A broad competence to respond to SHORT ROOT revealed by tissue-specific ectopic expression

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Summary

In plants, cell fate specification depends primarily on position rather than lineage. Recent results indicate that positional information can be transmitted through intercellular trafficking of transcription factors. The *SHORT ROOT* (*SHR*) gene, a member of the GRAS family of putative transcription factors, is involved in root radial patterning in *Arabidopsis*. Correct radial patterning depends on the positional information transmitted through limited SHR intercellular movement and translated into cell division and specification by competent target cells. To investigate the regulation of SHR movement and the competence to respond to it, we drove expression of a translational fusion SHR::GFP using four different tissue-specific promoters. In a wild-type background, SHR::GFP was not able to move from either phloem companion cells or epidermal cells, both of which have been shown to support movement of other proteins, suggesting a requirement for tissue-specific factors for SHR movement. When expressed from its native promoter in plants with multiple endodermal layers, SHR::GFP was not able to move beyond the first endodermal layer, indicating that movement is not limited by a mechanism that recognizes boundaries between cell types. Surprisingly, movement of SHR::GFP was observed when ectopic expression from an epidermal promoter was placed in a *scarecrow* (*scr*) mutant background, revealing a possible role for SCR in limiting movement. Analysis of the competence to respond to SHR-mediated cell specification activity indicated that it was broadly distributed in the epidermal lineage, while competence to respond to the cell division activity of SHR appeared limited to the initials and involved induction of SCR. The spatial distribution of competence to respond to SHR highlights the importance of tightly regulated movement in generating the root radial pattern.

Key words: *Arabidopsis*, Root, Radial pattern, *SHORT ROOT* (*SHR*), Protein movement, Intercellular trafficking

Introduction

Many lines of evidence indicate that cell fate determination in plants is based primarily on position, even though well-defined cell lineages exist (Kidner et al., 2000; van den Berg et al., 1995). Position-dependent fate determination must rely on highly coordinated intercellular interactions, which in plants are complicated by the presence of cell walls. Membrane-lined pores called plasmodesmata (PD) pierce the cell walls, creating a cytoplasmic continuum (symplast) that becomes a possible route of communication between cells in plants. Intercellular communication can thus occur either via secreted signals diffusing through the cell wall continuum (apoplast), or directly through the symplast. Positional information using either mode of cell-cell communication can be regulated by limiting the extent of travel of the signal (Lenhard and Laux, 2003), by the distribution of the competence to respond to the signal, or both.

Our previous work provided evidence that the SHORT ROOT (SHR) protein, a member of the GRAS family of putative transcription factors, acts as a positional signal essential to radial pattern formation. Genetic analyses in *Arabidopsis* first revealed a role for SHR in root radial patterning (Benfey et al., 1993). The radial organization of the root encompasses concentric rings of epidermis, cortex, endodermis and pericycle, surrounding a central vascular cylinder that together with the pericycle comprise the stele (Dolan et al., 1993). The radial pattern is generated through stereotyped asymmetric divisions of a set of stem cells ('initials') in the meristem and subsequent acquisition of different cell fates (Dolan et al., 1993). The endodermis and cortex cell layers (the ground tissue) are extended by two sequential divisions of the cortex/endodermis (co/en) initial cells. The first anticlinal division (i.e. with the plane of division perpendicular to the surface of the root) regenerates the initial cell and produces a daughter cell which then undergoes a periclinal division (i.e. with the plane of division parallel to the surface of the root) to form the first cells of the endodermis and cortex layers. In loss-of-function *shr* mutants, the periclinal division fails to take place (Benfey et al., 1993). This results in a single tissue that has only cortex characteristics (Benfey et al., 1993; Helariutta et al., 2000), indicating that SHR is necessary for both periclinal division of the co/en initial daughter and for endodermal specification. *SHR* transcripts were detected only in the stele, indicating that it acts in a non-cell-autonomous fashion (Helariutta et al., 2000). Evidence that SHR protein is able to move from the stele provided a mechanism by which it could act as a positional signal in radial

patterning (Helariutta et al., 2000; Nakajima et al., 2001). We have shown with GFP transcriptional and translational fusions, as well as with in situ mRNA hybridization and immunolocalization, that SHR moves from the stele of the root to the first adjacent cell layer (Nakajima et al., 2001).

Several other transcription factors have been shown to exert their non-cell-autonomous actions by intercellular movement, presumably through PD (for reviews, see Barton, 2001; Hake, 2001; Roberts and Oparka, 2003; Wu et al., 2002). These include the maize transcription factor KNOTTED1 (KN1) (Lucas et al., 1995), the *Antirrhinum* MADS domain proteins DEFICIENS (DEF) and GLOBOSA (GLO) (Perbal et al., 1996), the *Arabidopsis* floral identity protein LEAFY (LFY) (Sessions et al., 2000), and the MYB-related CAPRICE (CPC) protein (Wada et al., 2002).

Our previous results indicated that regulation of SHR signaling occurred both in the extent of travel of the signal (SHR protein moves into the endodermis but no further) as well as competence to respond to the signal (although SHR is present in stele cells they do not acquire endodermal characteristics) (Helariutta et al., 2000; Nakajima et al., 2001). Determining the mechanism by which SHR intercellular trafficking from the stele is limited to the next cell layer is essential to address how spatial control of the signal is regulated. Studying the distribution of competence to respond to SHR by either periclinal cell divisions or endodermal specification is important for understanding the role of cellular competence in generating radial positional information.

Previously, we attempted to address these issues by driving *SHR* expression with two different promoters. Expression by both the constitutive *35S* promoter and the *SCR* promoter, which is dependent on *SHR* activity for full activation and normally confers expression in the cell layer adjacent to the stele, resulted in supernumerary cell layers, many of which had endodermal characteristics (Helariutta et al., 2000; Nakajima et al., 2001). However, with both promoters it was impossible to determine if intercellular movement of SHR had occurred. Moreover, we could not determine the origins of those cells that responded to SHR by periclinal division or endodermal specification. For both movement and competence the problem was that one promoter is ubiquitously active and the other expresses in cells that normally contain SHR.

In this report, we investigate the regulation of SHR movement and the distribution of competence to respond to SHR using tissue-specific regulatory sequences from the *SUC2, GL2* and *WER* genes, as well as the native *SHR* promoter. These promoters were used to drive expression of the translational fusion SHR::GFP in the phloem companion cells, maturing atrichoblasts, the epidermis with its initials and in the stele, respectively. We analyzed the ability of SHR::GFP to move from different cell types, the extent of movement when it occurred and the relationship of movement to subcellular localization. We found that in a wild-type background, movement occurs only from certain cell types but that the extent of movement does not depend on cell type. We also discovered that SCR may play a role in restricting movement. Competence to respond to SHR-mediated cell specification activity was broadly distributed in the outermost layer of the root, while competence to respond to the cell division activity of SHR appeared limited to the initials and involved induction of SCR. This broad competence to respond to SHR highlighted the importance of restricted SHR movement to generate the normal radial pattern in the root. Moreover, these results showed that SHR movement is not a pre-condition for activity.

Materials and methods

DNA constructs

The translational fusion *SHR::GFP* was described in (Nakajima et al., 2001). The *SUC2* promoter (*pSUC2*) was a *Hin*dIII-*Bam*HI fragment from a *pSUC2::GFP* construct (*pMC720* from M. Cilia), where the *Hin*dIII and the *Bam*HI were 1988 bp and 2 bp upstream of the *SUC2* ATG, respectively. A *Bam*HI site was inserted 7 bp upstream of the ATG of the translational fusion *SHR::GFP* which was then inserted into the binary vector *pBI101* (Clontech) as a *Hin*dIII-*Sst*I fragment. The 5′ and 3′ regulatory regions (2.5 kb (*pWER5′*) and 1.1 kb (*pWER3′*) were from the *pWGFP3* plasmid described previously (Lee and Schiefelbein, 1999). The *pWER5′* was fused to *SHR::GFP* by overlap extension. A fragment composed of 682 bp from the 3′ end of *pWER5′* and 12 bp from the 5′ end of *SHR::GFP* was obtained by PCR with Pfu polymerase (Stratagene) from the *pWGFP3* plasmid with the forward primer TCCTTCTCACCTTCCAATGGG and reverse primer GAGAGTATCCATTGTTGATGTC (underlined region is from the *SHR* coding sequence). Then a fragment composed of 10 bp from the 3′ end of *pWER5′* and 452 bp from the 5′ end of *SHR*, including a unique *Sac*I site was amplified by PCR from *SHR::GFP* with the forward primer GACATCAACAATGGATACTCTC and the reverse primer, GAGCTCAGGACCGAGTCTGC. The two fragments were fused by overlap extension with the forward primer TCCTTCTCACCTTCCAATGGG and the reverse primer GAGCTC-AGGACCGAGTCTGC, obtaining a final product composed of 682 bp from *pWER5′* fused to 452 bp from the *SHR*-coding sequence and ending with a *Sac*I site. The resulting fusion has 8 bp without restriction sites between the end of the genomic *WER* 5′ regulatory region and the *SHR* ATG. From this, an 830 bp fragment spanning the fusion between *pWER5′* and *SHR* was inserted between the *Age*I and *Sac*I sites of a modified version of *pWGFP3* in which the only *Sac*I site left marks the beginning of the *pWER3′* regulatory region. Then, the 300 bp *Aat*II-*Sac*I region (where *Aat*II belongs to *SHR*) was substituted with the 2150 bp *Aat*II-*Sac*I fragment from the *SHR::GFP* fusion used elsewhere (Nakajima et al., 2001). Finally, the complete 5.8 kb *Kpn*I-*Sal*I construct *pWER5′::SHR::GFP::pWER3′* was inserted between *Kpn*I and *Xba*I of the binary vector *pCGN1547* (McBride and Summerfelt, 1990). To construct *pGL2::SHR::GFP*, the entire *SHR::GFP* fragment described previously (Nakajima et al., 2001) was isolated between the *Spe*I site 3 bp upstream of the *SHR* ATG and a *Sac*I site immediately adjacent to the *GFP* stop codon. The *Spe*I site was filled in and this fragment was inserted in place of the *GUS* cassette in the *pGL2::GUS* construct described elsewhere (Szymanski et al., 1998) between the *Sma*I and *Sac*I sites in the binary vector *pBI101*. To construct *pGL2::*YFP_{ER} we first isolated YFP_{ER} from *pBERYFP1* [a gift from Drs B. Scheres and R. Heidstra, made through modifications of the *H2B::YFP* construct described elsewhere (Boisnard-Lorig et al., 2001)] between a *Bam*HI site 15 bp upstream of the *YFP* ATG and an *Eco*RV site 20 bp downstream of the *YFP* stop codon, and then inserted in place of the *GUS* cassette in *pGL2::GUS* between *Bam*HI and *Sac*I [the latter made blunt with *Eco*ICRI (Promega)], upstream of the nopaline synthase terminator in the binary vector *pBI101*.

Plants strains, transformation, and crosses

Arabidopsis seeds were surface sterilized and grown as described previously (Benfey et al., 1993).

The constructs in binary vectors were electroporated into *Agrobacterium*, which were then used to transform *Arabidopsis* (Columbia ecotype) following the floral dip method (Clough and Bent, 1998). Transgenic seedlings were selected on MS agar plates

with 1% sucrose and 50 μg/ml kanamycin. For all the transgenes discussed, numerous individuals from at least three independently transformed lines were analyzed.

The line *pWER::SHR::GFP* in a *scr* mutant background was obtained by crossing a homozygous *pWER::SHR::GFP* line with a homozygous *scr-4* line (Fukaki et al., 1998). The progeny was followed to the second generation and genotyped for presence of the *scr-4* allele and absence of wild-type *SCR*.

The line *pWER::SHR::GFP, pGL2::YFP*$_{ER}$ was obtained by crossing a homozygous *pWER::SHR::GFP* line with a homozygous *pGL2::YFP*$_{ER}$ line and analyzing the F1 generation.

The line *pWER::SHR::GFP, pSCR::YFP*$_{ER}$ was obtained by crossing a homozygous *pWER::SHR::GFP* line with a homozygous *pSCR::YFP*$_{ER}$ line (a gift from Dr B. Scheres) and analyzing the F1 generation.

Confocal microscopy

Roots were counterstained in 10 μg/ml propidium iodide (PI) for 1 minute. Confocal images were obtained using a 63× water-immersion lens on a Leica TCS SP2 spectral confocal laser-scanning microscope. In 'GFP+YFP' mode, we used the 488 nm Argon laser line to excite and collected in the range 493-536 nm (rendered in green) and 587-731 nm (rendered in red, collecting emission from PI). In 'YFP' mode, we used the 514+543nm Argon laser lines to excite and collected in the ranges 555-587 nm (rendered in yellow) and 587-731 nm (rendered in red, collecting emission from PI).

Histochemical staining, immunolocalization and in situ hybridization

Roots from 4-7 day post-germination seedlings were fixed and embedded as described previously (Fukaki et al., 1998). For Casparian strip detection, 6 μm sections on slides were incubated overnight in 0.1% berberine hemisulfate (Sigma) at room temperature, rinsed with water, counterstained for 10 minutes in 0.5% Aniline Blue WS (Polyscience) at room temperature, rinsed with water and transferred for 5-10 minutes to 0.1% $FeCl_3$ in 50% glycerol. The slides were mounted with a drop of the same 0.1% $FeCl_3$ solution (Brundrett et al., 1988; Scheres et al., 1995). Samples were analyzed with a Leica DMRA2 epifluorescence microscope with FITC filters. For immunolocalization, 6 μm sections were processed for staining with the JIM13 antibody (Knox et al., 1990) as described previously (Di Laurenzio et al., 1996). The samples were then analyzed by epifluorescence microscopy, as above. In situ hybridization analysis was performed as described previously (Di Laurenzio et al., 1996). The GFP-specific probe was first amplified by PCR to make the template spanning nearly the entire coding region. The reverse primer was designed to contain a T3 promoter site that was used to generate the antisense probes.

Results

SHR::GFP is not able to move from the phloem companion cells

The translational fusion SHR::GFP was previously shown to be able to move from the stele into the adjacent tissue layer, but not further, when expressed under the control of the *SHR* promoter (*pSHR)* in a wild-type background (Nakajima et al., 2001). One possible model was that movement from the stele is by passive diffusion. If this were the case then one might expect that SHR::GFP could move from any cell in which it is expressed. To test this model, we investigated movement from cells known to be symplastically well connected to their neighbors.

Within the stele, symplastic connections have been documented between the phloem companion cells (CC) and

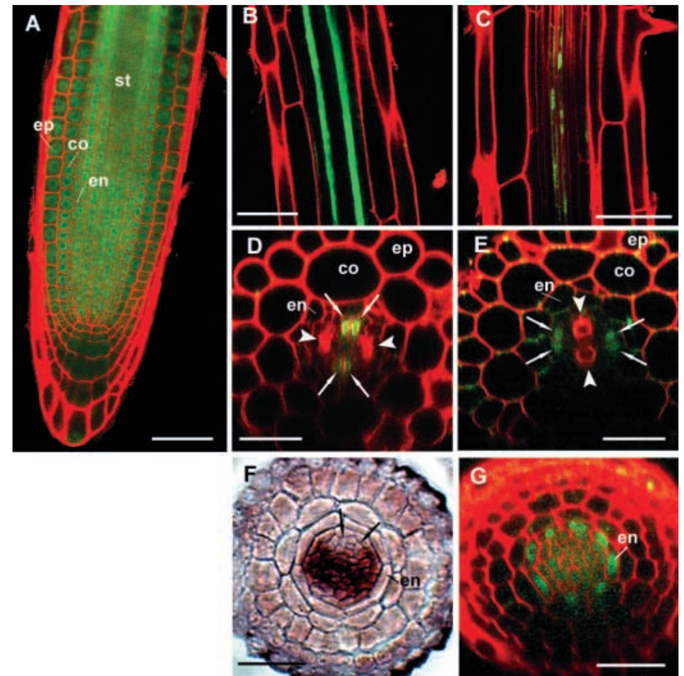

Fig. 1. SHR::GFP does not move from phloem companion cells (CC), where the native *SHR* promoter is not active. Longitudinal (A-C) and transverse (D,E,G) confocal images of GFP fluorescence of *pSUC2::GFP* (A), *pSUC2::GFP*$_{ER}$ (*GFP*$_{ER}$ contains ER targeting and retention signals) (B,D), *pSUC2::SHR::GFP* (C,E) and *pSHR::SHR::GFP* (G) transgenic roots. (F) In situ hybridization with *GFP* antisense probe on transverse section from *pSHR::SHR::GFP* transgenic root. The *SUC2* promoter is active in the phloem CC (B and arrows in D). Non-targeted GFP can move from the CC to the epidermis (A), while the fusion protein SHR::GFP does not leave the CC (C and arrows in E). The native *SHR* promoter is not active in the phloem CC (arrows in F), yet the SHR::GFP fusion protein can be found throughout the stele and in the endodermis when transcribed under the same promoter (G). Arrowheads in D and E indicate protoxylem. ep, epidermis; co, cortex; en, endodermis; st, stele. Scale bars: 50 μm in A-C; 25 μm in D-G.

sieve elements (SE) (for reviews, see Oparka and Turgeon, 1999; Ruiz-Medrano et al., 2001; Van Bel, 2003). The filament protein Phloem Protein 1 (PP1, 96 kDa) has been shown to move from the phloem CC to SE in *Cucurbita maxima* leaves (Clark et al., 1997; Leineweber et al., 2000). In addition, GFP (27 kDa) is able to move from the phloem CC throughout the root meristem when expressed under the promoter of the *SUC2* sucrose-H^+ symporter gene (*pSUC2*), which is known to be active in the phloem CC (Imlau et al., 1999; Truernit and Sauer, 1995) (Fig. 1A, compare with Fig. 1B,D).

SHR RNA is not normally expressed in the phloem CC. Analysis of *pSHR::GFP* transcriptional fusions (data not shown) as well as in situ hybridization, both with a *SHR* antisense probe in wild type (data not shown) and with a *GFP* antisense probe in *pSHR::SHR::GFP* transgenic lines (Fig. 1F), indicates that *SHR* RNA and *SHR::GFP* RNA, respectively, are excluded from the phloem CC and adjacent cells in the more mature regions of the meristem. Because in *pSHR::SHR::GFP* transgenic lines the SHR::GFP protein is found throughout the stele in these regions (Fig. 1G), this provides evidence that, in addition to movement from the stele

to the adjacent layer, SHR is normally able to move within the stele.

To determine if SHR::GFP is able to move from the phloem CC, we produced transgenic lines where the SHR::GFP fusion protein (87 kDa) is driven by the *SUC2* promoter. In the root of *pSUC2::SHR::GFP* lines, fluorescence was localized to the nuclei of the phloem CC (Fig. 1C,E). We did not detect any signal in other tissues (Fig. 1C,E, compare with Fig. 1B,D), indicating that the fusion protein is not able to move. This lack of movement from cells that support movement of other proteins raises the possibility that factors present where SHR is normally expressed are required for SHR::GFP movement. An alternative explanation is that factors inhibiting SHR::GFP movement are present in the phloem CC.

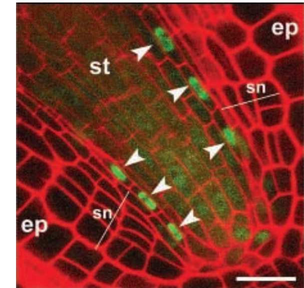

Fig. 2. SHR::GFP moves only into the first of the supernumerary endodermis-like layers. Longitudinal confocal image of a *pSCR::SHR, pSHR::SHR::GFP* transgenic root. The *pSCR::SHR* background produces supernumerary layers as described previously (Nakajima et al., 2001) and the fusion protein SHR::GFP is detected in the stele and in the first layer in contact with it (arrowheads). ep, epidermis; sn, supernumerary layers; st, stele. Scale bar: 25 μm.

SHR::GFP moves only one cell distance through supernumerary endodermal layers

We next addressed the role of cell type in limiting the extent of movement. SHR moves from the stele to the endodermis, but does not move into the adjacent cortex. This suggested that there might be some restriction in symplastic connectivity between endodermis and cortex that limits the extent of radial movement of SHR. A prediction from this hypothesis would be that SHR should be able to move from one presumptive endodermal cell to another. We tested this in plants with multiple ground tissue layers all of which acquire endodermal characteristics. This occurs when *SHR* is driven by the *SCR* promoter (*pSCR*) (Nakajima et al., 2001). Into these plants we introduced *SHR::GFP* driven by the native *SHR* promoter.

We detected the fusion protein in the stele and in the first layer of ground tissue in contact with it, but not in the tissues further away (Fig. 2). At the subcellular level, we detected SHR::GFP in both nuclei and cytoplasm within the stele but only in nuclei of the first ground tissue layer (arrowheads in Fig. 2). In a few instances, a faint signal was detected in a second cell next to the first ground tissue layer. This could be attributed to the fact that after a periclinal division, SHR is known to persist for a brief time in both daughter cells (Nakajima et al., 2001).

We conclude that even when there are multiple layers of presumptive endodermis, SHR::GFP movement from the stele is still limited to the distance of one cell. This result suggests that movement of SHR from the stele is limited not by differences in symplastic connections but by some other attribute of the cells from or into which it moves.

SHR::GFP is not able to move from the epidermis in a wild-type background

To further investigate the role of cell type in regulating SHR movement we asked if SHR::GFP is able to move from a cell type that does not normally contain SHR protein. We chose to focus these experiments on the epidermis because there are well-characterized promoters that confer expression in subsets of epidermal cells in different stages of development. The promoter of the homeobox gene *GLABRA2* (*GL2*) is active in the root epidermis, with preferential expression in the atrichoblasts (non-hair cells). Expression becomes detectable in the meristematic zone, but not in the initials, and is maintained in the elongation zone and in at least part of the differentiation zone (Lin and Schiefelbein, 2001; Masucci et al., 1996) (inset in Fig. 3A).

In plants carrying a *pGL2::SHR::GFP* transgene the fusion protein was primarily localized to the atrichoblast cells (data not shown) in the epidermis, and the fusion protein did not appear to move into the neighboring cortex layer (Fig. 3A,B). Moreover, the GFP signal appeared to be localized preferentially to the nuclei of those cells (Fig. 3B).

Competence to respond to SHR-mediated cell division is limited to initial cells

From the observation of four independent *pGL2::SHR::GFP* transgenic lines, we saw no evidence of perturbation in radial patterning, indicating that cell division processes had not been altered in these plants (Fig. 3A). We conclude that cells of the atrichoblast lineage above the initials do not appear to be competent to respond to the cell division promoting activity of SHR.

We hypothesized that competence to respond to SHR by generating periclinal divisions may reside in the initial cells in which the *GL2* promoter does not confer detectable expression. To test this hypothesis, we drove expression of the *SHR::GFP* translational fusion using the 5′ and 3′ regulatory sequences from the *WEREWOLF* (*WER*) gene, which is transcribed in the epidermis, a region of the lateral root cap (LRC) and the epidermal/LRC (ep/LRC) initials (Lee and Schiefelbein, 1999) (inset in Fig. 3C).

The *pWER::SHR::GFP* transgenic lines showed a dramatic perturbation of the radial pattern with an excess of periclinal cell divisions (Fig. 3C-E). The resulting supernumerary tissues appeared organized in concentric layers (Fig. 3E), although the number of layers varied between independent transgenic lines. These results together suggest that, in the epidermal lineage, only the initials are competent to respond to SHR with periclinal divisions.

SHR::GFP does not move from the epidermis even in the presence of supernumerary layers

We then asked if SHR::GFP was now able to move within these ectopic layers. In observations of three independent transgenic lines, we detected GFP signal only in the outermost tissue, where SHR::GFP appeared localized to the nuclei (Fig. 3C,D). In the very few cases when SHR::GFP was detected in a cell not in the outermost layer, the morphology always suggested that it resulted from a recent cell division of a neighboring cell in the outermost tissue layer, which also contained SHR::GFP. In conclusion, SHR::GFP does not move from the epidermis even in the presence of supernumerary layers.

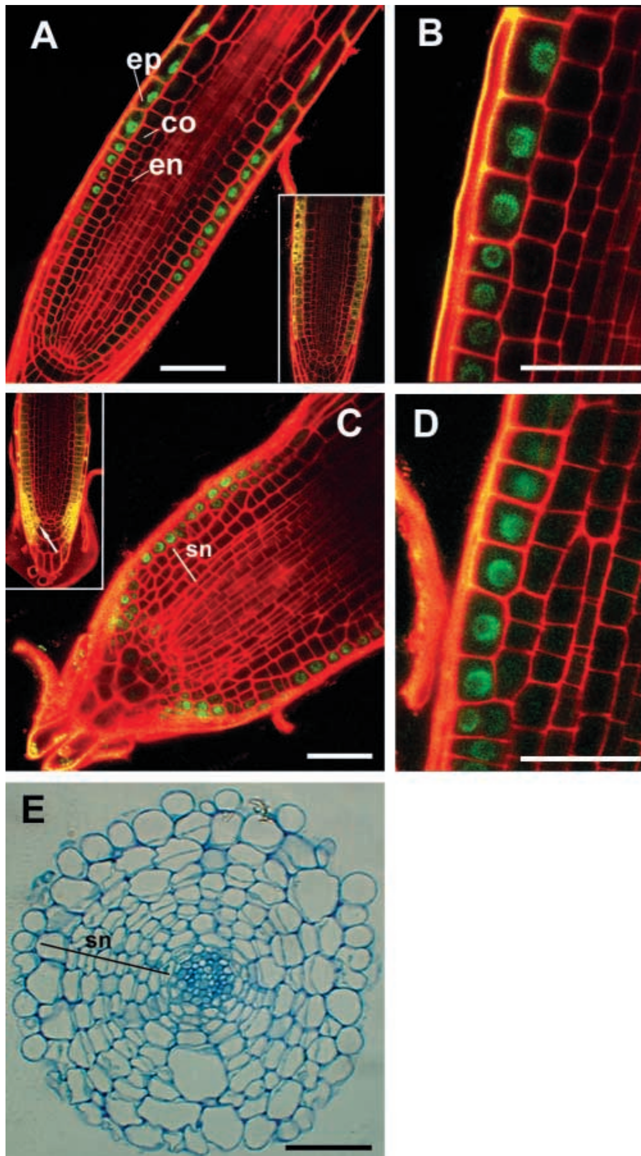

Fig. 3. SHR::GFP does not move from the epidermis even when supernumerary layers are present. Longitudinal confocal images (A-D) and transverse section (E) of *pGL2::YFP*$_{ER}$ (inset in A), *pWER::YFP*$_{ER}$ (inset in C), *pGL2::SHR::GFP* (A,B) and *pWER::SHR::GFP* (C-E) transgenic roots. *pWER::SHR::GFP* induces a pattern perturbation resulting in supernumerary layers (C,E), while *pGL2::SHR::GFP* does not alter the pattern (A). SHR::GFP does not appear to move from the epidermis in either *pGL2::SHR::GFP* (B) or in *pWER::SHR::GFP* (D) transgenic roots. Arrow in the inset of C indicates ep/LRC initial. ep, epidermis; co, cortex; en, endodermis; sn, supernumerary layers. Scale bars: 50 µm in A,C,E; 25 µm in B,D.

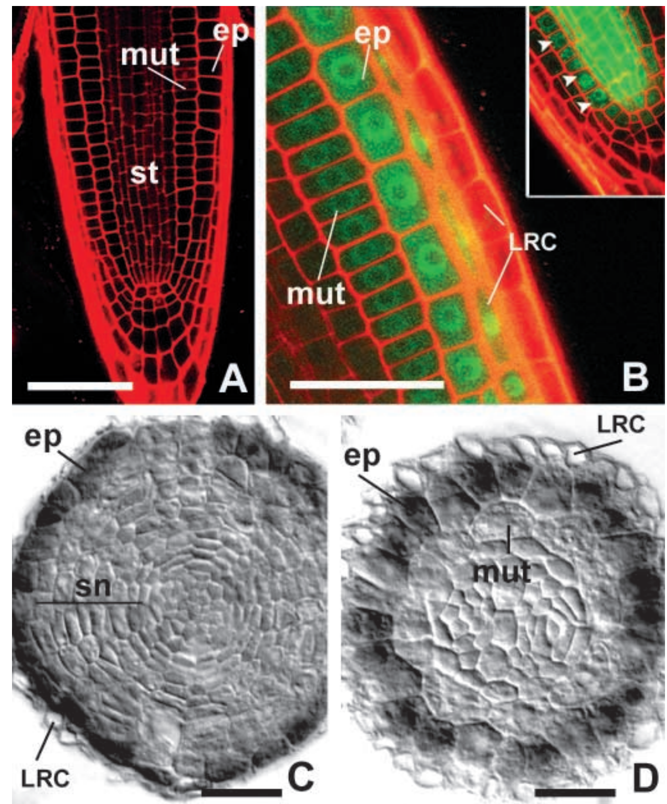

Fig. 4. SCR is required for *pWER::SHR::GFP*-mediated ectopic divisions and movement of SHR::GFP from the epidermis occurs in a *scr* mutant background. Longitudinal confocal images of *pWER::SHR::GFP* transgenic roots in a *scr-4* background (A, red channel only) and (B) a *pSHR::SHR::GFP* transgenic root in *scr-1* background (inset in B), and in situ hybridizations with *GFP* antisense probe on transverse sections from *pWER::SHR::GFP* transgenic roots in wild-type (C) and *scr-4* (D) backgrounds. The resulting radial pattern (A) is identical to that of *scr* (Di Laurenzio et al., 1996). The fusion protein SHR::GFP is found in the epidermis as well as in the mutant ground tissue layer (B), whereas no RNA expression of *SHR::GFP* is detectable in the mutant layer of the *scr-4* root (D), indicating movement of the fusion protein from the epidermis to the mutant layer. SHR::GFP is detected both in the nuclei and in the cytoplasm of the mutant layer, when moving either from the epidermis [as in *pWER::SHR::GFP* in *scr-4* (B)] or from the stele [as in *pSHR::SHR::GFP* in *scr-1* (inset in B)]. mut, mutant layer of *scr*; LRC, lateral root cap; ep, epidermis; sn, supernumerary layers; st, stele. Arrowheads in the inset indicate the mutant layer. Scale bars: 50 µm in A; 25 µm in B-D.

Competence to respond to SHR-mediated cell divisions is through SCR

SHR has been shown to act through SCR to induce the periclinal division that generates endodermis and cortex (Helariutta et al., 2000). Moreover we have previously shown that the supernumerary divisions induced by expression of *SHR* behind a *SCR* promoter are dependent on active SCR (Helariutta et al., 2000; Nakajima et al., 2001). In plants expressing *SHR::GFP* driven by the *WER* regulatory elements, we asked whether the resulting ectopic periclinal divisions were dependent on SCR. To address this question, we crossed a *pWER::SHR::GFP* line exhibiting a large number of supernumerary layers into the *scr-4* mutant background. We observed a *scr* phenotype, lacking not only the supernumerary layers but also one of the two ground tissues (Fig. 4A), demonstrating that SCR is required for the periclinal divisions induced in the ep/LRC initials by ectopic SHR::GFP.

Because *SCR* is not expressed in the ep/LRC initials in wild-type plants this result suggests that the expression of *SHR::GFP* from the *WER* promoter is sufficient to induce ectopic expression of *SCR*. To determine if this indeed is the case, we

crossed a *pWER::SHR::GFP* line with a reporter *pSCR::YFP$_{ER}$* line (*YFP$_{ER}$* contains ER targeting and retention signals). The lines were analyzed using two different confocal microscope settings (see Materials and methods). In GFP+YFP mode, fluorescent signals from both GFP and YFP are detected, while in YFP mode only YFP is detected. In the double transgenic plants, we observed YFP in a pattern similar to the wild-type expression pattern of *SCR* [endodermis, co/en initial and daughter and QC (Di Laurenzio et al., 1996)] (Fig. 5B). However, we did not observe YFP in the epidermis above the initials, although SHR::GFP was clearly visible in their nuclei (Fig. 5A). Strikingly, both YFP and SHR::GFP were detected in cells in a position corresponding to the ep/LRC initials (Fig. 5D, arrowhead). In all cases where YFP was detected outside the wild-type *SCR* expression pattern, we also observed nuclear localized SHR::GFP (Fig. 5C-F). Taken together, these results indicate that the competence to respond to SHR lies in the ep/LRC initials and that the generation of ectopic cell layers in response to SHR in these cells is through induction of *SCR*.

SHR::GFP is able to move from the epidermis in a *scr* mutant background

When we analyzed the localization of SHR::GFP driven by the *WER* promoter in the *scr* background, we observed fluorescence in both the epidermal layer as well as the single ground tissue layer (Fig. 4B). To be certain that this apparent movement was not due to a change in tissue-specificity of the *WER* promoter in the *scr* background, we performed in situ hybridization using antisense *GFP* sequence as a probe. The in situ results confirmed that the *SHR::GFP* mRNA was confined to the epidermis of this line (Fig. 4D, compare with Fig. 4C), indicating that the protein had moved from the epidermis to the mutant cell layer. Another striking observation was that GFP fluorescence was localized both to the nuclei and to the cytoplasm in the epidermis as well as the mutant layer (Fig. 4B). This last result is compatible with our previous observation (Nakajima et al., 2001) that SHR::GFP is present in both the cytoplasm and nucleus in the mutant layer of the *scr* background, when the fusion protein was expressed under the control of the *SHR* endogenous promoter (this is particularly evident in the meristematic zone, as shown in the inset of Fig. 4B). We conclude that the *scr* mutant background alters the SHR::GFP subcellular localization when expressed in the epidermis, as well as its potential for intercellular movement. These effects may argue for a role for SCR in restricting movement or may result indirectly from the altered radial pattern in the *scr* mutant root.

Competence to respond to SHR-mediated cell specification is widely distributed in the epidermis

To determine the competence to respond to SHR-mediated cell specification activity, we looked for two independent endodermis-specific markers in roots of *pGL2::SHR::GFP* and *pWER::SHR::GFP* transgenic lines. We used a histochemical stain that reveals the suberin in the endodermis-specific hydrophobic cell wall deposit known as Casparian strip (as well as the lignin in differentiating xylem cells) (Brundrett et al., 1988) (Fig. 6A) and the JIM13 monoclonal antibody specific for an arabinogalactan epitope found in the endodermis (as well as in a subset of stele cells) (Dolan and Roberts, 1995) (Fig. 6B). In both transgenic lines, evidence of Casparian strip

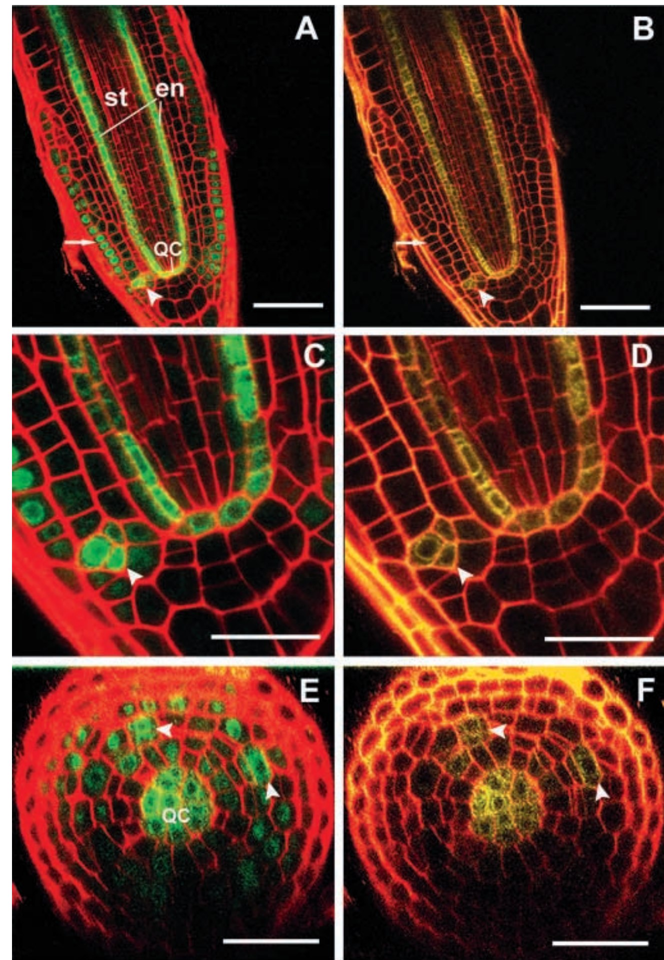

Fig. 5. *SCR* is induced only in initials by *pWER::SHR::GFP*. Longitudinal (A-D) and transverse (E,F) confocal images of the same *pSCR::YFP$_{ER}$*, *pWER::SHR::GFP* transgenic root. In the GFP+YFP mode (see Materials and methods), signals from both GFP and YFP$_{ER}$ are rendered in green (A,C,E); in the YFP mode, the signal from YFP$_{ER}$ alone is rendered in yellow (B,D,F). The *SCR* promoter is active in its normal expression pattern (endodermis, en/co initial and daughter and QC) and in cells in a position that corresponds to the location of the ep/LRC initials (arrowheads) (B,D,F). The *SCR* promoter is not active in the outermost tissue, even though SHR::GFP is visible in its nuclei (arrow in A,B). QC, quiescent center; st, stele; en, endodermis. Scale bars: 50 μm in A,B; 25 μm in C-F.

deposition was found not only in the normal location of the endodermis (arrowheads in Fig. 6C,E) but also in some cells of the outermost tissue layer (arrows in Fig. 6C,E). JIM13 immunostaining was detected in even a greater number of cells in the outermost tissue (Fig. 6D,F), confirming the endodermal character of these cells. These results indicate that the outer layer cells are competent to respond to the cell specification role of SHR. Moreover, SHR::GFP is synthesized in these cells, rather than moving into them, and yet is able to induce endodermal characteristics. This argues against a model in which movement is a prerequisite for activity.

To investigate whether a complete transformation from epidermal to endodermal fate had occurred, we looked for epidermal characteristics in the outer layer. Root hairs are

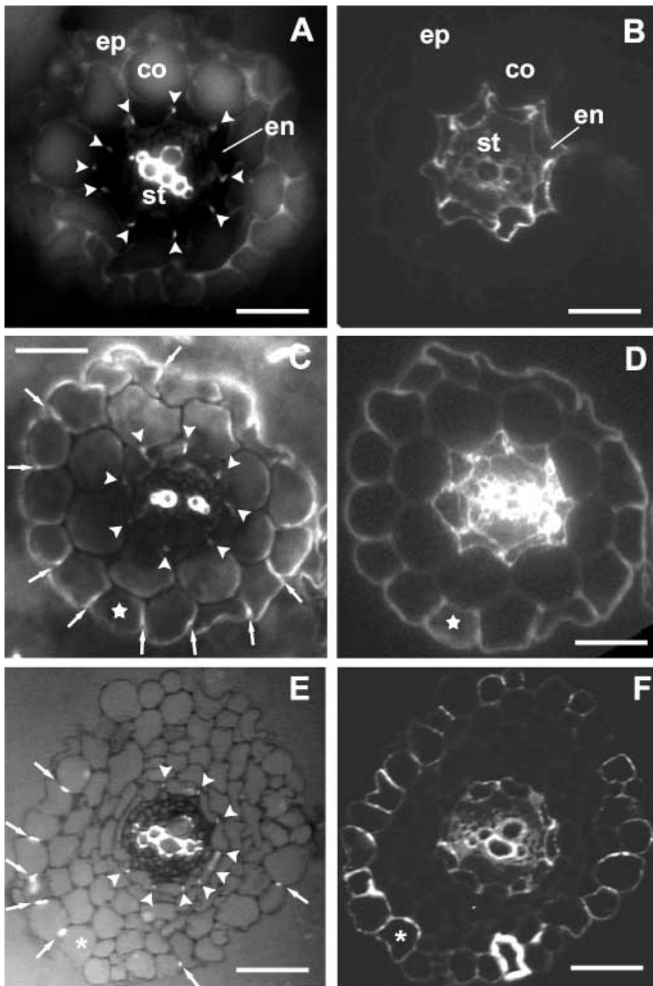

Fig. 6. Competence to respond to SHR::GFP-mediated cell fate changes. Endodermis-specific markers. Casparian strip histochemical staining (A,C,E) and JIM13 immunostaining (B,D,F) of transverse sections of wild-type (A,B), *pGL2::SHR::GFP* (C,D) and *pWER::SHR::GFP* (E,F) transgenic roots. C,D and E,F are consecutive sections (the star and the asterisk indicate the same cell in C,D and E,F, respectively). In both transgenic lines, Casparian strip is detected in the normal location of the endodermis (arrowheads in A,C,E), as well as in some cells of the outermost tissue (arrows in C,E). Lignin in the xylem is also stained. JIM13 is also detected in the normal location of the endodermis (B,D,F) and in some cells of the outermost tissue (D,F). Some cells in the stele are also stained by JIM13. ep, epidermis; co, cortex; en, endodermis; st, stele. Scale bars: 25 μm.

developed by epidermal cells in the trichoblast position in wild type and there is evidence that signaling from atrichoblasts is required for their correct placement (Lee and Schiefelbein, 2002). In our transgenic lines, root hairs were indeed produced and, at least in the *pGL2::SHR::GFP* lines, appeared to be in the expected positions (data not shown). A second marker of epidermal fate is the *GL2* promoter activity itself, which is atrichoblast specific. It clearly remains active in the *pGL2::SHR::GFP* line, suggesting that the presence of the fusion protein in the nuclei of atrichoblasts is not sufficient to shut down this aspect of their epidermal fate. To determine if expression of *pWER::SHR::GFP* has an effect on *GL2* expression, we crossed the *pGL2::YFP$_{ER}$* reporter construct into the transgenic line *pWER::SHR::GFP* and performed an analysis with confocal microscopy using the settings described above. In the GFP+YFP mode, we detected SHR::GFP in the nuclei of the ep/LRC initials and cells in the outermost tissue (Fig. 7A,B), while in the YFP mode we observed ER-localized YFP in the outermost tissue starting a few cells above the initials (Fig. 7C) in a pattern similar to that of the *GL2* promoter in wild type (data not shown). These observations indicate that although endodermal characters are induced by the ectopic expression of SHR::GFP in the outermost layer, epidermal cell-fate is not completely lost.

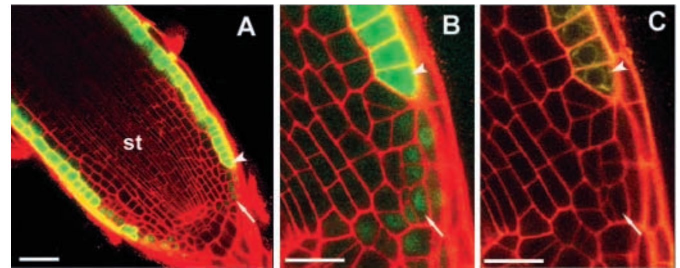

Fig. 7. Epidermal cell fate is not lost when SHR is expressed in the epidermis. Longitudinal confocal images of the same *pGL2::YFP$_{ER}$*, *pWER::SHR::GFP* transgenic root (A-C). In the GFP+YFP mode (see Materials and methods) signals from both GFP and YFP$_{ER}$ are rendered in green (A,B) while in the YFP mode the signal from YFP$_{ER}$ alone is rendered in yellow (C). The epidermis-specific *GL2* promoter is active in cells above the initials containing SHR::GFP (arrowheads), while younger cells contain SHR::GFP but do not show any YFP$_{ER}$ (arrows). st, stele. Scale bars: 50 μm in A; 25 μm in B,C.

Discussion

Intercellular movement of SHR::GFP is regulated by tissue-specific factors

In principle, a mechanism of positional information requires a spatial distribution of a signal that is then detected and translated into the appropriate output. The distributions of both the signal and the competence to detect and translate it can be targets of regulation. To characterize the role of SHR in transmitting positional information required for root radial patterning, we investigated both the regulation of its intercellular movement and the competence to respond to it.

It has been proposed that symplastic movement of molecules can occur by two mechanisms: simple diffusion is possible if the molecule is smaller than the basal size exclusion limit (SEL) of the PD, while active movement requiring a dilation of the PD can occur for bigger molecules (Zambryski and Crawford, 2000). The process involved in triggering PD dilation is not understood, but it appears that some form of specific interaction between the trafficking protein and the PD apparatus is required (Haywood et al., 2002).

When the SHR::GFP fusion protein is expressed in the stele under the control of the *SHR* promoter, it moves into the adjacent tissue layer, but not further (Nakajima et al., 2001) (Fig. 8A,C). One hypothesis for this limited trafficking is that SHR::GFP can move passively from any cell in which it is expressed, and that movement is limited by a symplastic barrier at the endodermal/cortex boundary. Neither part of this

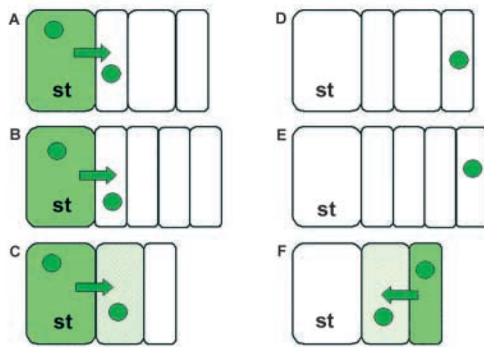

Fig. 8. Summary of the SHR::GFP localization experiments along the radial axis. *pSHR::SHR::GFP* in wild type (A) (Nakajima et al., 2001); *pSHR::SHR::GFP* in *pSCR::SHR* (B); *pSHR::SHR::GFP* in *scr-1* (C) (Nakajima et al., 2001); *pGL2::SHR::GFP* in wild type (D); *pWER::SHR::GFP* in wild type (E); *pWER::SHR::GFP* in *scr-4* (F). Green circle represents nuclear GFP; (strong or weak) green cell represents (strong or weak accumulation of) cytoplasmic GFP. st, stele. Refer to the text for discussion.

hypothesis could be tested using the constitutive *35S* promoter or the *SCR* promoter. Here, we first used the *SUC2* promoter to express SHR in the phloem CC, which have been shown to be symplastically connected to other cells (Oparka and Turgeon, 1999; Ruiz-Medrano et al., 2001; Van Bel, 2003). The lack of any movement of the fusion protein from the phloem CC, was our first indication that factors present in the source tissue may play an important part in determining the ability of SHR::GFP to move. This was confirmed by expression of SHR::GFP in the epidermal lineage, which did not support movement (Fig. 8D,E). The lack of SHR::GFP movement from both phloem CC and epidermal cells cannot be attributed to impaired symplastic connections between these cells and their neighbors, as non-targeted GFP driven by the same promoters is able to move throughout all root tissues (Imlau et al., 1999) (M. Cilia, personal communication). This suggests that factors required for SHR::GFP movement are absent in the phloem CC, the epidermis and the endodermis. Alternatively, factors inhibiting movement might be present in these cells.

Next we addressed the importance of cellular connectivity in limiting movement by showing that even in a situation where there are multiple cell layers specified as endodermis (Nakajima et al., 2001), SHR::GFP moves only from the stele to the first ground tissue layer (Fig. 8B). This demonstrates that the mechanism that limits SHR::GFP movement does not make use of differences between endodermis and the neighboring cortex.

This result also provides new insight into the generation of the ectopic cell layers in plants containing the *pSCR::SHR* construct (Nakajima et al., 2001). The lack of movement of SHR::GFP from the endodermis to the adjoining endodermal layers would suggest that the ectopic layers are not produced by movement from one layer to the next. Immunolocalization of SHR in wild-type roots indicated that SHR was normally partitioned in roughly equal amounts after the periclinal division of the co/en initial daughter (Nakajima et al., 2001). This would suggest that the extra divisions in the *pSCR::SHR* lines are likely to be the result of increased levels of SHR in the external daughter (produced by a positive-feedback loop of SHR on the *SCR* promoter). These increased levels would then trigger another round of division, and this process could be repeated.

A role for SCR in limiting intercellular movement

A surprising result was that SHR::GFP moved from epidermal cells in the *scr* mutant background and that movement correlated with a change in subcellular localization (Fig. 8F). Because we showed that in those transgenic lines the *SCR* promoter is not active in the epidermal tissue above the initials, formally this suggests a non-cell autonomous effect of *SCR* on SHR::GFP movement. This could be achieved through perdurance of SCR protein produced in the initials. A more plausible explanation is that there is normally an indirect effect either of SCR expressed in the initials or expressed in the endodermis resulting in nuclear localization of SHR::GFP, which inhibits movement. Alternatively, the change of radial pattern in the *scr* mutant could have an effect on subcellular localization and movement of SHR::GFP.

Subcellular localization has been suggested to be one relevant parameter in the regulation of intercellular protein movement (Crawford and Zambryski, 2000). A recent analysis of a number of GFP-tagged proteins expressed in the shoot apical meristem showed a positive correlation between cytoplasmic accumulation and intercellular movement (Wu et al., 2003). In particular, a functional translational fusion between GFP and the transcription factor LEAFY has been proposed to move by diffusion in the shoot apical meristem (Sessions et al., 2000; Wu et al., 2003). The fact that SHR::GFP accumulation in the cytoplasm of the source tissue correlates with its movement suggested that this might be a necessary and sufficient condition for movement. However, analysis of a mutant form of SHR suggests that although necessary for movement, cytoplasmic localization is not a sufficient condition (K. L. Gallagher, A. J. Paquette, K. Nakajima and P.N.B., unpublished). This raises the possibility that either tissue-specific factors or post-translational modifications of SHR could be involved in regulating movement. Interestingly, protein phosphorylation has been associated with the regulation of the tobacco mosaic virus movement protein TMV-MP intercellular trafficking (Citovsky et al., 1993; Waigmann et al., 2000) and a similar mechanism has been recently proposed for KN1 (Kim et al., 2003).

There is a broad competence to respond to SHR-mediated cell specification

Because cell specification has been shown to be dependent on position in most plant tissues (Kidner et al., 2000; van den Berg et al., 1997), it follows that cues are required to define a location along the radial axis. SHR is expressed in the stele and it is necessary for the correct differentiation of the endodermal tissue, into which it moves (Helariutta et al., 2000; Nakajima et al., 2001). In the simplest scenario, SHR could then be the positional cue 'instructing' the ground tissue in contact with the stele to acquire endodermal fate. This raises the question of whether a pre-pattern of competence exists to respond to SHR or whether SHR alone is sufficient to induce endodermal fate in any cell in which it is present. We know that SHR is not sufficient to induce endodermal differentiation in the stele (Helariutta et al., 2000). However, we have shown that it is sufficient to induce at least some aspects of endodermal fate determination in the supernumerary layers between the stele and the epidermis in plants expressing *pSCR::SHR* (Nakajima

et al., 2001). To extend this analysis to the outermost layer of the root, we asked if cells of the epidermal lineage are able to acquire endodermal characteristics in response to SHR. At least two independent endodermal markers could be found in cells of the epidermal lineage when *SHR::GFP* is expressed there. Thus, competence to develop endodermal characteristics in response to SHR appears to exist in all root tissues along the radial axis external to the stele. Moreover, expression of SHR::GFP by the *GL2* promoter solely in maturing epidermal cells is sufficient to confer endodermal fate. This broad competence to respond to SHR indicates that development of a single endodermal layer in contact with the stele depends on tight regulation of SHR movement. Without this tight regulation, endodermal characteristics would be found throughout the radial axis of the root external to the stele. The ability to induce endodermal characteristics in the epidermal lineage in the absence of SHR::GFP movement also provides evidence that movement is not a pre-condition for activity.

It is interesting to note that expression of SHR::GFP in the epidermal lineage did not result in a complete transformation of this tissue to endodermis. Root hairs were still made by trichoblasts and the atrichoblast-specific reporter *pGL2::YFP*$_{ER}$ was active. We previously reported that, in *pSCR::SHR*, *pGL2::GUS* transgenic roots, a very small number of cells in the outermost position of the supernumerary layers contained SHR protein in their nuclei and did not express *GUS* from the *GL2* promoter (Nakajima et al., 2001). In these plants, there appeared to have been a more complete transformation to endodermis. A likely explanation is that the cells containing SHR originated in the internal supernumerary layers (expressing SHR and not *GL2*), and were 'pushed' into the outermost position due to cell division events associated with the strongly perturbed radial pattern.

There is a restricted competence to respond to SHR-mediated periclinal cell divisions

We have previously shown that either ubiquitous *SHR* expression driven by the *35S* promoter or more restricted expression with the *SCR* promoter resulted in a perturbed radial pattern with supernumerary cell layers (Helariutta et al., 2000; Nakajima et al., 2001). In both cases, *SCR* expression was shown to be induced in the supernumerary layers as well as in the co/en initials (Helariutta et al., 2000; Nakajima et al., 2001). Moreover, induction of ectopic cell layers by the *pSCR::SHR* transgene was dependent on active SCR (Nakajima et al., 2001).

We have extended our understanding of the potential for SHR to alter radial patterning by showing that the ep/LRC initials are competent to respond to SHR by expressing *SCR* and producing supernumerary cell layers. By contrast, expression of *SHR::GFP* restricted to more mature epidermal cells did not result in *SCR* expression, suggesting that only the initials are competent to express *SCR* upon SHR induction. In the case of *pWER::SHR::GFP*, production of the supernumerary tissues disappeared in a *scr* mutant background, indicating that the competence to respond to SHR by periclinal cell divisions is mediated by SCR in these initial cells that normally never see SHR or SCR.

We cannot formally exclude the possibility that instead of acting in the ep/LRC initials, small amounts of SHR::GFP moved into the co/en initials (where endogenous SHR is already present) and there induced *SCR*-dependent extra

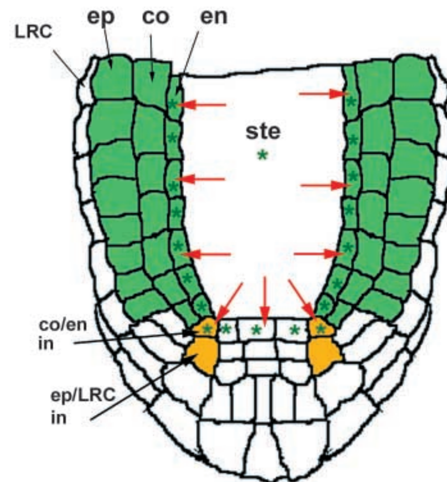

Fig. 9. Relationship between SHR movement and competence to respond to SHR in regulating root radial patterning. Movement of SHR protein (asterisks) from the stele is limited to the adjacent layer (red arrows), while the competence to respond to SHR extends beyond the zone of movement. Cells that are competent to respond to SHR-mediated cell specification are rendered in green (cortex competence has yet to be directly tested); initial cells competent to respond to SHR-mediated cell periclinal divisions are rendered in yellow. The fact that competence to respond to SHR exists outside of the zone of SHR movement highlights the crucial role of regulated SHR movement in root radial patterning. in, initial; ep, epidermis; co, cortex; en, endodermis; st, stele; LRC, lateral root cap.

periclinal cell divisions. However, whenever the pattern allowed us to morphologically recognize the exact location of the co/en initials, we did not detect any GFP in these cells. Moreover, in *pSHR::SHR::GFP* transgenic roots in a wild-type background, where the fusion protein moved into the co/en initials, we never observed any significant alteration of the radial pattern (data not shown). The difference in competence to respond to SHR in different cell types suggests the presence of factors distributed in a tissue-specific manner. These factors could for example dimerize with SHR to either activate or inhibit the transcription of target genes, or modify SHR activity by producing post-translational modifications.

In conclusion, our analysis of tissue-specific ectopic expression of SHR::GFP has revealed a complex picture of the regulation of its intercellular trafficking. Movement by diffusion seems insufficient to explain the features we observed. Rather, tissue-specific factors seem likely to play a role in regulating movement of SHR::GFP. Restriction of SHR movement to the first layer external to the stele is crucial to radial patterning, as competence to respond to SHR-mediated cell specification appears widespread along the radial axis, while competence to respond to SHR-mediated periclinal cell divisions resides in initial cells (Fig. 9). This non-uniform distribution of competence to respond to SHR also suggests tissue-specific localization of factors essential for SHR activity.

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pSUC2::GFP construct; and D. Jackson and M. Cilia for the seeds from the $pSUC2::GFP_{ER}$ and *pSUC2::GFP* transgenic lines, for the *pMC720* plasmid containing the regulatory region of *SUC2* and for sharing unpublished data about *pWER::GFP*. We thank K. Birnbaum, K. Gallagher, Y. Helariutta, J. E. Malamy, A. J. Paquette and T. Vernoux, for helpful comments and suggestions related to this work. This work was supported by a grant from the NIH (RO1-GM43778).

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