Current Biology 21, 1559–1564, September 27, 2011 ©2011 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2011.08.013

Report

An Essential Protein that Interacts with Endosomes and Promotes Movement of the SHORT-ROOT Transcription Factor

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Summary

Plant cells can communicate through the direct transport of transcription factors [1–7]. One of the best-studied examples of this phenomenon is SHORT-ROOT (SHR), which moves from the stele cells into the endodermis and root tip of *Arabidopsis*, where it specifies endodermal cell identity and stem cell function, respectively [8–10]. In the endodermis, SHR upregulates the transcription factors SCARECROW (SCR) [2] and JACKDAW (JKD), which in turn inhibit movement of SHR from the endodermis [11]. Although much is known about the regulatory pathways that mediate expression and activity of SHR [1, 8–14], little is known about the factors that promote its movement or the movement of other transcription factors. We have identified a novel protein, SHORT-ROOT INTERACTING EMBRYONIC LETHAL (SIEL), that interacts with SHR, CAPRICE (CPC), TARGET OF MONOPTEROUS 7 (TMO7), and AGAMOUS-LIKE 21 (AGL21). Null alleles of SIEL are embryonic lethal. Hypomorphic alleles produce defects in root patterning and reduce SHR movement. Surprisingly, both SHR and SCR regulate expression of SIEL, so that *siel/scr* and *siel/shr* double mutants have extremely disorganized roots. SIEL localizes to the nucleus and cytoplasm of root cells where it is associated with endosomes. We propose that SIEL is an endosome-associated protein that promotes intercellular movement.

Results and Discussion

In order to identify proteins that promote trafficking, we used SHORT-ROOT (SHR) as bait in a yeast two-hybrid screen that identified approximately 30 proteins. The At3g08800 protein was chosen for further analysis, because it is a single gene in *Arabidopsis* and it is predicted to contain both ARM (Armadillo) and HEAT (Huntington, elongation factor 3, PR65/A, TOR) domains [15]. Many HEAT-domain-containing proteins are involved in intracellular protein transport pathways (http://www.arabidopsis.org). Direct interaction of SHR with At3g08800 was verified using protein complementation assays (bimolecular fluorescence complementation [BiFC]; see Figure S1 available online). Based on its mutant phenotype, we named this gene *SIEL* for *SHORT-ROOT INTERACTING EMBRYONIC LETHAL*.

To determine the biological function of *SIEL*, we obtained and analyzed four alleles of At3g08800 (Figure 1; Figure S2). Homozygosity for either the *siel-1* or *siel-2* allele resulted in embryonic lethality (Figures S1D–S1F). Plants heterozygous for *siel-1* or *siel-2* were indistinguishable from wild-type. Approximately 60% of the *siel-3* homozygotes arrested during embryo development (Figures S1G–S1J). The remaining 40% had a very poorly developed primary root (Figure 1A) and generally died within 10 days of germination. *siel-4* plants had short roots (Figure 1A) and ectopic root hairs (Figures 1B and 1C). The aerial portions of the plant appeared normal (Figure S1K).

The Anatomy of *siel-3* and *siel-4* Roots

To determine whether the *siel* mutations affect patterning of the root meristem, we examined *siel-3* and *siel-4* seedlings. Wild-type roots contain single layers of endodermis and cortex (collectively the ground tissue; Figures 1D and 1H; Figure S2E), each of which is composed of eight cells (Figure 1G). They also have a well-defined quiescent center (QC) (Figure 1H; Figure S2E) [16]. *shr-2* mutants have a single ground tissue layer and show ectopic divisions in the presumptive QC, which correlate with cessation of root growth [9, 10]. In contrast, plants that are heterozygous for *shr-2* show ectopic cell divisions in the ground tissue that lead to extra cells in each ground tissue layer, as well as extra cell layers (Figure 1I). Examination of the primary roots of *siel-3* (Figures 1E and 1J) and *siel-4* homozygotes (Figures 1F, 1G, and 1K) revealed changes both in the number of ground tissue layers and in the number of cells in the layer. The defects in the homozygous *siel-4* roots were similar to *shr-2* heterozygotes (Figure 1I). Transverse sections through *siel-3* and *siel-4* roots confirmed that hair cells formed in non-hair-cell positions (Figures 1E and 1F). *siel-3* and *siel-4* mutants also showed aberrant divisions in the presumptive QC. In *siel-3* plants, there was a general disorganization of all cell layers of the root tip. The patterning defects were variable in both *siel-3* and *siel-4*, with *siel-3* plants always more severe than *siel-4*.

SHORT-ROOT Movement Is Affected in *siel* Mutants

The phenotype of the *siel-4* mutants is consistent with a reduction in SHR activity (Figure 1I). To test whether SIEL plays a role in SHR activity by affecting its movement, we examined SHR-GFP [4, 10] in *siel-3* and *siel-4* mutant backgrounds. The aberrant patterning in *siel-3* plants made it difficult to assess SHR movement. However, SHR signal was either absent or reduced in the presumptive endodermis (Figure 2A). In *siel-4* plants, SHR-GFP was absent or reduced in regions of the endodermis that correlated with aberrant cell divisions (Figures 2B and 2D; Figure S3A). This lack of signal was not due to differences in the location of the nuclei because we took serial sections through the root and did not detect signal in these endodermal cells. Nor can this lack of signal in the endodermis be attributed to reduced SHR expression in the *siel-4* mutant background, because the level of SHR-GFP expression in stele cells based upon GFP fluorescence and real-time qRT-PCR did not differ from wild-type (data not shown).

Although short, approximately 40% of *siel-4* roots had wild-type or nearly wild-type patterning of the meristem. When we looked at SHR-GFP in these roots, there was strong signal in the endodermis (Figure 2F). To quantify the SHR-GFP signal in these roots, we took multiple serial confocal images in which the nuclei of endodermal cells were clearly visible

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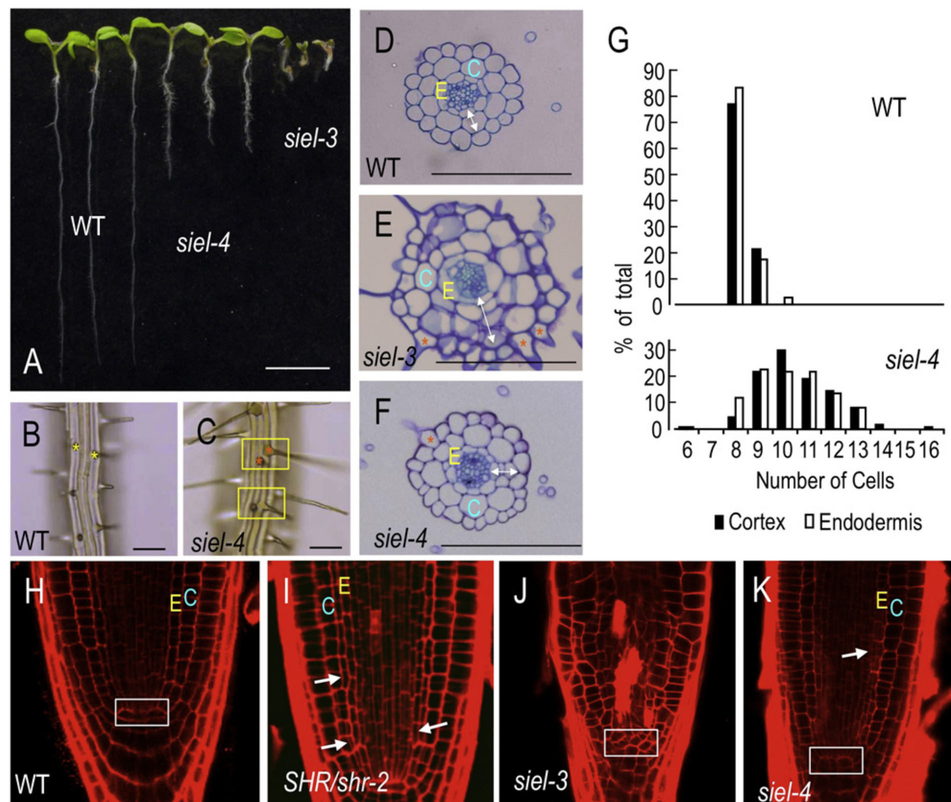

Figure 1. *SIEL* Is Required for Normal Patterning of the *Arabidopsis* Root

(A) Morphology of wild-type (WT; Col-0), *SHORT-ROOT INTERACTING EMBRYONIC LETHAL* (*siel*)-*4*, and *siel-3* seedlings.

(B and C) Close-up images of WT (B) and *siel-4* (C) root hairs (yellow and orange asterisks, respectively). Root hairs are often found in immediately adjacent epidermal files in *siel-4* (yellow boxes), whereas in WT there is an intervening epidermal cell layer.

(D–F) Cross-sections through WT (D), *siel-3* (E), and *siel-4* (F) roots stained with toluidine blue. Orange asterisks mark abnormally positioned root hairs. Double white arrows mark the extent of the ground tissue, which in WT is confined to two cell layers.

(G) Quantification of the number of cells in the cortex and endodermis in WT and *siel-4* roots.

(H–K) Longitudinal confocal cross-sections through the tips of WT (H), *SHORT-ROOT* (*SHR*)/*shr-2* (I), *siel-3* (J), and *siel-4* (K) roots that were stained with propidium iodide. Arrows indicate abnormal or ectopic cell divisions. The boxed areas in (H), (J), and (K) correspond to the presumptive quiescent center (QC) along with the cortical endodermal initials. The following abbreviations are used: E, endodermis; C, cortex. Scale bars represent 0.5 cm in (A) and 100 μm in (B)–(F).

and compared them to wild-type (Figure 2E). We used these images to calculate the ratio of GFP signal in the endodermis as compared to the stele [3]. Using these ratios, we found that even in *siel-4* roots with normal patterning, the SHR-GFP endodermis to stele ratio was only 80% of wild-type (t test $p = 1.8 \times 10^{-6}$). These results are consistent with a decrease in SHR movement. To directly test this, we selected *siel-4* roots with normal or nearly normal root patterning and quantified fluorescent recovery after photobleaching. The recovery of SHR-GFP fluorescence in the *siel-4* roots was only 40% of that seen in wild-type roots ($p = 0.009$; Figure 2G; Figure S3B), indicating that even in *siel-4* seedlings with normal root morphology, SHR movement is reduced.

Double Mutants of *siel-4* and Either *shr-2* or *scr-4* Show Synthetic Defects in Root Development

To test for genetic interactions between *SIEL* and *SHR*, *SCARECROW* (*SCR*), or *JACKDAW* (*JKD*) transcription factors, we generated double mutants. Null mutations in either *SCR* or *SHR* result in short roots that lack a ground tissue layer (Figures 3E and 3G) [9, 17]. Mutations in *JKD* cause extra cell divisions in the ground tissue (Figure 3C) and produce a phenotype similar to but less severe than *siel-4* (Figure 3B) [11, 18]. Double mutants between *siel-4* and *jkd-4* (Figure 3D) were indistinguishable from *siel-4* single mutants (Figure 3B), indicating that *siel-4* is epistatic to *jkd-4*. In contrast, the roots of the *siel-4 scr-4* (Figures 3F, 3I, and 3J) and the *siel-4 shr-2* (Figures 3H, 3K, and 3L) double mutants were significantly more abnormal than either of the single mutants alone. These defects were not restricted to the ground tissue and the stele; instead, all cell layers were affected. The *shr-2 siel-4* individuals with the most severe phenotype lacked all internal cell layers (Figures 3K and 3L) and had determinate roots as evidenced by formation of root hairs very close to the root tip (Figure 3K). These results suggest synthetic interactions between *siel-4* and *shr-2* and between *siel-4* and *scr-4*.

Because the roots of the *siel-4 shr-2* and *siel-4 scr-4* double mutants resembled those with a strong *siel-3* phenotype, we asked whether SIEL expression is regulated by SHR and/or SCR. Indeed, in both the *shr-2* and *scr-4* background, *SIEL* mRNA was significantly reduced (>4-fold) compared to wild-type (Figure S2D). As a control, we also measured *SIEL* mRNA levels in *jkd-4* mutants and saw no effect on *SIEL* expression. These results indicate that *SIEL* expression is downstream of SHR and SCR but not JKD.

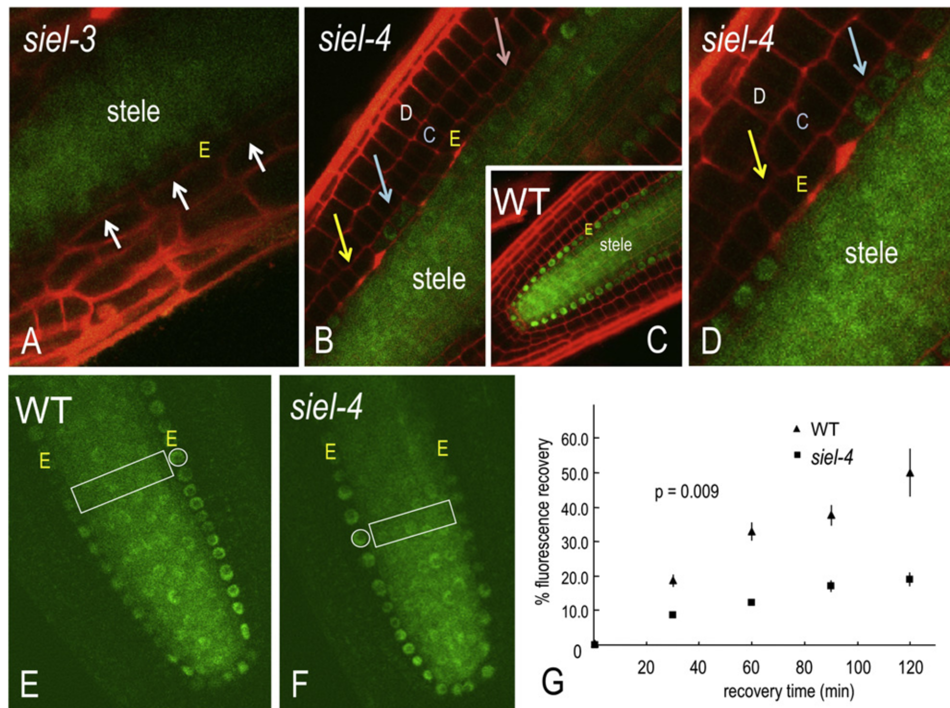

Figure 2. *siel* Mutants Have Reduced Movement of SHR-GFP

(A–F) Longitudinal confocal sections of 5-day-old, *siel-3* (A), *siel-4* (B, D, and F), and WT (C and E) roots expressing *pSHR::SHR:GFP*. In all panels, arrows point to the endodermis. (B) and (D) are sections through the same *siel-4* root at different magnifications. With the exception of the endodermal cells indicated by the light blue arrow, there is little or no SHR-GFP in this endodermal cell file. The colored arrows in (B) and (D) correspond to the position of the cross-sections in Figure S4A.

(E and F) SHR-GFP in a wild-type root (E). The root shown in (F) had a very weak *siel-4* phenotype, with mild defects in the QC. Roots of the type shown in (E) and (F) were used for calculation of the ratio of endodermal (circles) to stele (rectangle) signal to quantify movement [3]. Multiple measurements were made for each root and more than 20 roots of each genotype were used for these calculations.

(G) Fluorescent recovery after photobleaching of the SHR-GFP signal in the endodermis of WT and *siel-4* roots. Error bars show standard error.

SIEL Interacts with *SHR* and Additional Non-Cell-Autonomous Proteins

Because the phenotype of the *shr-2 siel-4* double mutants is not restricted to cells that contain SHR, SIEL must affect other root expressed proteins. CAPRICE (CPC) [5, 19], TARGET OF MONOPTEROUS 7 (TMO7) [20], and five additional transcription factors [6, 21, 22] move between cells in the root. In addition, the transcription factors SCR, JKD, and MAGPIE (MGP) interact with SHR [2, 11] in part to regulate movement of SHR. JKD also has non-cell-autonomous functions in root development [18]. To assess whether SIEL interacts with any of these proteins, we performed BiFC assays [23] (Figure S1B; Table S1). SIEL interacted with proteins known to regulate SHR movement: MGP, SCR, and JKD, as well as several non-cell-autonomous transcription factors: CPC, TMO7, and AGAMOUS-LIKE 21 (AGL21). However, SIEL did not interact with LEAFY (LFY), which diffuses between cells in the shoot meristem [24]. Nor does it interact with SHOOT MERISTEMLESS (STM), which is a member of the family of homeodomain transcription factors, some of which can move between animal cells in addition to plant cells [25].

SIEL Localizes to Nuclei and Endosomes

To determine how the SIEL protein promotes SHR movement, we expressed YFP-SIEL from the CaMV35S promoter in leaf epidermal cells of *Nicotiana benthamiana* (tobacco) and in *Arabidopsis* roots. In these cells, YFP-SIEL localized to the nucleus and the cytoplasm. In the cytoplasm we often saw localization of YFP-SIEL in vesicular structures. These vesicles were found outside of the nucleus and in the cytoplasm (Figure 4). Live imaging of these cells revealed rapid movement of YFP-SIEL (Movie S1A; Movie S1C). The protein was also mobile when complexed with SHR in BiFC assays (Movie S1B). To avoid potential mislocalization caused by overexpression of the YFP-SIEL transgene, we selected roots for analysis that had YFP signals below what we observed for expression of *pSHR:SHR-GFP*. In fact, the level of YFP-SIEL expression in the meristem of most roots was so low that localization was done using anti-GFP antibodies on fixed tissue (Figures 4C–4E; Figure S4C). To show that the *35S:YFP-SIEL* was functional, we used it to rescue *siel-4* roots (Figures S4A and S4B).

Short-term incubation of YFP-SIEL expressing roots with FM4-64 showed partial overlap between the YFP and FM4-64 signals suggesting that SIEL associates with endosomes (Figures 4F and 4G). To further characterize SIEL localization, we performed immunoelectron microscopy using anti-GFP antibodies on stele cells expressing YFP-SIEL. Outside of the nucleus, SIEL was most often associated with vesicles and the endoplasmic reticulum (ER) (Figure S4C). To further test whether SIEL associates with the endomembrane, we examined YFP-SIEL localization in seedlings expressing mCherry-tagged proteins that localize to various cellular compartments [26]. We found preferential localization of SIEL to endosomes. In particular, SIEL was associated with the RabA5d-mCherry

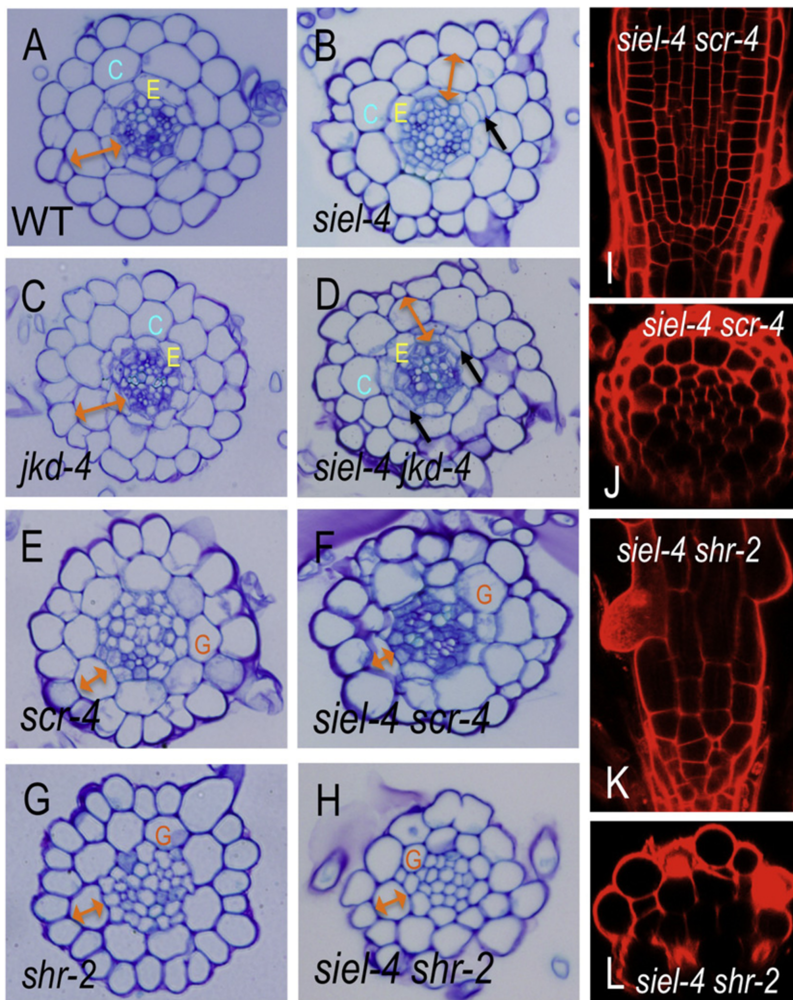

Figure 3. *shr-2* and *scr-4* Mutations Enhance the *siel-4* Phenotype

(A–H) Cross-sections through single and double mutants (as labeled).

(A)–(E) show representative images. (F) and (H) show moderate *siel-4 SCARECROW* (*scr*)*-4* and *siel-4 shr-2* double-mutant phenotypes. In particular, the *shr-2 siel-4* double mutants were difficult to section and prepare for light microscopy as a result of the short length and bending of the root. Therefore, the more severe individuals were analyzed by confocal microscopy as shown in (I)–(L). The following abbreviations are used: E, endodermis; C, cortex; G, ground tissue of undetermined identity. Arrows point to ectopic cell divisions. Double arrows indicate the extent of the ground tissue throughout.

marker, which localizes to endosomes/recycling endosomes (Figure 4H; Figure S4D).

Intercellular movement of transcription factors is essential for normal plant development. Here we identified an essential protein, SIEL, that promoted the movement of SHR and interacted with several unrelated non-cell-autonomous transcription factors. Interestingly, SIEL expression was regulated by both SHR and SCR. Because SCR is not expressed in stele cells, these findings suggest a non-cell-autonomous role for SCR in SHR movement (Figure S4E). Work by Sena et al. previously showed a non-cell-autonomous role for SCR in nuclear localization and movement of SHR in the epidermis, when SHR is expressed from the *WEREWOLF* promoter [7].

In the endodermis, SHR directly upregulates both *SCR* and *JKD*, which in turn restrict SHR movement [2, 11]. However, the finding that SHR promotes *SIEL* expression suggests that SHR may actively promote its own movement. In 5-day-old roots, the average SHR-GFP signal in the endodermis is ~1.5-fold the level in the stele (Figure 2E). This pathway could then provide a mechanism by which the SHR protein specifically increases its own concentration in the endodermis. As *shr-2* heterozygotes have ectopic cell divisions in the ground tissue, the concentration of SHR may be critical for proper function, similar to what was shown for the

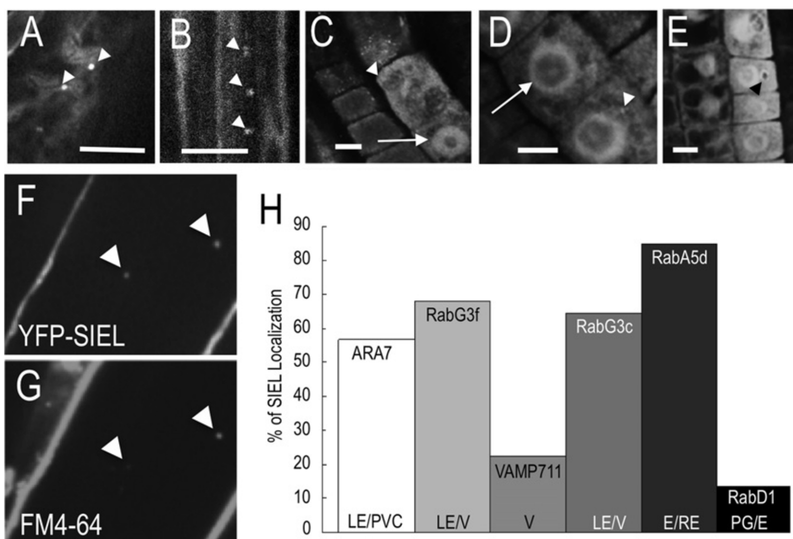

Figure 4. YFP-SIEL Localizes to the Nucleus and Cytoplasm of Root Cells Where It Associates with Endosomes

(A and B) Micrograph of YFP-SIEL (SHORT-ROOT INTERACTING EMBRYONIC LETHAL) localization in live WT roots showing epidermal (A) and stele cells (B).

(C–E) Localization of YFP-SIEL in cortex cells of fixed tissue using anti-GFP antibodies. Arrowheads indicate vesicle structures; arrows point to nuclei throughout. Scale bars for (A)–(E) represent 10 μm.

(F and G) YFP-SIEL (F) and FM4-64 (G) signal in the same cell of a wild-type root. Arrows point to the same structures in (F) and (G).

(H) Percentage of punctate YFP-SIEL signal that colocalized with the indicated markers. These calculations are based upon based upon images like those shown in Figure S4D. The following abbreviations are used: LE, late endosome; PVC, prevacuolar compartment; V, vacuole; E, endosome; RE, recycling endosome; PG, post Golgi.

PLETHORA (PLT) proteins in the root meristem, where high levels of PLT promote quiescence or stem cell fate and moderate levels induce mitosis [27].

The phenotypes of the *siel-4* double mutants and the strong *siel* alleles suggest that more than SHR movement is regulated by SIEL. Indeed, we found that SIEL can interact with several non-cell-autonomous transcription factors. Interaction of SIEL with CPC is consistent with the root hair patterning defects in the *siel* mutants. Likewise, double mutants between *siel-4* and *cpc* look like the *cpc* single mutants (data not shown), indicating that *siel-4* acts through *cpc* to affect root hair patterning.

The association of SIEL with endosomes suggests that endogenous plant proteins can interact with components of the endomembrane without being targeted to the secretory pathway. Various viral movement proteins associate with the ER, Golgi, or recycling endosome in order to target the plasmodesmata [28–31]. In animal cells, the endosome promotes intercellular movement of cargo proteins including ENGRAILED through a process of unconventional secretion [32–35]. We propose that SIEL, via its association with endodomes, acts as an intracellular shuttle that promotes SHR movement (Figure S4E). This model for SHR movement is akin to the "grab a Rab" model for movement of plant viral proteins that Oparka proposed [29] and the model proposed by Lewis and Lazarowitz in which viral movement proteins essentially "grab" endocytic and exocytic vesicle to access plasmodesmata [31].

Supplemental Information

Supplemental Information includes four figures, two tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at doi:10.1016/j.cub.2011.08.013.

Acknowledgments

We thank P.N. Benfey, in whose laboratory the original yeast two-hybrid screen was conducted, and R.S. Poethig and D. Weijers for comments on the manuscript. D. Weijers, I. Bilou, and B. Scheres provided seeds or DNA and shared unpublished data on protein interactions and protein movement. T. Wada provided the bait library. We thank T. Byford, K. Miyahara, R. Schlarp, and C. Nicoletti for technical assistance; A. Stout, L. Peachey, and T. Svitkina for microscopy expertise; and D. Wagner, R.S. Poethig, B.D. Gregory, and members of their laboratories for technical assistance and comments. The Salk Institute Genomic Analysis Laboratory provided the sequence-indexed *Arabidopsis* T-DNA insertion mutants. S.W. and K.K. are partially supported by National Science Foundation grant 0920327 awarded to K.L.G.

Received: May 20, 2011
Revised: July 13, 2011
Accepted: August 5, 2011
Published online: September 15, 2011

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