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## Both the conserved GRAS domain and nuclear localization are required for SHORT-ROOT movement

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### Summary

Transcription factor movement is well established in plants. Since the initial report of KNOTTED movement, more than a dozen transcription factors have been shown to move in plants. However, the developmental significance of movement is not known. Using the SHORT-ROOT (SHR) transcription factor as a tool for studying cell-to-cell trafficking, we show that movement of SHR from its site of synthesis is necessary for normal development of the Arabidopsis root. We identify multiple regions of SHR that are required for intra- and intercellular movement of SHR, including a region that is necessary for movement but not activity. We made the surprising discovery that the capacity for intercellular movement may be conserved among other GRAS family proteins. Finally, we provide evidence that movement requires both cytoplasmic and nuclear localization, strongly suggesting a mechanistic link between nuclear transport and cell-to-cell movement.

### Keywords

SHORT ROOT; Arabidopsis root development; intercellular protein movement; plasmodesmata; protein trafficking

### Introduction

In plants, cell-to-cell communication is possible by the direct trafficking of transcription factors between cells. Transcription factor movement was initially described by Lucas *et al.* (1995), who showed that the maize KNOTTED1 (KN1) protein is found beyond the domain of *KN1* RNA transcription. Since then, additional transcription factors have been shown to traffic between cells, including KNAT1/BREVIPEDICELLUS (KNAT1/BP), SHOOT MERISTEMLESS (STM) (Kim *et al.*, 2003), DEFICIENS (DEF) (Perbal *et al.*, 1996), CAPRICE (CPC) (Wada *et al.*, 2002), LEAFY (LFY) (Sessions *et al.*, 2000) and SHORT-ROOT (SHR) (Nakajima *et al.*, 2001).

Transcription factor movement is possible in plants due to the presence of plasmodesmata (Lucas, 1995; Lucas *et al.*, 1995; Maizel, 2006; Perbal *et al.*, 1996; Ruiz-Medrano *et al.*, 2004; Wu *et al.*, 2003; Zambryski and Crawford, 2000). Plasmodesmata are membrane-lined channels that connect nearly all plant cells (Gunning and Overall, 1983). Functionally they are similar to gap junctions (or ring canals), in that they allow passage of small molecules between cells. However, unlike gap junctions, plasmodesmata selectively allow passage of macromolecules between cells. When tested using GFP, the size-exclusion limit of

plasmodesmata in most growing tissues was found to range from approximately 30–50 kDa (Crawford and Zambryski, 2001; Kim *et al.*, 2005a). In theory, transcription factors could either diffuse through open plasmodesmata or actively dilate plasmodesmata to facilitate their own movement (Crawford and Zambryski, 2001). However, recent results with KNOTTED1, in which Tassetto *et al.* (2005) showed that the KNOTTED1 homeodomain can traffic between animal cells in culture, suggest that at least some transcription factors may selectively move between cells by a plasmodesmata-independent pathway.

While the precise mechanisms regulating intercellular trafficking are not known, structure/function analysis of several of these transcription factors as well as other plant-derived non-cell-autonomous proteins revealed two primary modes of cell-to-cell movement (Aoki *et al.*, 2002; Kim *et al.*, 2005b; Kurata *et al.*, 2005; Lucas *et al.*, 1995; Wu *et al.*, 2003). The first, which is referred to as selective movement, relies upon sequences within the transported protein for movement (Lucas *et al.*, 1995). For example, within CPC, two motifs are necessary for transport: one in the N-terminus and the other in the MYB domain (Kurata *et al.*, 2005). Likewise, the KN1 homeodomain has been shown to mediate KNI movement (Kim *et al.*, 2005b). In SHR, substitution of a single amino acid in the VHIID domain inhibits movement (Gallagher *et al.*, 2004). In contrast, there is no requirement for movement sequences in proteins that move non-selectively between cells (i.e. by diffusion), as has been shown for LFY (Wu *et al.*, 2003). For all of the selectively transported proteins, the sequences relevant to protein movement appear to function only within the context of the protein as a whole. No short autonomous movement domain akin to the 9–11 amino acid HIV Tat transduction domain, which is able to cause movement to unrelated proteins (Chauhan *et al.*, 2007), has been found in plants. Recently, however, it was shown that 38 amino acids from the non-cell-autonomous Cm-PP16-1 phloem protein appear to confer movement to the normally cell-autonomous glutathione-S-transferase protein (Taoka *et al.*, 2007).

It is generally believed that cell-to-cell transport of transcription factors is developmentally relevant, because zones of symplastic connectivity change over developmental time (Gisel *et al.*, 1999; Kim *et al.*, 2002a,b; Rinne and van der Schoot, 1998) and because movement of specific transcription factors correlates with specific developmental events. However, direct evidence showing that movement is required for developmental patterning is lacking.

Here we examine SHR movement in the Arabidopsis root with respect to both the relevance of movement to development, and the mechanisms regulating cell-to-cell trafficking. SHR is a member of the plant-specific family of GRAS transcription factors. It is required for proper root development, moving from the stele into the adjacent ground tissue where it is required for an asymmetric cell division that creates the two distinct ground tissue layers, the cortex and endodermis, which are located between the pericycle and epidermal cell layers (Figure 1) (Nakajima *et al.*, 2001). Recent results have shown that *SHR* occurs directly upstream of *SCARECROW* (*SCR*) in a pathway that leads to this patterning of the ground tissue and maintenance of quiescent center (QC) cells (Nakajima *et al.*, 2001). However, *SHR* and *SCR* are expressed in non-overlapping domains. *SHR* is expressed only in the stele, whereas *SCR* is confined to the ground tissue. This raises the question of how *SHR* regulates *SCR* expression.

In the present study, we show that *SHR* movement from the stele is required for proper patterning of the root. In addition, we add to our earlier findings that *SHR* movement is selective and requires cytoplasmic localization (Gallagher *et al.*, 2004), identifying multiple domains that are required for *SHR* movement and for proper sub-cellular localization, and suggesting that both nuclear and cytoplasmic localization are required for *SHR* movement. These data expand upon our previous results (Gallagher *et al.*, 2004) and the results of Cui *et al.* (2007) who showed that *SCR* is required in endodermal cells to prevent movement of *SHR* into the cortical cell layer and expansion of the ground tissue. Collectively, these data allow us to

propose a model for SHR trafficking in which a balance between nuclear localization and nuclear export promotes SHR movement into the ground tissue, where further movement is blocked by SCR (Cui *et al.*, 2007).

## Results

The demonstration that SHR moves from the stele into the endodermis (Figure 1) and is responsible for up-regulating *SCR* expression (Nakajima *et al.*, 2001) strongly suggested that SHR movement is required for normal ground-tissue patterning. However, this has not been directly demonstrated. To address this issue, we determined whether SHR could rescue the radial patterning and root length of *shr-2* plants (shown in Figure 2a) without moving into the endodermis or QC.

Previously we have shown that efficient nuclear localization of SHR by addition of a canonical nuclear localization signal (NLS) (*SHR-NLSGFP*) inhibited movement into the endodermis when expressed in the stele of wild-type roots (Figure 2b), but did not affect the activity of SHR when expressed directly in the endodermis (Gallagher *et al.*, 2004). We used *SHR-NLSGFP* to test whether movement of SHR is required to rescue the phenotype of *shr-2*. *shr-2* plants have only one ground-tissue layer between the pericycle and epidermal cell layers, and have a disorganized QC (Figure 2a). The QC cells are thought to serve as a signaling center that maintains the stem-cell population (initial cells) in the root meristem (Helariutta *et al.*, 2000; Sabatini *et al.*, 1999, 2003). Loss of the QC population results in a shortened root due to exhaustion of the initial cells. Thus SHR is required both for correct radial patterning and maintenance of root growth.

The *SHR-NLSGFP* construct expressed from the *SHR* promoter (*SHR<sub>pro</sub>*) was transformed into plants heterozygous for the *shr-2* mutation, and T<sub>2</sub> generation seedlings with the *shr-2* phenotype were identified. In approximately 70% of the *shr-2* seedlings with *SHR-NLSGFP* expression, fluorescence due to GFP was restricted to the stele, and the radial pattern resembled that of *shr-2*, with one ground-tissue layer and a disorganized QC (Figure 2c–e). Approximately 30% of seedlings with short roots showed sporadic rescue of the ground tissue (Figure 2d,e). In all of these plants, *SHR-GFP* was detectable in the inner layer of ground tissue, indicating that the protein had moved. We did not observe rescue of the radial pattern without the presence of SHR in the inner ground-tissue layer. These results show that addition of an exogenous nuclear localization signal does not entirely abrogate movement in the *shr-2* background. More importantly, they indicate that movement of SHR out of the stele is consistent with normal radial patterning of the root.

In addition to being required for proper radial patterning, both SHR and SCR are required to prevent differentiation of the initial cells and maintain root growth, presumably due to their role in preventing loss of the QC (Sabatini *et al.*, 2003). In all of the T<sub>2</sub> lines expressing *SHR<sub>pro</sub>-SHR-NLSGFP* and segregating for *shr-2*, there was an increase in the expected ratio of long to short roots (from 3:1 to approximately 10:1), indicating that the *SHR<sub>pro</sub>-SHR-NLSGFP* transgene was often able to rescue *shr-2* root length. We examined seedlings with long roots and found two classes: those with aberrant root radial patterning, which were genotyped as *shr-2* homozygotes, and wild-type plants. In *shr-2* plants with a normal root length, the *SHR-NLSGFP* protein was often not detected in the QC cells (Figure 2f). These results suggest that expression of nuclear-localized SHR in the stele is sufficient to maintain continued divisions of the root initials in a *shr-2* background.

Like *shr-2* plants, *scr* plants also have aberrant QC cells and fail to maintain continued division of the root initial cells. As SCR is also required for maintenance of the QC, we tested whether expression of GFP-SCR in the stele could rescue *scr* root length, by expressing it from the

*SHR* promoter in *scr-5* (a null allele) plants (Paquette and Benfey, 2005). Analogous to the results with *SHR*, expression of GFP-*SCR* in the stele is able to rescue *scr-5* root length without detection of GFP in the QC cells (Figure 2g,h).

The above results suggest that *SHR* movement from the stele is required for correct radial patterning of the Arabidopsis root, as expression of largely cell-autonomous *SHR* in the stele tissue is not sufficient for rescue of the division that produces the separate cortex and endodermal cell layers. However, rescue of root length may not require movement of *SHR*. Instead, it appears that expression of cell-autonomous *SHR* or *SCR* in the stele is able to rescue the root length defect in *shr-2* or *scr-5* plants respectively.

### Identification of domains within *SHR* required for movement

We previously showed that threonine at position 289 (T289) is required for proper *SHR* localization and movement, and that, independent of localization and movement, T289 is also required for *SHR*'s ability to function (Gallagher *et al.*, 2004). Here we determined whether T289 is the critical residue for *SHR* movement, or whether other regions of the *SHR* protein are also required to promote cell-to-cell transport. In addition, we determined whether there is complete overlap between the sequences that are required for *SHR* movement and *SHR* activity.

The *SHR* protein can be divided into eight distinct domains based upon sequence homology with the other GRAS proteins (Figure 3a) (Pysh *et al.*, 1999). The functional significance of these regions of the GRAS domain is not known except for *SLENDER RICE*, in which the first set of leucine heptad repeats has been shown to be required for homodimerization (Itoh *et al.*, 2002). To determine the role of these sequences in *SHR* movement, we produced deletions within the *SHR* protein corresponding to the domain structure and then assayed the effects of these mutations on movement and activity in a wild-type Columbia background. Expression of the mutated proteins as GFP fusions from the *SHR* promoter allowed us to assess the ability of the protein to properly localize and move. To determine whether the mutated proteins can function independently of movement, we expressed them directly in the endodermis, using the *SCR* promoter (*SCR<sub>pro</sub>*) (Gallagher *et al.*, 2004). Expression of *SHR* from *SCR<sub>pro</sub>* in wild-type plants results in an increase in the number of ground-tissue layers (Gallagher *et al.*, 2004; Nakajima *et al.*, 2001). Mutated *SHR* proteins were considered to retain *SHR* activity if they caused an increase in the number of ground-tissue layers when expressed from *SCR<sub>pro</sub>*.

The results of the structure/function analysis are summarized in Figure 3b. In general, the deletion constructs fell into three distinct classes: (1) those that were undetectable and thus required other approaches to test their function, (2) those that lost the ability to move and function, and (3) one that did not move, but retained activity.

**Class 1: The *SHR* N-terminus and *SAW* domain appear to be required for protein stability**—Two different N-terminal (NT) deletions of *SHR* were produced (Figure 3). No GFP was detected in any of the lines expressing either construct ( $n > 10$ ). This was also the case for deletion of the *SAW* domain ( $n > 10$ ). These results suggest that the N-terminus and *SAW* domains are required for protein stability (Waldo *et al.*, 1999), and that other methods are required to assay their function.

**Class 2: Deletion of the *VHIID* or *PFYRE* domains disrupts movement and activity**—Deletion of either the *VHIID* domain (Figure 4) or the *PFYRE* domain affected subcellular localization and disrupted movement from the stele, with neither protein detected in the endodermis. As we had previously demonstrated that T289, within the *VHIID* domain, is required for *SHR* activity (Gallagher *et al.*, 2004), we did not express the *SHRDVHIID*

construct from *SCR<sub>pro</sub>* to test for activity, as deletion of the VHIID domain would eliminate this critical residue. Expression of the SHRΔPFYRE protein from the *SCR* promoter did not cause an increase in the number of ground-tissue layers (data not shown, Figure 5a–d). These results indicate that both the VHIID and PFYRE domains are necessary for movement and activity of the SHR protein. It is noteworthy that nuclear localization was reduced by these mutations.

**Class 3: Mutation of the leucine heptad repeat II disrupts movement, but not activity**—To assess the function of the second leucine heptad repeat in SHR movement, we disrupted the heptad repeat structure, substituting an LNELDV motif with a polyalanine (AAA) stretch. In the stele, there was a reduction in nuclear accumulation compared to wild-type SHR, with SHRΔLNEVDV distributed throughout the cells (compare Figure 5c,d). The mutant protein was not detected in the endodermis, quiescent center cells or the root initials.

To test for activity of the SHRΔLNEVDV protein, we expressed it directly in the ground tissue from *SCR<sub>pro</sub>* (Figure 5e). In this tissue, the protein localized to both the cytoplasm and the nucleus. We observed a multi-layered ground-tissue phenotype indistinguishable from that of wild-type SHR expression from *SCR<sub>pro</sub>*. Thus, in contrast to the other mutations that affected SHR movement, SHRΔLNEVDV is a functional SHR protein. These results suggest that the LNEVDV motif is required for SHR movement, but not its activity.

### The SCR GRAS domain has the capacity to move

As multiple regions within the conserved GRAS domain are required for trafficking of SHR, we hypothesized that the GRAS domains from other family members may also have the capacity for cell-to-cell movement. We tested this hypothesis using the SCR protein. The SCR protein is normally expressed in the endodermis, with low-level expression in the cortex. In these tissues, the SCR protein is nuclear-localized and cell-autonomous. When expressed in the stele, SCR–GFP is also nuclear-localized, and does not move into the endodermis, quiescent center cells or root initials (Gallagher *et al.*, 2004). To test whether the SCR GRAS domain alone (i.e. SCR without the N-terminus) has the capacity for movement, we expressed it as a GFP fusion from the *SCR* and *SHR* promoters. Neither SCR GRAS protein (SCRΔNTa or SCRΔNTb; see Figure 6) showed exclusive nuclear localization in the endodermis or the stele (Figure 7a–d) as is seen for wild-type SCR, indicating that the SCR N-terminus confers proper subcellular localization to the SCR GRAS domain. Localization is independent of the predicted NLS, as the SCRΔNTa construct lacks this region. The SCR GRAS protein was found throughout stele and endodermal cells, with some indication of weak protein aggregation. Significantly, the SCR GRAS domain was able to move from the stele into the endodermis, quiescent center cells and the cortical endodermal root initials, indicating that the SCR GRAS domain has the capacity to move (Figure 7b,c).

To quantify movement of the SCR GRAS domain, we measured GFP intensities in the stele and endodermis. The ratio of endodermal fluorescence to stele fluorescence was used as an indicator of the efficiency of transport from the stele into the endodermis (Figure 6b). Using such calculations, we found that movement of the SCR GRAS domain was approximately 50% of that observed for SHR–GFP. This suggests that the SCR GRAS domain is not as efficient in promoting movement as SHR, perhaps due to sequences within the SHR N-terminus that promote movement. In addition, the fact that SCR can move when the SCR N-terminus is removed suggests that the SCR N-terminus inhibits movement. To test these hypotheses, we performed domain swaps between the SHR and SCR N-termini.

The N-terminus of SHR was fused to the C-terminus of SCR (the SCR GRAS domain) to produce SHRNT–SCR–GFP (see Figure 6). This construct was then expressed from the *SHR* promoter (Figure 7e–g) or the *SCR* promoter (data not shown). In the stele, the SHRNT–SCR–

GFP protein localized similarly to SHR, with GFP detected in both the nucleus and cytoplasm of individual stele cells (Figure 7e–g), suggesting that the SHR N-terminus is, at least in part, responsible for SHR subcellular localization. There was no evidence, however, that the SHR N-terminus increased the ability of the SCR GRAS domain to move. In fact, movement of the SHRNT–SCR–GFP construct was 80% of that of the SCR GRAS domain alone, indicating that, if anything, the SHR N-terminus reduces the efficiency with which the SCR GRAS domain moves.

To test the ability of the SCR N-terminus to inhibit movement, we performed the reciprocal swap (SCRNT–SHR), removing the N-terminus of SHR and replacing it with the N-terminus of SCR, which does not include the predicted NLS. The chimeric protein was fused to GFP and expressed from the *SHR* and *SCR* promoters. In both the stele (Figure 7h) and endodermis (data not shown), the SCRNT–SHR protein was predominantly nuclear-localized. When expressed from *SHR<sub>pro</sub>*, we rarely detected the chimeric SCRNT–SHR protein in the endodermis, indicating very limited movement of the nuclear-localized SCRNT–SHR protein. However, addition of a nuclear export signal (NES) allowed the SCRNT–SHR protein to accumulate in the cytoplasm of stele cells, and, more importantly, to move into the endodermis (Figure 7i,j). Movement of the SCRNT–SHR chimera with the NES was approximately 50% of that of wild-type SHR. These results suggest that the role of the SHR N-terminus in potentiating SHR movement may derive primarily from its ability to stabilize the SHR GRAS domain and provide nuclear export activity. For SCR, the N-terminus also controls subcellular localization, which is likely to play a role in inhibiting movement of the intact SCR protein.

### The SHR SAW domain is required for proper localization and movement

As removal of the SAW domain from SHR–GFP appeared to result in an unstable protein, swaps with the SCR SAW domain were performed to assess the role of this region in SHR movement. Removal of the SCR SAW domain has no effect on SCR localization and does not allow SCR to move (data not shown). The SHR SAW domain was removed and replaced with the equivalent region from SCR to produce SHR–SCRSAW–GFP (Figure 7k,l). Localization of the chimeric protein was similar to the localization observed for the class 2 and class 3 mutations. In addition, there was no indication that the protein moved from the stele into the endodermis (Figure 7k), suggesting that the SHR SAW domain is required for SHR movement, and that the SCR domain cannot substitute for the SHR domain in this context.

For the reciprocal swap, the SCR SAW domain was replaced with the equivalent domain from SHR (SCR–SHRSAW–GFP) (Figure 7m,n). No effect on SCR localization was seen when expressed in either the stele (Figure 7m) or the endodermis (Figure 7n). The protein was detected only in nuclei, and there was no movement into neighboring cells.

Collectively, these results indicate that the SHR SAW domain is required for stability of the SHR protein, and, independent of stability, for nuclear accumulation and movement. The SHR SAW domain is not, however, sufficient for movement, as it did not confer movement to SCR.

### Both cytoplasmic and nuclear localization are required for SHR movement

Previous results have shown that cytoplasmic localization of SHR is required for cell-to-cell movement. The results of the structure/function analysis, however, show a positive correlation between nuclear accumulation and the ability of SHR to move between cells. All mutations that significantly reduced nuclear accumulation also prevented cell-to-cell transport (indicated in bold in Figure 3 and Figure 6). However, for modifications that resulted in a decrease in the cytoplasmic localization of SHR, addition of an NES promoted movement. Together, these results suggest that both nuclear and cytoplasmic localization are required for cell-to-cell transport of SHR. To further explore the relationship between subcellular localization and SHR

movement, we attempted to rescue movement of one of the class 2 mutations by restoring nuclear localization.

### Increasing nuclear localization can enhance movement

If nuclear localization is mechanistically required for SHR movement, then at least some of the mutations that disrupted both nuclear localization and movement of SHR might be primary defects in nuclear localization that indirectly affect protein movement. For example, conversion of T289 to an isoleucine (I) resulted in reduction of nuclear accumulation of SHR and a lack of SHR movement (Gallagher *et al.*, 2004). These results suggest that T289 is independently required for both nuclear localization and movement of SHR. However, the alternative is that T289 is necessary for nuclear localization, which is in turn necessary for cell-to-cell trafficking. If the latter scenario were correct, then one would expect that restoration of nuclear localization could (at least partially) restore movement. We tested this by adding NLSGFP to the mutated SHRTAI protein (T289 mutation) and expressing the resulting *SHRTAI-NLSGFP* construct from the *SHR* promoter in wild-type plants (Figure 8a,b). In these lines (*SHR<sub>pro</sub>-SHRTAI-NLSGFP*), the modified protein was found in both the nucleus and cytoplasm of stele cells, and we consistently detected SHRTAI-NLSGFP in the endodermis, indicating that protein movement was restored. These results indicate that T289 is not specifically required for SHR movement, and that instead this residue is required for nuclear accumulation, which is in turn required for SHR movement. These data indicate a mechanistic link between nuclear localization of SHR and movement.

### Decreasing nuclear localization can inhibit SHR movement

If nuclear accumulation is a prerequisite for cell-to-cell movement of SHR, then reducing that ability of SHR to localize to the nucleus should inhibit movement. It was previously shown that *SCR* is required for nuclear accumulation of SHR both in the endodermis and when ectopically expressed in the epidermis (Sena *et al.*, 2004). In an effort to dramatically reduce nuclear accumulation of SHR in stele cells (essentially phenocopy the class 2 or 3 mutations), we expressed SHR fused to NESGFP (*SHR<sub>pro</sub>-SHR-NESGFP*) in the stele in the *scr-5* null background (Paquette and Benfey, 2005) (Figure 8c,e,f). In these plants, there was a reduction in nuclear accumulation of SHR-NESGFP in both stele and in the mutant layer of cells compared to unmodified SHR or SHR-NESGFP in the wild-type background (Figure 8d). As predicted if nuclear accumulation is required for cell-to-cell transport, there was a strong reduction in the amount of SHR-NESGFP detected in the mutant layer (Figure 8e,f), indicating a reduction in movement. These results provide further evidence that nuclear accumulation is necessary for optimal SHR movement, and that the nuclear localization signal in SHR does not directly serve as a movement sequence, but that the sequences required for nuclear localization promote movement as a consequence of nuclear accumulation of the SHR protein. These results also extend the results of Sena *et al.* (2004) who showed a non-cell-autonomous role for SCR (that is not dependent upon SCR movement) in promoting nuclear accumulation (or inhibiting nuclear export) of SHR in epidermal tissues.

## Discussion

Recent evidence indicates that there are many transcription factors that traffic between plant cells. Estimates by Lee *et al.* (2006) suggest that as many as 17% of transcription factors move in the root. To date, very little is known about the developmental significance of movement, or the sequences and mechanisms controlling cell-to-cell trafficking. Using SHR as a model for transcription factor movement, we have shown that SHR movement from the stele is essential for radial patterning of the Arabidopsis root. Through a structure/function analysis, we have identified a strong correlation between SHR nuclear localization and movement, and

show that the GRAS domain is the important region for intercellular trafficking. Further, we provide evidence that SHR movement is controlled in part by nuclear/cytoplasmic partitioning.

### SHR movement is required for normal root development

Many groups have shown that cell-to-cell movement of transcription factors, as well as other macromolecules, is developmentally regulated (Crawford and Zambryski, 2001; Imlau *et al.*, 1999; Kim *et al.*, 2003; Kurata *et al.*, 2005; Sena *et al.*, 2004; Sessions *et al.*, 2000; Wu *et al.*, 2003; Zambryski, 2004). However, conclusive evidence that movement is required for normal development has been lacking. Our data indicate that SHR movement from the stele is necessary for normal patterning of the Arabidopsis root. In *shr-2* roots, in which SHR–NLSGFP was expressed and only detected in the stele, the ground tissue failed to divide and the QC was often disorganized. Rescue of *shr-2* radial patterning correlated with the cell-to-cell movement of SHR–NLSGFP, indicating that movement of SHR is developmentally relevant.

Surprisingly, we observed rescue of root length in *shr-2* plants with abnormal patterning of the ground tissue and no detectable GFP signal in the presumptive QC. These results raise the possibility that SHR movement is not required to maintain the root meristem. Based upon the results of Sabatini *et al.* (2003), we had anticipated that SHR would be required in the QC for maintenance of the stem-cell niche. However, our results suggest that expression of nuclear-localized SHR or SCR in the stele is sufficient to maintain continued divisions of the root initials in *shr-2* and *scr-5* mutant backgrounds, respectively. SCR expression in the stele correlates with ectopic divisions above the QC in the stele cells, perhaps resulting in reorganization of the QC signaling center to a more basal region of the root. This is not unlike what was found when the QC was laser-ablated (Xu *et al.*, 2006). Initially, SHR–GFP became nuclear-localized and SCR was expressed in the provascular cells immediately above the ablated QC, then a new QC signaling center was organized in what was the apical region of the stele (Sabatini *et al.*, 1999; Xu *et al.*, 2006).

An alternative explanation for the above results is that there was low-level movement of SHR or SCR into the QC cells that was below the level of detection. As addition of the NLS to SHR–GFP did not completely abrogate movement in *shr-2*, and as our data indicate that the SCR GRAS domain does have the capacity to move, it is possible that the GFP-tagged proteins moved into the QC cells of the rescued roots. If this is the case, then the levels of SHR and SCR required for QC maintenance are likely to be lower than those needed for radial patterning. In support of this second hypothesis is the finding by Mace *et al.* (2006) that low levels of GFP expression are difficult to detect.

Previously, Wu *et al.* (2003) presented data suggesting that movement of LFY, a transcription factor required for the transition from vegetative to floral development, is required for LFY function. Movement-compromised GLFY (LFY with a GFP insertion at amino acid 31) does not completely rescue the *lfy-11* phenotype. This is in contrast to constructs with GFP at either the N- or C-terminus, which are movement-competent, and rescue *lfy-11*. While these results strongly suggest that LFY movement is required for its function, the possibility cannot be excluded that GLFY is compromised in its ability to fully function as a transcription factor independently of movement. If movement of GLFY were restored, and GLFY was shown to rescue the *lfy-11* phenotype, this would provide conclusive evidence that LFY movement is required for function.

A significant distinction between LFY and SHR movement is that LFY is expressed throughout the entire region in which the protein moves. Thus cell-to-cell trafficking of LFY does not increase its effective domain, whereas SHR movement allows SHR to function in cells in which it is not normally expressed. The LFY results suggest that movement *per se* is required for



LFY function. This is not the case for SHR, for which we have no evidence that movement in and of itself is required for function (Gallagher *et al.*, 2004; Levesque *et al.*, 2006).

### Multiple regions within the SHR GRAS domain are necessary for movement

Our data indicate that multiple regions within the GRAS domain are required for nuclear accumulation and cell-to-cell movement of SHR. Experiments in which the N-termini of SHR and SCR were swapped (or deleted in the case of SCR) suggest that the conserved GRAS domain, which defines the family, is the important region for cell-to-cell transport. This is analogous to what has been shown for the KN1 and ENGRAILED2 (EN2) proteins (Tassetto *et al.*, 2005), of which the conserved homeodomain has the capacity to move. It has been suggested that intercellular transfer may be an evolutionarily conserved feature of many DNA-binding homeoproteins (Tassetto *et al.*, 2005). Sequences outside the conserved homeodomain either promote or inhibit movement. Likewise, for SCR, sequences outside the GRAS domain probably regulate movement. Further analysis of the GRAS domains from additional GRAS proteins will allow us to determine how widespread the potential for cell-to-cell movement is for this family.

### There is not complete overlap between domains required for movement and activity

Previously we identified a single amino acid in the VHIID domain as being necessary for SHR movement and activity (Gallagher *et al.*, 2004). In the present study, most of the mutations that inhibited movement also disrupted activity. Significantly, the LNELDV motif in LHR2 is not required for SHR activity but is required for movement, indicating that there is not complete overlap in the sequences that are required for movement and activity. This is in contrast to KN1 and CPC, for which all of the sequences that have been identified to date as being involved in movement (when examined) are also required for activity (Kim *et al.*, 2005b; Kurata *et al.*, 2005; Lucas *et al.*, 1995). The overlap between movement and activity has made determination of the mechanism of cell-to-cell trafficking problematic. Loss of both movement and activity could be attributed to improper protein folding rather than a specific movement sequence. However, for some of these proteins, such as KN1, the capacity for specific sequences to confer movement has been directly demonstrated (Kim *et al.*, 2005b; Tassetto *et al.*, 2005). This has not been possible for SHR (Gallagher *et al.*, 2004). However, the ability to separate movement and activity in SHR allows us to confidently conclude that specific sequences within SHR are required for movement, and that loss of movement is not a consequence of improper folding.

### Correlation between nuclear localization and movement

An interesting similarity between CPC, KN1 and SHR movement is that all mutations that diminish nuclear localization also reduce movement (Gallagher *et al.*, 2004; Kim *et al.*, 2005b; Kurata *et al.*, 2005; Lucas *et al.*, 1995; Prochiantz and Joliot, 2003; Tassetto *et al.*, 2005). Mutation of the Myb domain in CPC inhibited both nuclear localization and movement (Kurata *et al.*, 2005). Mutation of the predicted NLS in KN1 blocked movement (Lucas *et al.*, 1995; Prochiantz and Joliot, 2003; Tassetto *et al.*, 2005). Likewise, for SHR, all of the mutations within the GRAS domain that diminished nuclear accumulation also prevented movement. These results suggest that either there is broad overlap between the sequences required for nuclear localization and those required for movement, or that nuclear localization mechanistically promotes movement. The latter hypothesis is supported by the results showing that reduction of nuclear localization of SHR-NESGFP in *scr-5* decreased movement, and, more importantly, that movement of the SHRT289I mutant could be rescued by restoration of nuclear localization.

Interestingly, for the KN1 homeodomain, the correlation between nuclear localization and movement has been extended to transport between animal cells. Tassetto *et al.* (2005) showed that the KN1 homeodomain can traffic between animal cells in culture. The mutation within

the NLS that blocked trafficking *in planta* also inhibited nuclear localization in and transport between animal cells. Movement of the mutant KN1 homeodomain could be restored by addition of a heterologous NLS.

Both nuclear and cytoplasmic localization are required for SHR movement. The finding that promoting either nuclear or cytoplasmic localization at the expense of the other inhibits movement suggests that a balance between nuclear import and export must be maintained for continued movement. The movement of many endogenous animal proteins that traffic by non-conventional secretion, including thioredoxin, galectin, HMGB1 and homeodomain proteins is also correlated with nuclear localization (Prochiantz and Joliot, 2003; Tassetto *et al.*, 2005). Secretion of the EN2 homeodomain protein requires both nuclear import and nuclear export. The significance of subcellular localization for EN2 protein movement is not known. However, Prochiantz and Joliot (2003) have speculated that specific modifications occur in the nucleus, which confer competence to move. They have also suggested that nuclear localization could promote intercellular trafficking by bringing the cargo proteins into proximity with factors that facilitate movement or by allowing access to the secretory compartment. These are possible explanations for the correlation that we see between SHR subcellular localization and movement.

While these data collectively suggest parallels between SHR movement and secretion of homeodomain proteins (such as KN1 and EN2), we have no evidence to suggest that SHR moves through an unconventional secretory pathway. Similarities between transport via plasmodesmata and nuclear–cytoplasmic transport have long been suggested (Lee *et al.*, 2000; Lucas, 1995; Lucas *et al.*, 1995), as has a possible mechanistic link between nuclear localization and movement (Oparka, 2004). This is not to suggest that the NLS equates to a plasmodesmata-targeting signal, as nuclear-localized proteins clearly do not traffic between cells and we were able to diminish SHR movement by reducing nuclear localization without disrupting the nuclear localization sequences. However, the correlation between nuclear localization and cell-to-cell movement may provide clues as to the proteins and processes involved in intercellular transport of non-cell-autonomous proteins *in planta*, and perhaps movement through plasmodesmata.

### Model for SHR movement

Our previous data suggested a model for SHR movement in which cytoplasmically localized SHR protein was selectively trafficked between cells, based upon sequences within the SHR protein. These data further suggested that cytoplasmic localization of SHR was necessary but not sufficient for SHR movement (Gallagher *et al.*, 2004). Our current data, together with recent findings by Cui *et al.* (2007), allow us to expand upon this model to reflect a requirement for nuclear accumulation of SHR in cell-to-cell movement. However, it appears that there needs to be a balance between nuclear import and nuclear export, as movement is inhibited by either a strong NLS (nuclear trapping) (Cui *et al.*, 2007; Gallagher *et al.*, 2004) or loss of nuclear accumulation. These results suggest that nuclear accumulation of SHR may in some way confer onto SHR a movement-promoting modification that is then recognized in the cytoplasm. The requirement for multiple domains in the nuclear localization of SHR indicates the likelihood that protein–protein interaction is required for nuclear localization of SHR. In the endodermis, SCR is involved in nuclear accumulation of SHR, and therefore plays a role in regulating SHR movement (Cui *et al.*, 2007). Thus nuclear localization of SHR appears to play both positive and negative roles in SHR trafficking. As we discover the proteins that regulate the subcellular localization of SHR, and perhaps the downstream targets of SCR, we will learn more about the factors that control intercellular protein movement.

## Experimental procedures

### Construction of transgenes

Standard molecular biology techniques were used for all cloning procedures. The *SHR* promoter, *SCR* promoter and NLS–GFP constructs used were as previously described (Gallagher *et al.*, 2004; Nakajima *et al.*, 2001). mGFP5 was used for translational fusions not requiring an additional NLS (Haseloff *et al.*, 1997). All GFP fusions are to the N-terminus of mGFP5. To aid in cloning, a *Bam*HI site was added to the 5' end of mGFP5 that encodes an aspartate–proline–glycine linker. The NES is identical to that used by Haasen *et al.* (1999). For all constructs containing *SHR*, a modified coding sequence was used that silently eliminates the internal *Spe*I and *Sac*I sites. In addition, an *Spe*I site was created just upstream of the ATG, and the stop codon was replaced in-frame with a *Bam*HI site (Nakajima *et al.*, 2001). The junctions of the deletion and fusion constructs are as shown in Figure 3 and Figure 6. Specific information about the primer sequences used is available upon request.

### Plant materials, transformation and growth conditions

The *shr* and *scr* lines used have been described previously (Nakajima *et al.*, 2001; Paquette and Benfey, 2005; Sena *et al.*, 2004). All lines are in the Columbia background. Plants used for confocal imaging were grown vertically in Percival growth chambers (<http://www.percival-scientific.com>) on MS medium containing 1.0% sucrose and 1.0% granulated agar (BBL, <http://www.bd.com>). All plant transformations were performed using standard dipping protocols.

### Confocal microscopy

All confocal images were obtained on 4–7-day-old roots using either a Zeiss LSM-510 microscope (<http://www.zeiss.com/>) or a Leica SP2 microscope (<http://www.leica-microsystems.com>) equipped with an argon–krypton ion laser with the appropriate filter sets for visualizing GFP and propidium iodide. Prior to visualization, the seedlings were incubated for 0.5–2 min in 0.01  $\mu\text{g ml}^{-1}$  propidium iodine in  $\text{H}_2\text{O}$ . Images were collected using a 60 $\times$  water immersion lens. Root cross-sections were obtained in line-imaging mode on the Zeiss LSM-510.

Percentage movement was calculated by comparing the ratio of GFP levels in the endodermis to levels in the stele, and then comparing these ratios between modified and unmodified SHR. These calculations were performed using root images with less than 5% saturated pixels in the GFP channel. In some cases, roots with more than 5% saturated pixels in the GFP channel are shown to make visualizing the signal in the endodermis easier. However, these images were not used to calculate the percentage movement.

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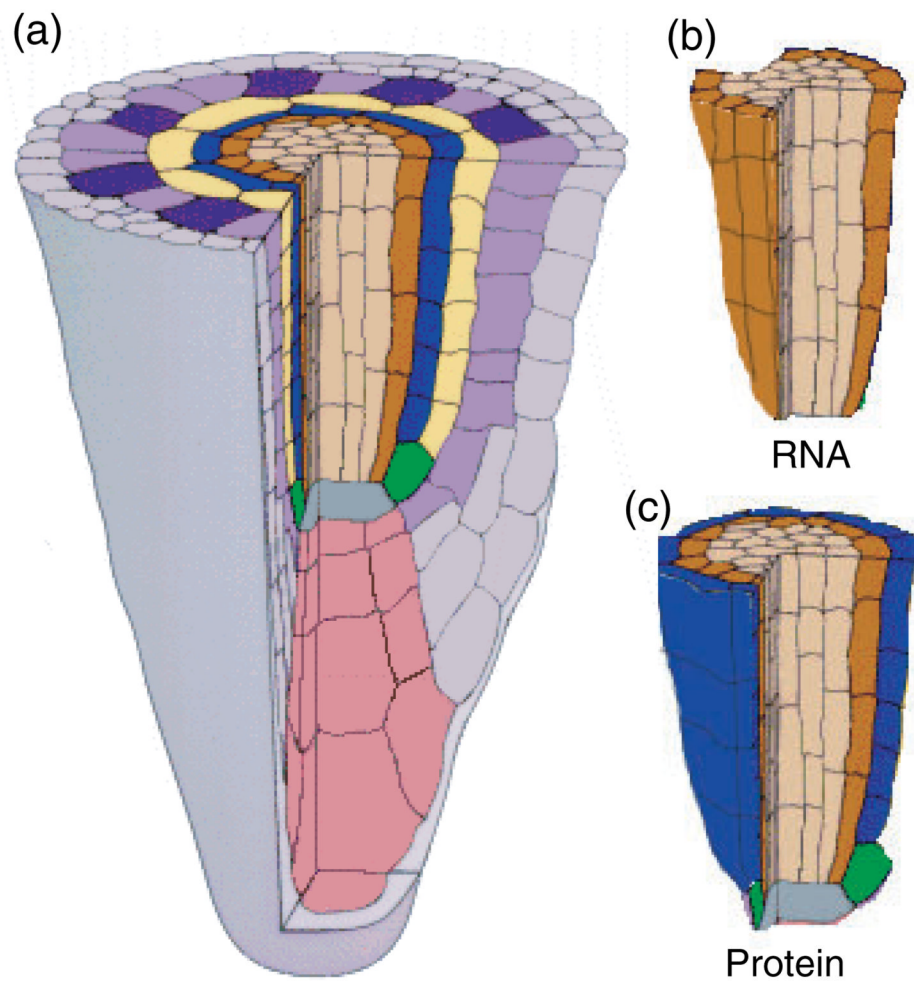
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 Cortical endodermal initials	 Quiescent center cells
Stele	 Vasculature  Pericycle
	Ground tissue
	 Cortex  Endodermis
 Lateral root cap	 Columella
	 Epidermal cells

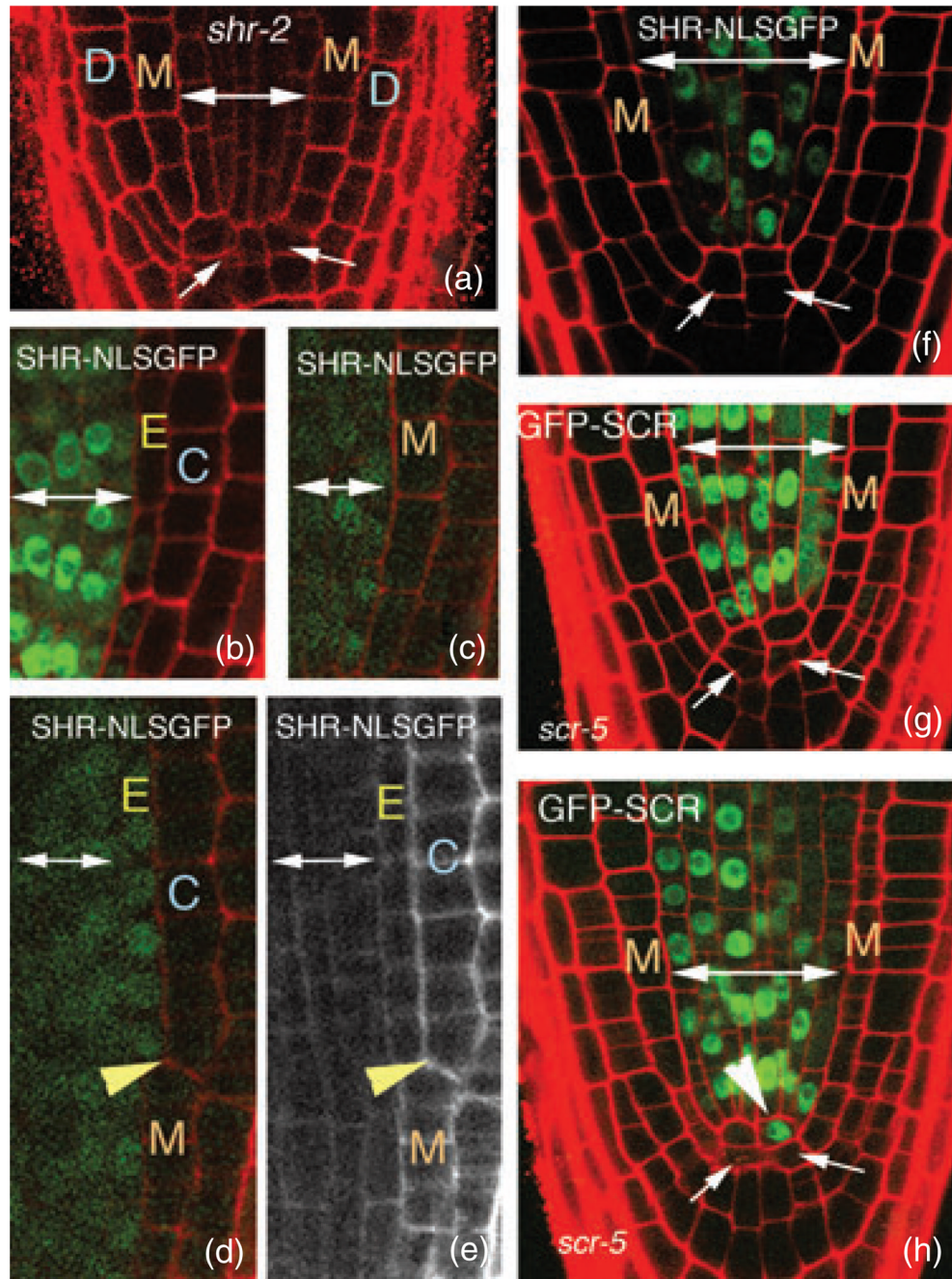
**Figure 1. Diagram illustrating SHR RNA and protein localization in the Arabidopsis root meristem**

(a) Cut-away view of the Arabidopsis root tip.

(b) The cells in which *SHR* RNA is detected.

(c) The cells in which *SHR* protein is detected.

This figure is modified from Benfey and Scheres (2000).



**Figure 2. Cell-autonomous SHR does not rescue *shr-2***

(a) Longitudinal confocal section through the tip of a *shr-2* root. Arrows indicate abnormal QC morphology.

(b) Longitudinal confocal section through the tip of a wild-type root expressing  $SHR_{pro}$ - $SHR$ - $NLSGFP$ .

(c-e) Longitudinal confocal micrographs of *shr-2* plants expressing  $SHR_{pro}$ - $SHR$ - $NLSGFP$ . (c) The cell-autonomous  $SHR$ - $NLSGFP$  protein is unable to rescue either the radial patterning or QC morphology. However, in (d), rescue of radial patterning is coincident with movement of the  $SHR$ - $NLSGFP$  protein into the ground tissue. The yellow arrowheads in (d) and (e) indicate the wall between the endodermis and cortex. Notice that above the yellow arrowhead

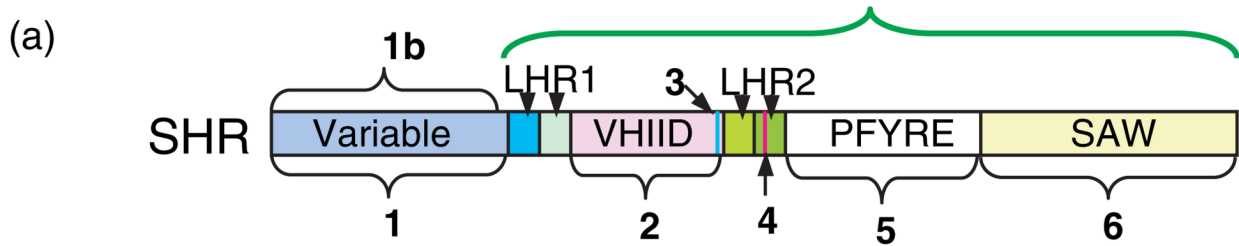


there are two distinct ground-tissue layers, and SHR-NLSGFP is clearly detected in the endodermal layer. (e) The red-channel image of the root in (d) is shown in gray scale to allow easier visualization of the rescue of the endodermal layer.

(f) *shr-2* root expressing  $SHR_{pro}$ -SHR-NLSGFP. This root has aberrant radial patterning and QC morphology, but normal root length despite a lack of detectable GFP in the QC cells.

(g,h) *scr-5* roots expressing  $SHR_{pro}$ -GFP-SCR. Expression of SCR in the stele does not rescue the *scr-5* radial patterning defects, but these roots do have normal lengths. In (h), there is an indication of divisions at the base of the stele (arrowhead) above the abnormal QC (arrows) forming a population of cells that morphologically resemble a new QC.

Double-headed arrows delineate stele cells throughout. M, mutant ground-tissue layer; D, epidermis; E, endodermis; C, cortex.



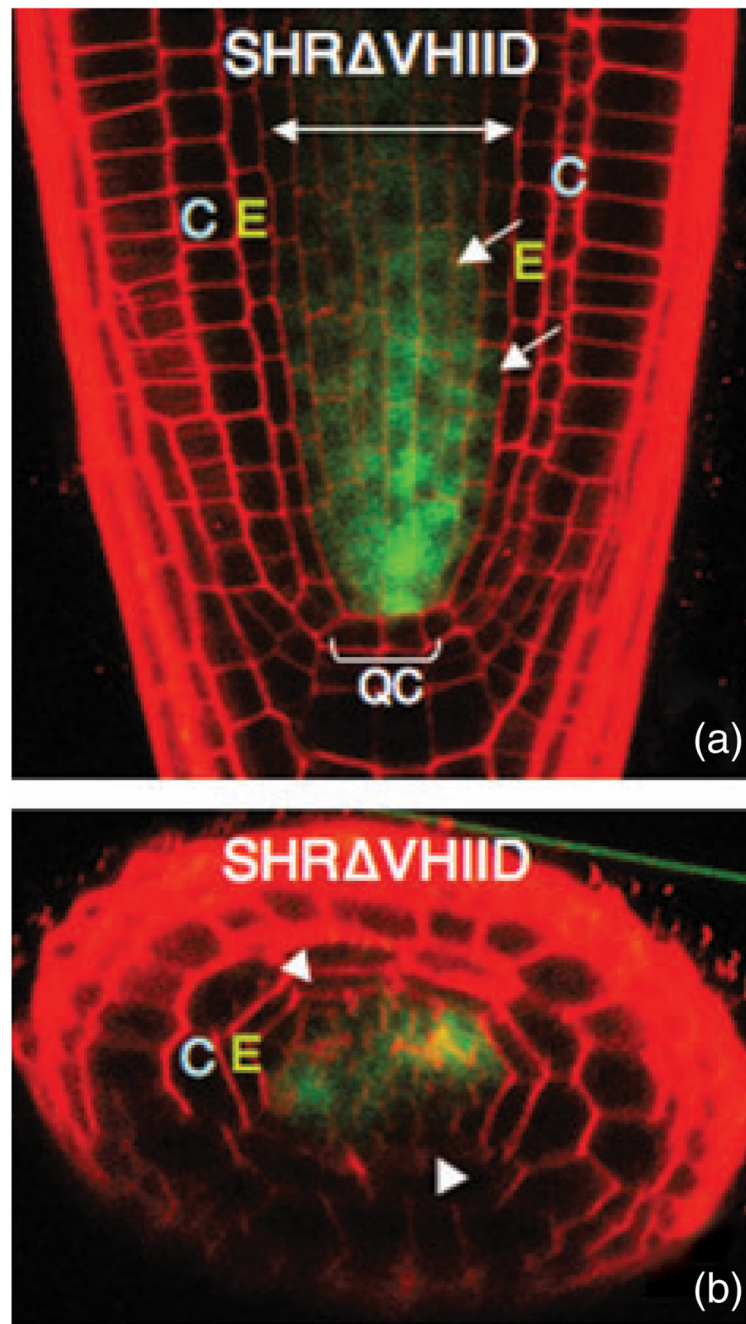
(b)

ID	GFP	Accumulation		Protein moves	Active
		Nuc	Cyt		
SHR (unmodified)	Yes	Yes	Yes	Yes	Yes
SHR $\Delta$ Nta [-1] 1-162	No				
SHR $\Delta$ NTb [-1b] 1-139	No				
SHR $\Delta$ SAW [-6] 451-531	No				
<b>SHR<math>\Delta</math>VHIID [-2] 222-294</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>N/D</b>
<b>SHR<math>\Delta</math>PFYRE [-5] 350-454</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>No</b>
<b>SHR T289 [3] 289</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>No</b>
<b>SHR<math>\Delta</math>LNELDV [-4] 341-346</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>

**Figure 3. SHR deletion constructs**

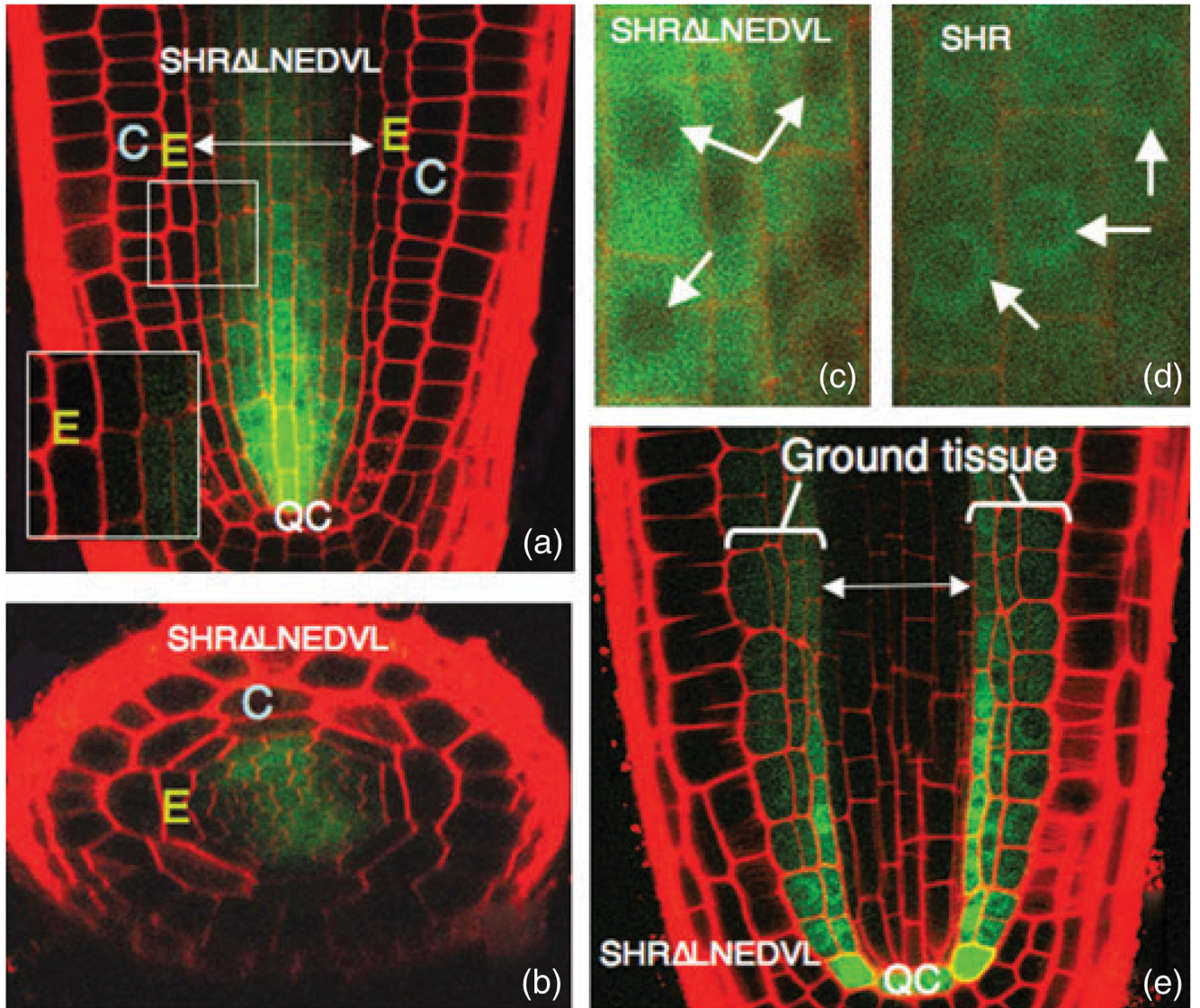
(a) Diagram of the domains deleted in SHR for analysis of SHR movement and activity. Arrow 3 indicates the single conserved threonine (T289) in the VHIID domain, which we previously showed was required for movement. Arrow 4 indicates the LNELDV motif. The green bracket shows the region designated as the SHR GRAS domain.

(b) Summary of the results of the deletion analysis. The bracketed numbers in the ID column refer to the numbered domains in (a). The numbers in italics refer to the amino acids deleted or mutated in each construct. Entries in bold show disruptions in both nuclear accumulation and movement.



**Figure 4. The SHR VHIID domain is required for SHR movement**

(a) Longitudinal and (b) transverse confocal sections through a wild-type root expressing *SHR<sub>pro</sub>-SHRΔVHIID-GFP*. The mutant protein is not excluded but does not accumulate in the nuclei of stele cells (arrows). No protein is detected in the endodermis. Arrowheads in (b) indicate the endodermal cells. E, endodermis; C, cortex; QC, quiescent center.



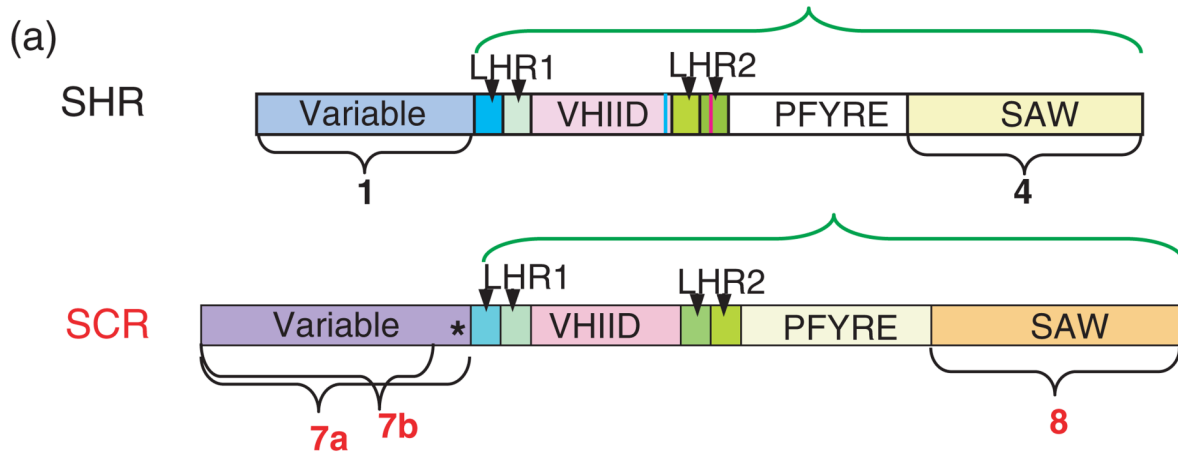
**Figure 5. Mutation of the LNEDLV residues within LHR2 (arrow 4 in Figure 3a) results in loss of SHR movement, but not SHR function**

(a) Longitudinal and (b) transverse confocal sections through a wild-type root expressing *SHR<sub>pro</sub>-SHRΔLNEDLV-GFP*. No protein is detected in the endodermis.

(c,d) Compared to wild-type SHR in (d), the mutant *SHRΔLNEDLV* protein in (c) does not accumulate in the nuclei (indicated by arrows) of stele cells.

(e) When expressed directly in the endodermis from *SCR<sub>pro</sub>*, the *SHRΔLNEDLV* protein increases the number of ground-tissue layers in a manner consistent with wild-type SHR function.

E, endodermis; C, cortex; QC, quiescent center.



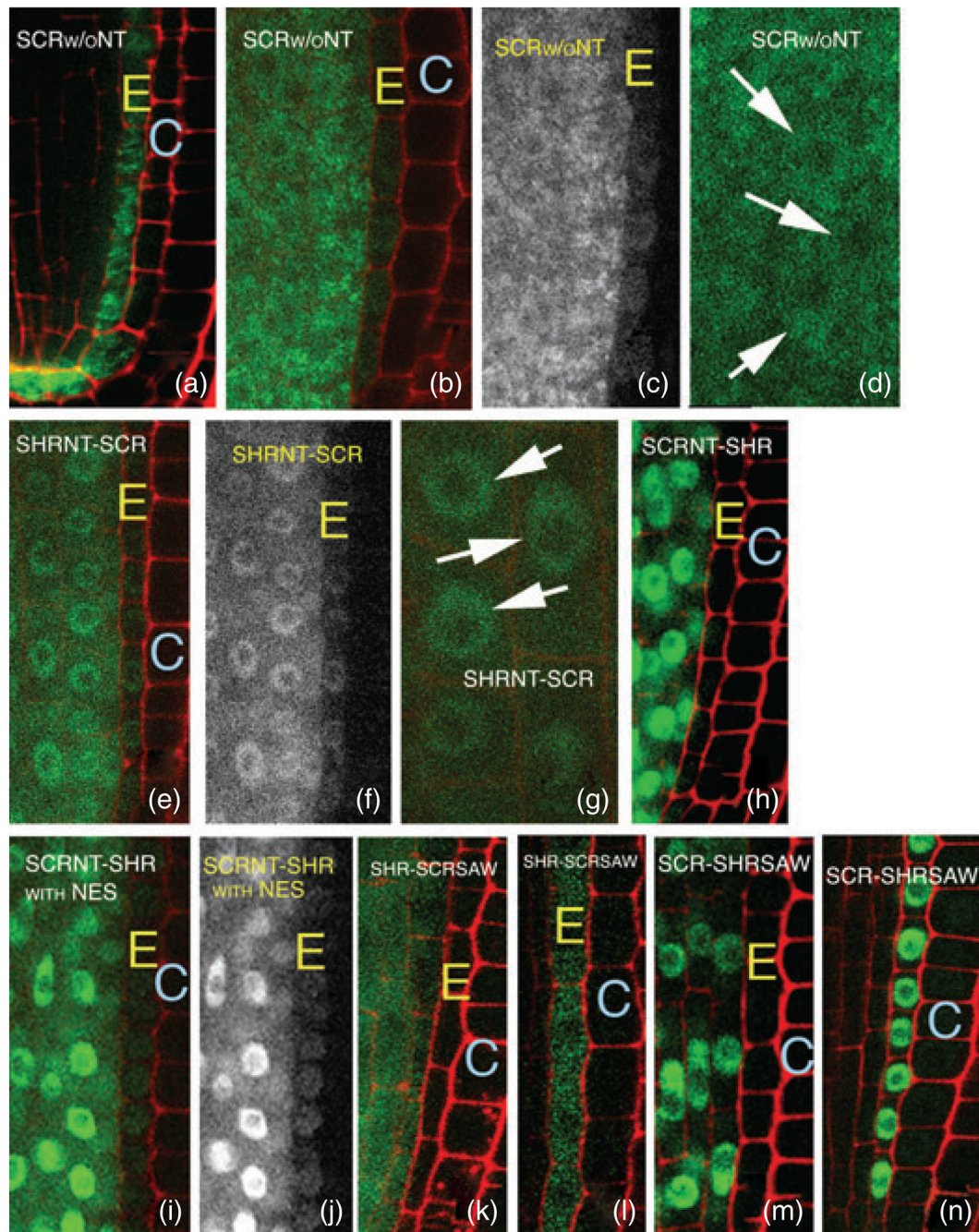
(b)

ID	GFP	Accumulation		Protein moves
		Nuc	Cyt	
SCR (unmodified)	Yes	Yes	No	No
SCRΔNTa [-7a] 1-282	Yes	Yes	Yes	Yes (51%)
SCRΔNTb [-7b] 1-256	Yes	Yes	Yes	Yes (53%)
SCRΔSAW [-8] 581-653	Yes	Yes	No	No
SHRNT-SCR [7b replaced by 1]	Yes	Yes	Yes	Yes (44%)
SCRNT-SHR [1 replaced by 7b]	Yes	Yes	No	Limited
SCRNT-SHR [1 replaced by 7b]+NES	Yes	Yes	Yes	Yes (46%)
<b>SHR-SCRSAW [4 is replaced by 8]</b>	<b>Yes</b>	<b>No</b>	<b>Yes</b>	<b>No</b>
SCR-SHRSAW [8 is replaced by 4]	Yes	Yes	No	No

**Figure 6. Key to the domain swaps**

(a) Diagrams of SHR and SCR proteins indicating relevant domains. In both, the GRAS domains are indicated by a green bracket and include the entire protein minus the N-terminus. The asterisk in SCR represents the predicted NLS.

(b) Summary of the results of the SCR deletions and domain swaps between SHR and SCR. The bracketed ID numbers refer to the numbered domains in (a). The numbers in italics refer to the amino acids deleted or mutated in each construct. SCR is indicated in red and SHR in black. The entry in bold shows disruption in both nuclear accumulation and movement. The percentages listed in the column 'Protein moves' are relative to SHR-GFP movement in the wild-type background in the root meristem.



**Figure 7. The SHR and SCR GRAS domains are necessary for movement**

(a–d) Expression of the SCR GRAS domain in the endodermis (a) and the stele (b–d). Loss of the SCR N-terminus independent of the predicted NLS results in localization of the SCR GRAS protein throughout the cell and movement of the protein from the stele into the endodermis when expressed from *SHR<sub>pro</sub>* (as in b–d). (c) GFP channel only from (b) to facilitate visualization of the GFP signal in the endodermis. (d) Higher-resolution image of localization of the SCR GRAS protein in stele cells. Arrows indicate nuclei.

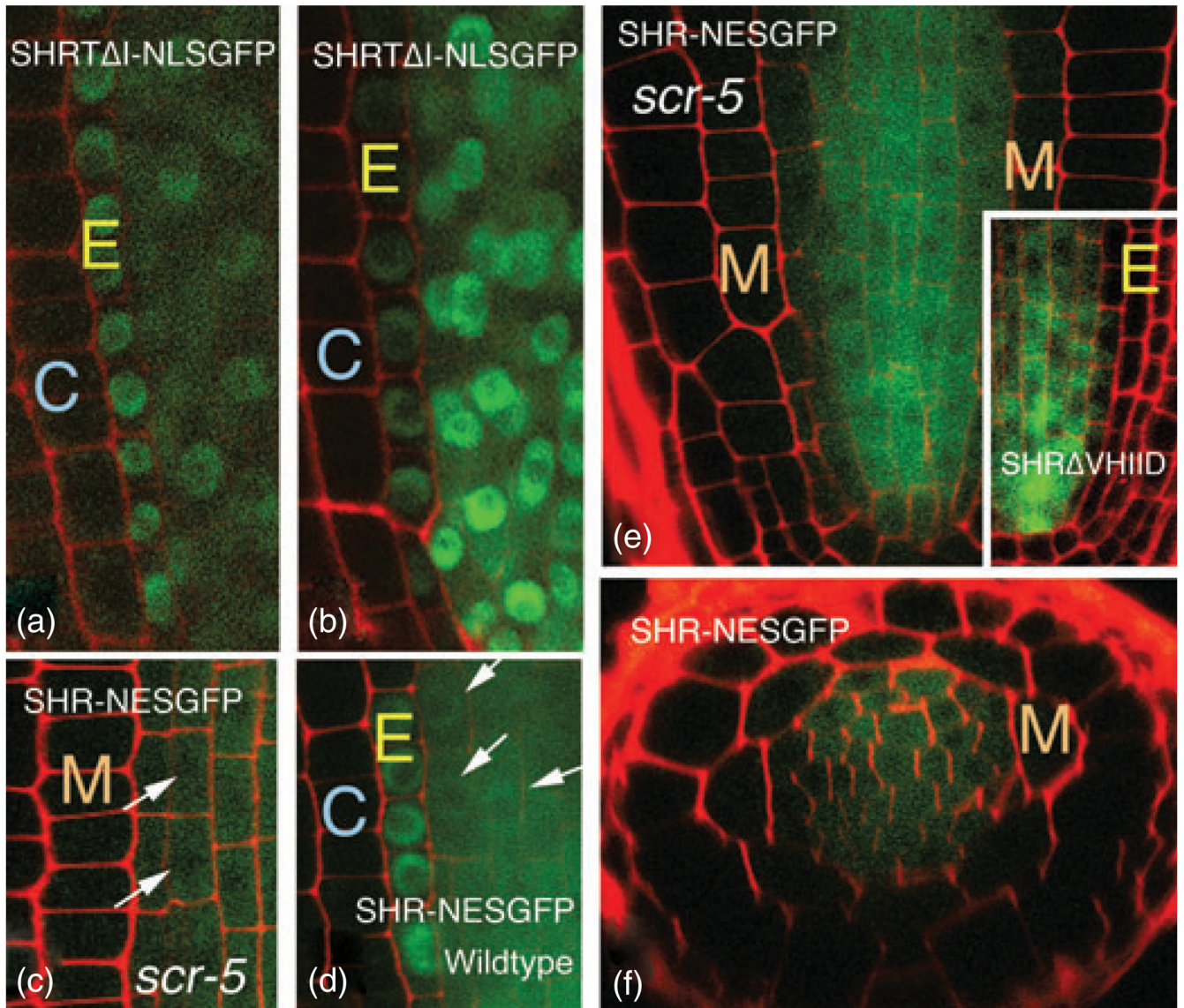
(e–g) Replacement of the SCR N-terminus with the SHR N-terminus (designated 7b/1 in Figure 6) results in both nuclear and cytoplasmic localization in the stele without increasing the efficiency of movement of the SCR GRAS domain [see (b) for comparison]. (f) GFP channel

only from (e) to facilitate visualization of the GFP signal in the endodermis. (g) Higher-resolution image of localization of the SHRNT–SCR protein in stele cells. Arrows indicate nuclei.

(h–j) Exchange of the SHR N-terminus with the SCR N-terminus (designated 1/7b in Figure 6) independent of the predicted NLS leads to nuclear localization of the chimeric protein (h). Addition of an NES (i,j) allows movement of the SCR–SHR chimera. (j) GFP channel only from (i) to facilitate visualization of the GFP signal in the endodermis.

(k,l) Expression of the SHR–SCRSAW protein (designated 8/4 in Figure 6) from the SHR (k) and SCR promoters (l). Replacement of the SHR SAW domain with the SCR SAW domain results in mislocalization of SHR in the stele (k) and the endodermis (l), and loss of movement from the stele (k).

(m,n) Replacement of the SCR SAW domain with the SHR SAW domain (designated 4/8 in Figure 6) does not affect subcellular localization in either the stele (m) or the endodermis (n), indicating that the SHR SAW domain does not represent an autonomous movement domain. E, endodermis; C, cortex. For all images, expression in the stele is achieved using the *SHR* promoter and expression in the endodermis is achieved using the *SCR* promoter.



**Figure 8. Nuclear accumulation correlates with the ability to move**

(a,b) Movement of the T289 mutation is rescued by addition of an NLSGFP construct. Two different roots expressing the *SHR<sub>pro</sub>-SHRAT289-NLSGFP* construct are shown. GFP is detected in the endodermis in both roots.

(c-f) Expression of *SHR<sub>pro</sub>-SHR-NESGFP* in *scr-5* resulted in SHR localization similar to that for *SHR<sub>pro</sub>-SHRΔVHIID* [for comparison, see inset in (e)] and a reduction in SHR movement from the stele into the endodermis. The effect of the NES on SHR localization is more pronounced in *scr-5* (c,e,f) than in wild-type plants (d).

M, mutant ground-tissue layer; E, endodermis; C, cortex. Arrows indicate nuclei.