therefore are consistent with a reduction in SHR inducing the expression of *CYCD6;1* and periclinal cell divisions in the inner ground tissue layer of the *SCRi* lines. However this model cannot explain the full extent of cell divisions observed in the *SCRi* lines. Thus based upon the collective data, we conclude that both the reduced concentration and the ectopic activation models are required to explain the extra cell layers in the *SCRi* lines. A reduced concentration of SHR in the inner ground tissue layer induces middle cortex-like divisions in this cell layer, and ectopic movement of SHR into the cortex triggers periclinal divisions in the outer ground tissue layer (**Fig. 3D**). Interestingly, we never saw more than a total of 4 ground tissue layers in the *SCRi* lines, which is far fewer than what was seen when SHR-GFP was expressed directly in the endodermis from the *SCR* promoter (in some roots over 10 additional layers),[7,13,15,16] suggesting that there may limit to the number of ground tissue layers that can be generated through ectopic movement of SHR. Also of note, and in contrast to the *SCRi* lines, when Sozzani et. al. (2011) directly expressed CYCD6;1-YFP in the endodermis and cortex of wild-type roots using the J0571 enhancer trap line, periclinal cell divisions were induced in the endodermis, but not the cortex. As SCR-GFP is present in the cortex of wild-type roots expressing *pSCR:SCR-GFP* (**Fig. 3E and F**), these results may point to a role for SCR in inhibiting cell divisions in the cortex that is independent of CYCD6;1.

Collectively these results show that SCR plays a critical role in restricting SHR movement from the endodermis. A reduction in SCR allows SHR to move into the outer ground tissue layers where SHR induces ectopic periclinal cell divisions in these cells. A loss or reduction of SCR also leads to a reduction of SHR in the endodermis, which activates periclinal cell divisions in the endodermis. The reduction in SHR in the endodermis is likely the result of movement of SHR into the cortex and epidermis. In addition, as SCR enhances the expression of *SIEL*, which promotes movement of SHR out of the stele[12] SCR may also maintain SHR level in the endodermis by enhancing SHR movement from the stele.

Materials and Methods

Plant Materials and Growth Conditions. *scr-4*, *SCR-RNAi*, *pSHR:SHR-GFP*, *pSCR:SCR-GFP* *pSCR:erGFP* and *pCYCD6;1:GFP-GUS* lines were described previously.[6,7,9,11,17] Surface sterilized seeds were sown on plates containing 0.5–1.0 x Murashige and Skoog salt, 0.05% (w/v) Mes (pH 5.7), 1% (w/v) sucrose, and 0.8–1.5% (w/v) Granulated agar (DIFCO). Plants were incubated vertically in a growth chamber at 23°C under 16 h light/8 h dark cycle.

Microscopic observation. Confocal images were obtained using a Leica TCS SL microscope. Before visualization, the roots were stained in 0.01 μg/mL PI in water. For GFP quantification of *pSHR:SHR-GFP*, images were collected on the same day using identical confocal settings. The average GFP intensity was measured in ImageJ software (http://rsbweb.nih.gov/ij/) or Adobe Photoshop (Adobe Systems Inc.) as described in the text and in[13] on unmodified root images. For all assays a minimum of 15 roots with 3 different focal planes were used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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