SEUSS Integrates Gibberellin Signaling with Transcriptional Inputs from the SHR-SCR-SCL3 Module to Regulate Middle Cortex Formation in the Arabidopsis Root[1][OPEN]

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A decade of studies on middle cortex (MC) formation in the root endodermis of Arabidopsis (*Arabidopsis thaliana*) have revealed a complex regulatory network that is orchestrated by several GRAS family transcription factors, including SHORT-ROOT (SHR), SCARECROW (SCR), and SCARECROW-LIKE3 (SCL3). However, how their functions are regulated remains obscure. Here we show that mutations in the *SEUSS* (*SEU*) gene led to a higher frequency of MC formation. *seu* mutants had strongly reduced expression of *SHR*, *SCR*, and *SCL3*, suggesting that SEU positively regulates these genes. Our results further indicate that SEU physically associates with upstream regulatory sequences of *SHR*, *SCR*, and *SCL3*; and that *SEU* has distinct genetic interactions with these genes in the control of MC formation, with *SCL3* being epistatic to *SEU*. Similar to *SCL3*, *SEU* was repressed by the phytohormone GA and induced by the GA biosynthesis inhibitor paclobutrazol, suggesting that SEU acts downstream of GA signaling to regulate MC formation. Consistently, we found that SEU mediates the regulation of *SCL3* by GA signaling. Together, our study identifies SEU as a new critical player that integrates GA signaling with transcriptional inputs from the SHR-SCR-SCL3 module to regulate MC formation in the Arabidopsis root.

The root of Arabidopsis (*Arabidopsis thaliana*) consists of four concentric rings of tissue that surround the central vascular tissue, namely the epidermis, cortex, endodermis, and pericycle, from the outside inwards (Benfey and Scheres, 2000). Each of these tissues has typically a single cell layer at early seedling stage. A middle cortex (MC) layer, however, will form as the root ages (Baum et al., 2002). This extra layer is produced via periclinal divisions of the endodermal cells situated a small distance basal from stem cells of the cortex and endodermis.

During the last decade, several interconnected mechanisms have been proposed to be responsible for the MC formation (Paquette and Benfey, 2005; Cui et al., 2007; Heo et al., 2011; Koizumi et al., 2012a; Koizumi et al., 2012b; Koizumi and Gallagher, 2013). Two GRAS family transcription factors, SHORT-ROOT (SHR; Helariutta et al., 2000) and SCARECROW (SCR; Di Laurenzio et al., 1996), have been shown to play crucial roles in shaping these mechanisms. *SHR* is transcribed in the vascular tissue and the pericycle, collectively known as the stele. SHR protein, which localizes to the cytoplasm and the nucleus in the stele, is able to move from the cytoplasm of the stele cells to the adjacent *SCR*-expressing layer (Nakajima et al., 2001; Gallagher et al., 2004), including stem cells of the cortex/endodermis, their daughter cells, the endodermis, and the quiescent center. SHR protein becomes exclusively localized to the nucleus in cells of the *SCR*-expressing layer, where SHR directly up-regulates *SCR* to limit its own movement via SCR-dependent nuclear sequestration, thereby preventing its further movement into the cortex

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and maintaining its levels in the endodermis (Cui et al., 2007; Koizumi et al., 2012a). High levels of SHR in the endodermis inhibit the formation of the MC, whereas intermediate levels of SHR, which occur physiologically during root development or if induced by a reduction or loss of SCR activity, promote MC formation (Koizumi et al., 2012b). GA function, which decreases in the meristem starting at 5 d postgermination under physiological conditions (Paquette and Benfey, 2005; Moubayidin et al., 2010; Heo et al., 2011), is thought to influence the abundance of SHR and subsequently the formation of MC (Koizumi et al., 2012b). In addition, GA has also been shown to repress *SCARECROW-LIKE3* (*SCL3*), a SHR and SCR target (Levesque et al., 2006; Cui et al., 2007) that encodes another GRAS family transcription factor and positively regulates GA-mediated control of MC formation (Heo et al., 2011). The repressive effect of GA on *SCL3* is most likely accomplished through GA-mediated degradation of DELLA domain GRAS family transcription factors, which are critical negative regulators of GA signaling and direct upstream activators of *SCL3* (Zentella et al., 2007; Heo et al., 2011).

SEUSS (*SEU*) encodes an Arabidopsis protein with a conserved central domain that shares sequence homology with the dimerization domain of the LIM-domain-binding transcriptional coregulators (Franks et al., 2002), which play fundamental roles in animal development (Matthews and Visvader, 2003; Bronstein et al., 2010; Bronstein and Segal, 2011). *SEU* is widely expressed in Arabidopsis and functions in many developmental processes, including floral organ identity specification, gynoecium, ovule and embryo development, and lateral root initiation (Franks et al., 2002; Pfluger and Zambryski, 2004; Sridhar et al., 2006;

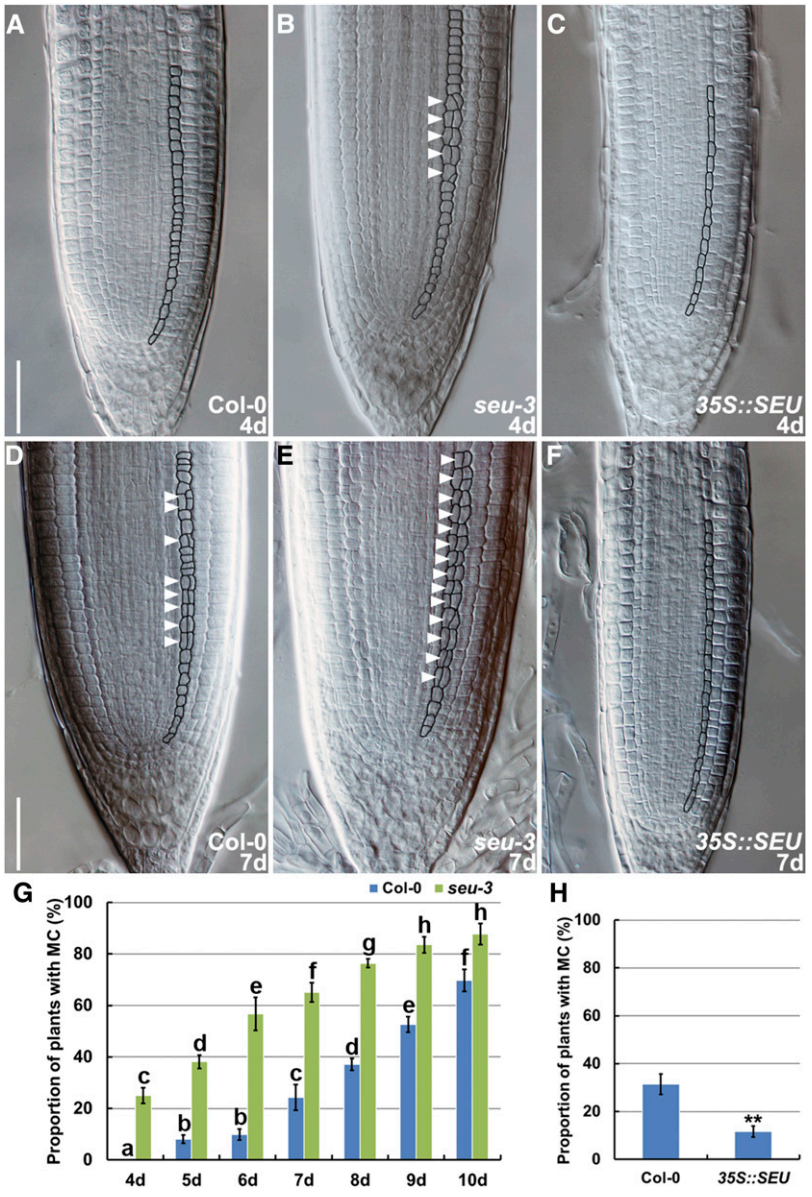

Figure 1. SEU represses MC formation in the root endodermis. A to F, Occurrence of MC formation in roots of Col-0 (A and D), *seu-3* (B and E), and *35S::SEU* (C and F) at the indicated time points (4 d or 7 d after sowing). The black outlines and white arrowheads highlight the occurrence of MC formation in the root endodermis. Scale bars = 50 μm. G, Time-course analysis of proportion of plants with MC formation in roots of Col-0 and *seu-3* seedlings. Error bars represent SD of three independent experiments ($n \geq 25$). Bars with different letters are significantly different at $P < 0.05$, ANOVA. H, Quantitative analysis of proportion of plants with MC formation in roots of Col-0 and *35S::SEU* seedlings 7 d after sowing. Error bars represent SD of three independent experiments ($n \geq 25$). **, $P < 0.01$, Student's *t* test.

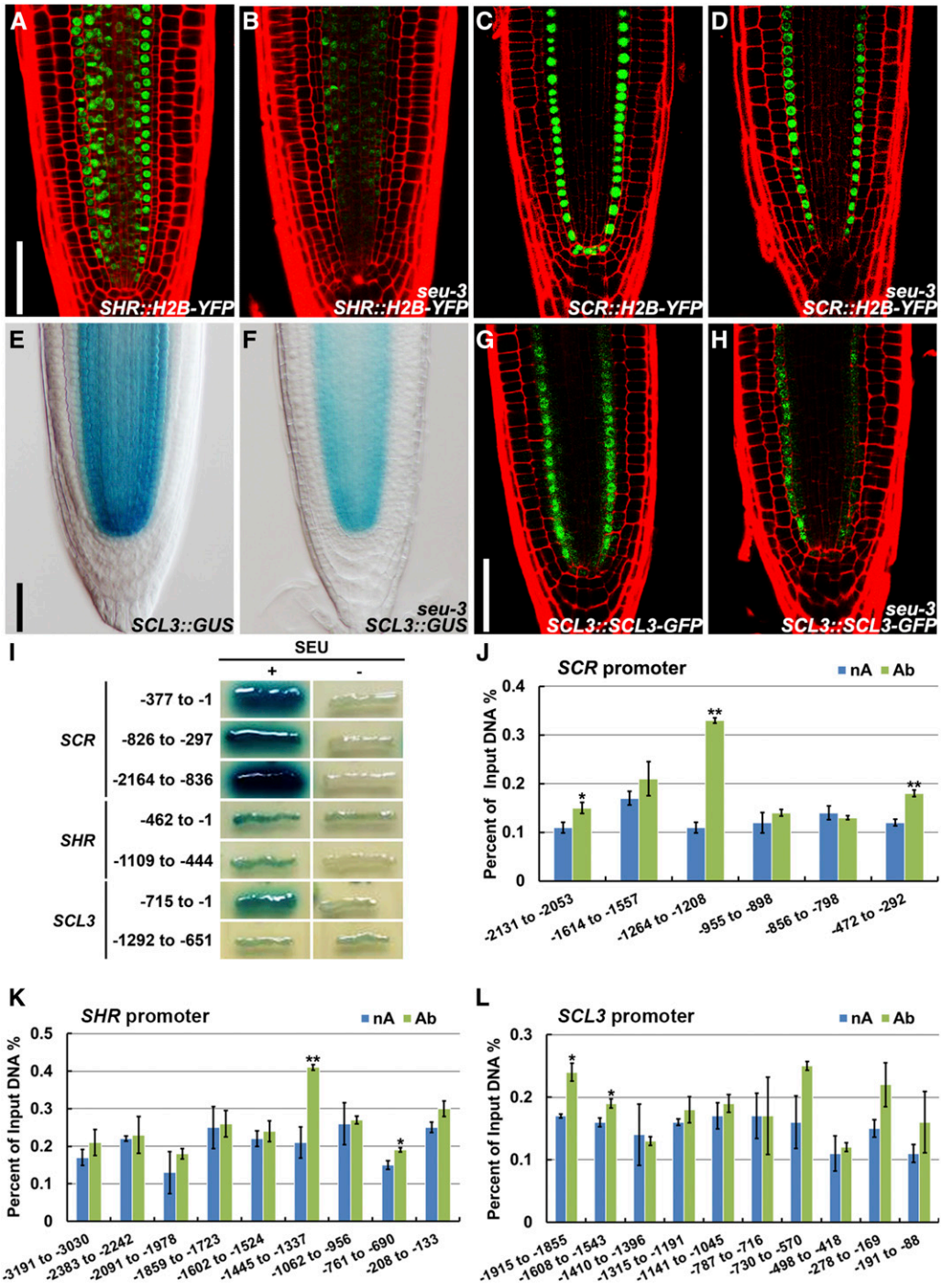

Figure 2. SEU physically associates with promoter regions of *SHR*, *SCR*, and *SCL3* and positively regulates their expression. A to H, Expression of *SHR*::*H2B-YFP* (A and B), *SCR*::*H2B-YFP* (C and D), *SCL3*::*GUS* (E and F), and *SCL3*::*SCL3-GFP* (G and H) in root tips of Col-0 (A, C, E, and G) and *seu-3* (B, D, F, and H) seedlings. Scale bars = 50 μm. I, Yeast one-hybrid assay. The boundaries of promoter regions of *SCR*, *SHR*, and *SCL3* used for reporter constructs are shown. Yeast cells cotransformed with each combination of reporter and *SEU-GAL4-AD* constructs (+) were tested for β-galactosidase assay (blue color). Yeast cells transformed only with each of the reporter constructs were used as controls (−). J to L, ChIP-qPCR assay. The relative ChIP recovery of each region in the *SEU*::*SEU-GFP* line was expressed as percentage of input DNA (*y* axis). Error bars represent SD of three independent experiments. Bars with different letters are significantly different at $P < 0.05$, ANOVA. nA, No antibody; Ab, GFP antibody.

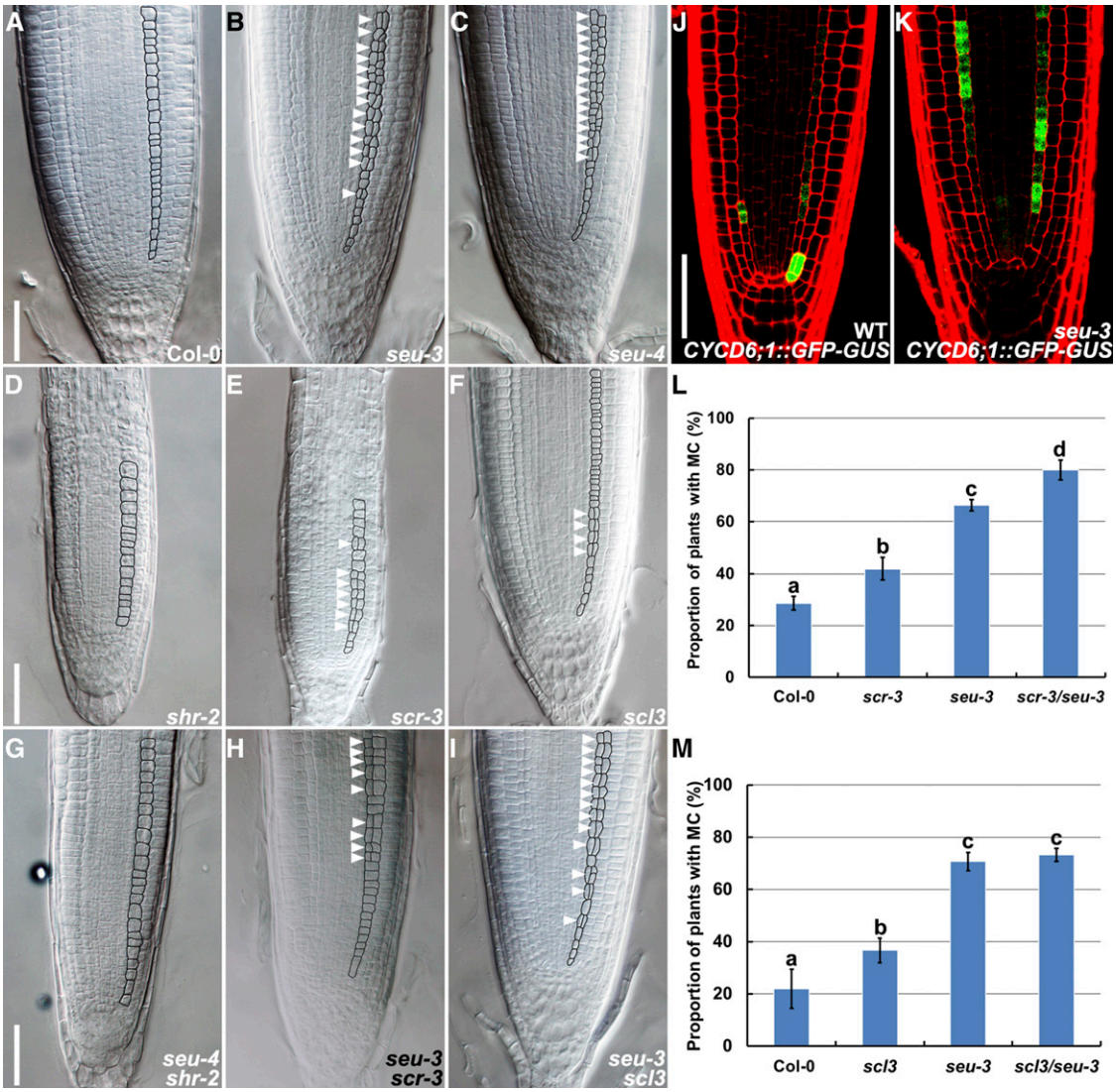

Figure 3. *SEU* has distinct genetic interactions with *SHR*, *SCR*, and *SCL3*. A to I, Occurrence of MC formation in roots of Col-0 (A), *seu-3* (B), *seu-4* (C), *shr-2* (D), *scr-3* (E), *scl3* (F), *seu-4 shr-2* (G), *seu-3 scr-3* (H), and *seu-3 scl3* (I) 7 d after sowing. The black outlines and white arrowheads highlight the occurrence of MC formation in the root endodermis. Scale bars = 50 μm. J and K, Expression of *CYCD6;1-GFP-GUS* in roots of wild-type (WT; J) and *seu-3* (K) seedlings 5 d after sowing. Scale bar = 50 μm. L and M, Quantitative analysis of proportion of plants with MC formation in roots of Col-0, *seu-3*, *seu-3 scr-3*, and *seu-3 scl3* seedlings 7 d after sowing. Error bars represent SD of three independent experiments ($n \geq 15$). Bars with different letters are significantly different at $P < 0.05$, ANOVA.

Azhakanandam et al., 2008; Bao et al., 2010; Nole-Wilson et al., 2010; Lee et al., 2014). Interestingly, although SEU does not appear to possess a recognizable DNA-binding motif, it was shown by chromatin immunoprecipitation (ChIP) to associate with cis-regulatory elements located in the second intron of the floral homeotic gene *AGAMOUS* (*AG*; Sridhar et al., 2006) and with cis-regulatory elements of miR172 genes (Grigorova et al., 2011). Hence, SEU likely regulates gene expression through interactions with DNA-binding transcription factors.

Here we report the discovery of a novel function of SEU in Arabidopsis. Specifically, we show that SEU functions to repress MC formation in the root endodermis. SEU positively regulates the expression of *SHR*, *SCR*, and *SCL3*, all of which encode GRAS family transcription factors involved in the control of MC formation. As previously observed with *SCL3*, *SEU* is repressed by GA and induced by the GA biosynthesis inhibitor paclobutrazol (PAC), suggesting that SEU participates in the GA signaling pathway to regulate MC formation.

RESULTS

SEU Represses MC Formation in the Root Endodermis

Given the broad expression and function of *SEU* in Arabidopsis (Franks et al., 2002; Pfluger and Zambryski, 2004; Sridhar et al., 2006; Azhakanandam et al., 2008;

Bao et al., 2010; Nole-Wilson et al., 2010; Lee et al., 2014), we initiated studies to determine the potential role of SEU in root development. Two *seu* mutants, *seu-3* and *seu-4*, which were previously identified in searches for floral organ patterning mutants (Pfluger and Zambryski, 2004), were utilized for these studies. The *SEU* gene in *seu-3* contains an early stop codon caused by a C-to-T transition at amino acid 127 in the first exon (Supplemental Fig. S1A), and *seu-4* has a T-DNA insertion at codon 199 (Supplemental Fig. S1A). Both *seu-3* and *seu-4* display significantly reduced *SEU* transcript expression (Supplemental Fig. S1B) and were previously reported to be strong *seu* alleles (Pfluger and Zambryski, 2004).

Phenotypic analyses of *seu-3* and *seu-4* roots revealed that loss of *SEU* function altered the timing and extent of MC formation (Fig. 1, A, B, D, and E; Supplemental Fig. S2, A–C). Under our experimental conditions, MC formation was first observed in approximately 8% of 5-d-old wild-type roots (Fig. 1G, and reached about 24% at day 7 (Fig. 1G). By contrast, MC formation was already evident in approximately 25% of 4-d-old *seu-3* roots (Fig. 1G), and reached about 38% at day 5 and 65% at day 7 (Fig. 1G), suggesting that loss of SEU function led to an earlier onset and a higher proportion of plants with MC formation. This was further supported by a similar phenotype in *seu-4* (Supplemental Fig. S2, A–C). Consistently, overexpression of *SEU* by the 35S promoter (*35S::SEU*; Supplemental Fig. S1B) markedly inhibited MC formation (Fig. 1, C, F, and H).

SEU Is an Upstream Activator of *SHR*, *SCR*, and *SCL3*

We next examined whether SEU regulates the expression of the GRAS family transcription factors involved in MC formation. Quantitative reverse transcription PCR (qRT-PCR) assays showed that the expression levels of *SHR*, *SCR*, and *SCL3* were reduced in *seu* roots (Supplemental Fig. S3A) but increased by overexpression of *SEU* (Supplemental Fig. S3A), indicating that SEU positively regulates the transcription of *SHR*, *SCR*, and *SCL3*. Consistently, we found that the expression levels of *SHR::H2B-YFP* and *SHR::SHR-GFP* (Nakajima et al., 2001) were markedly reduced in *seu* mutant roots compared with the wild type (Fig. 2, A and B; Supplemental Fig. S3, B–D). A reduction in the expression level of *SCR::H2B-YFP* (Heidstra et al., 2004) was also observed in *seu* mutant roots (Fig. 2, C and D; Supplemental Fig. S3, B, E, and F). Expression of *SCR::H2B-YFP* was found in new MC cells derived from asymmetrical division of the endodermal cells but was not maintained in other MC cells (Supplemental Fig. S3F). The expression levels of *SCL3::GUS* and *SCL3::SCL3-GFP* (Heo et al., 2011) were both decreased in *seu-3* roots (Fig. 2, F and H;

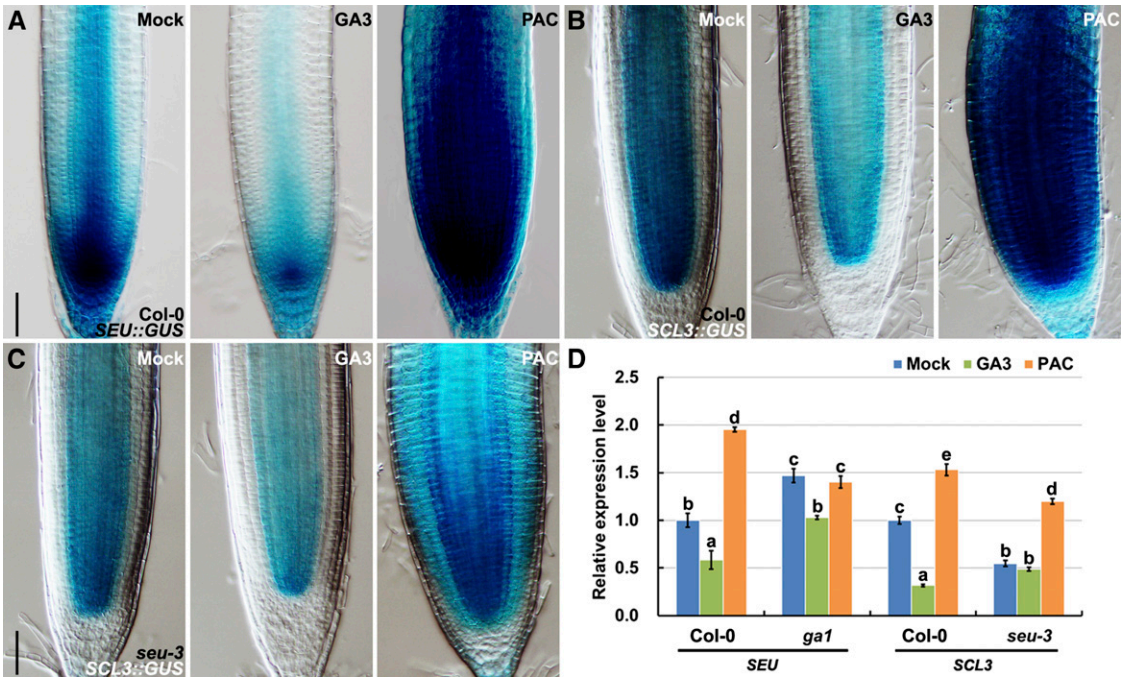

Figure 4. SEU mediates the regulation of *SCL3* by GA signaling. A to C, Expression of *SEU::GUS* (A) and *SCL3::GUS* (B and C) in root tips of Col-0 (A and B) and *seu-3* (C) seedlings treated with mock, 10 μM GA_3, or 10 μM PAC. Scale bars = 50 μm. D, qRT-PCR analysis of *SEU* and *SCL3* transcript levels in roots of Col-0, *ga1*, and *seu-3* seedlings treated with mock, 10 μM GA_3, or 10 μM PAC. Transcript levels from the mock were set to 1. *GAPC* (*AT3G04120*) was chosen as the reference gene. Error bars represent SD of three biological replicates. Bars with different letters are significantly different at $P < 0.05$, ANOVA.

Supplemental Fig. S3B) compared with wild-type roots (Fig. 2, E and G; Supplemental Fig. S3B), suggesting that both the transcription and protein levels of SCL3 are positively regulated by SEU.

SEU was previously shown to physically associate with the cis-regulatory regions within the second intron of the floral homeotic gene *AG* in vivo (Sridhar et al., 2006). We thus performed yeast one-hybrid and ChIP-qPCR assays to investigate whether SEU physically associates with *SHR*, *SCR*, and *SCL3* to activate their transcription. Results from both assays indicated that SEU associates with upstream regulatory sequences in the promoter regions of *SHR*, *SCR*, and *SCL3* (Fig. 2, I–L). Thus, SEU may act directly on these genes to inhibit the formation of MC.

SEU Has Distinct Genetic Interactions with *SHR*, *SCR*, and *SCL3*

As *seu* mutants exhibit a higher frequency of MC formation (Fig. 3, B and C, compared with A) and a reduced expression of *SHR*, *SCR*, and *SCL3* (Fig. 2; Supplemental Fig. S3), we next examined whether *SEU* genetically interacts with *SHR*, *SCR*, and *SCL3* to control MC formation. We generated *seu-4 shr-2*, *seu-4 scr-3*, *seu-3 scr-3*, and *seu-3 scl3* double mutant combinations and compared their MC formation phenotypes with those of the corresponding single mutants (Fig. 3; Supplemental Fig. S4).

In *seu-4 shr-2* double mutants, a single ground tissue layer was observed (Fig. 3G), as seen in *shr-2* (Fig. 3D). This finding is consistent with previous reports showing that SHR is indispensable for MC formation (Paquette and Benfey, 2005; Heo et al., 2011). Since a reduction of SHR is necessary to permit the formation of MC (Koizumi et al., 2012b), we hypothesized that SEU is required to maintain high levels of SHR that prevent MC formation (Koizumi et al., 2012b). Accordingly, we found that in *seu-3* the expression of a cell cycle regulatory gene, *CYCD6;1*, which is a direct downstream target of SHR and SCR (Sozzani et al., 2010) but activated only by intermediate level of SHR during MC formation (Koizumi et al., 2012b), was significantly increased in regions of the endodermis where MC formation was frequently observed, as compared by the expression patterns of *CYCD6;1*:*GFP-GUS* in roots of the wild type (Fig. 3J) and *seu-3* (Fig. 3K).

The *seu-3 scr-3* and *seu-4 scr-3* double mutants displayed a significantly higher frequency of MC formation than either of the single mutants (Fig. 3L; Supplemental Fig. S4, A–E), suggesting that both SEU and SCR are needed to control the frequency of MC formation and that SCR-mediated control of MC formation is regulated by other factors in addition to SEU. By contrast, the MC formation phenotype in *seu-3 scl3* (Fig. 3, I and M) was similar to that in *seu-3* (Fig. 3, B and M), indicating an epistatic relationship of *SEU* to *SCL3* in the control of MC formation.

SEU Mediates the Regulation of *SCL3* Expression by GA Signaling

SCL3 expression is induced by DELLA proteins and repressed by GA (Heo et al., 2011; Zhang et al., 2011), which acts in conjunction with SHR and SCR to regulate the formation of MC (Heo et al., 2011). Because our results showed that *SCL3* is epistatic to *SEU*, we next examined whether GA signals through *SEU* to repress *SCL3* expression. We found that *SEU* expression was significantly reduced by GA_3 (Fig. 4, A and D; Supplemental Fig. S5), whereas GA deficiency, induced by either PAC or the loss of *GA1* (Sun and Kamiya, 1994) function, resulted in an increased expression of *SEU* in the root (Fig. 4, A and D; Supplemental Fig. S5). These characteristics are reminiscent to those reported for *SCL3* (Heo et al., 2011; Zhang et al., 2011), which were also observed under our experimental conditions (Fig. 4B; Supplemental Fig. S5). However, GA_3 was no longer able to suppress the expression of *SCL3* in *seu-3* (Fig. 4, C and D; Supplemental Fig. S5). In the presence

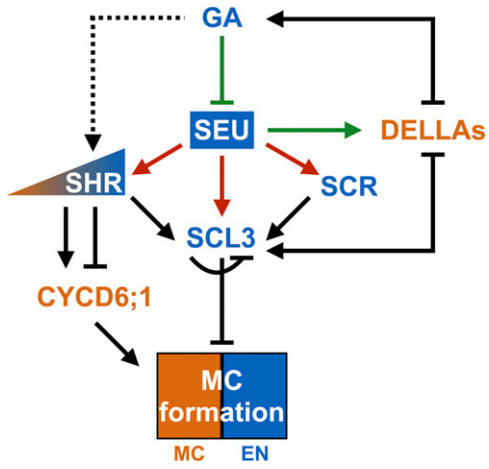

Figure 5. A simplified yet complex regulatory network for MC formation. In the Arabidopsis root meristem, MC forms by periclinal cell division in the endodermis in a developmental stage-dependent manner. Previous studies (black arrows and inhibition signs) have revealed that GA and several GRAS family transcription factors, including SHR, SCR, SCL3, and DELLAs, are involved in the precise control of MC formation. SHR (at high abundance, which inhibits transcription of its direct target CYCD6;1), SCR, and their direct target SCL3 (which represses its own transcription) inhibit MC formation, whereas SHR (at low abundance, which induces transcription of CYCD6;1) and DELLAs (which are direct upstream regulators of SCL3 and known to induce the expression of early GA biosynthesis genes) promote it. GA triggers degradation of DELLAs and may influence abundance of SHR, thus preventing MC formation. In this study (green arrow and inhibition sign or red arrows), we show that SEU is a critical new player that integrates GA signaling with the transcription inputs from the SHR-SCR-SCL3 module to regulate MC formation. SEU is transcriptionally repressed by GA. SEU is a transcriptional activator of SHR, SCR, SCL3, and DELLAs, and physically associates with promoter regions of *SCR*, *SHR*, and *SCL3* (red arrows). The colors orange and blue indicate factors that positively (orange) or negatively (blue) regulate MC formation, respectively. EN, Endodermis.

of PAC, the level of *SCL3* induction was also attenuated in *seu-3* (Fig. 4, C and D; Supplemental Fig. S5). These findings suggest that GA-mediated regulation of *SCL3* expression is subject to SEU function. Moreover, we found that expression of DELLA proteins (Feng et al., 2008), which directly activate *SCL3* (Zentella et al., 2007; Heo et al., 2011), were transcriptionally down-regulated in *seu-3* but up-regulated in *35S*::*SEU* (Supplemental Fig. S6), indicating that the regulation of *SCL3* by DELLA proteins is also subject to SEU function.

DISCUSSION

The ground tissue of the Arabidopsis root is initially composed of single layers of cortical and endodermal cells that arise from the periclinal divisions of stem cell daughters of the cortex/endodermis. Baum et al. (2002) discovered that as the root ages, however, a third layer of ground tissue, namely MC, arises from periclinal divisions of the endodermal cells located at the basal region of the root meristem. While the physiological function of MC remains elusive, the follow-up studies have demonstrated that MC formation can be used as a powerful experimental model for the understanding of how developmental patterns are created and maintained in plants (Paquette and Benfey, 2005; Long et al., 2015). These studies have revealed that MC formation is regulated by the GRAS family transcription factors SHR and SCR, which also play an essential role in formative divisions that create the cortex and endodermis (Paquette and Benfey, 2005; Cui and Benfey, 2009; Koizumi et al., 2012a; Koizumi et al., 2012b). SHR, SCR, and DELLA domain GRAS family transcription factors are direct upstream transcriptional activators of *SCL3* (Levesque et al., 2006; Cui et al., 2007), which encodes another GRAS family transcription factor that mediates the inhibitory effect of GA on MC formation (Heo et al., 2011). Conversely, GA represses *SCL3* (Heo et al., 2011) and is thought to influence the abundance of SHR (Koizumi et al., 2012b), indicating the existence of a complex regulatory network (Fig. 5) that integrates GA signaling homeostasis and the transcriptional activities of the SHR-SCR-SCL3 module to control MC formation in the Arabidopsis root endodermis.

In this study, we provide further insight into the complexity of the MC formation network (Fig. 5). We show that SEU is a repressor of MC formation. Surprisingly, while previous studies indicate that SEU is a component of the repressor complex that prevents ectopic AG transcription in flowers (Franks et al., 2002; Sridhar et al., 2006), our results in the root show that SEU acts as an upstream activator of *SHR*, *SCR*, and *SCL3*. Moreover, association of SEU proteins with several promoter regions of *SHR*, *SCR*, and *SCL3* were shown by yeast one-hybrid analysis and further confirmed by ChIP-qPCR assay, indicating that SEU physically associates with upstream regulatory sequences in the promoter regions of *SHR*, *SCR*, and *SCL3*. Furthermore, we show that *SEU* has distinct genetic interactions with *SHR*, *SCR*, and *SCL3*. *seu-4 shr-2* double mutants have a *shr-2* phenotype and fail to form MC, in agreement with previous studies showing that SHR is indispensable for MC formation (Paquette and Benfey, 2005; Heo et al., 2011). Given that MC formation occurs at intermediate but not high SHR levels (Koizumi et al., 2012b) and SEU positively regulates *SHR* transcription but negatively impacts MC formation, we conclude that SEU is required to maintain high levels of SHR that prevent MC formation (Koizumi et al., 2012b). In support of this conclusion, the expression of *CYCD6;1*, which is up-regulated in response to a reduction of SHR levels in regions of the endodermis where MC forms (Koizumi et al., 2012b), was found to be significantly induced by the loss of SEU function. By contrast, our analyses of *seu-3 scr-3*, *seu-4 scr-3*, and *seu-3 scl3* double mutant combinations indicate that (1) SCR-mediated control of MC formation is regulated by both SEU and additional factors, and that (2) *SCL3* is epistatic to *SEU* in the control of MC formation. The epistatic relationship of *SEU* to *SCL3* was further supported by our results showing that *SEU*, similar to *SCL3* (Heo et al., 2011), is repressed by GA and induced by PAC, and that SEU mediates the effects of GA signaling on *SCL3* expression. Thus, SEU appears to be a critical new player that integrates GA signaling with the transcription inputs from the SHR-SCR-SCL3 module to regulate MC formation in the root endodermis. The fact that *SEU* expression itself is regulated by GA signaling indicates that SEU may function in the feedback loop of GA signaling to maintain a homeostasis required for proper cellular patterning (e.g. MC formation) in Arabidopsis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Columbia plants (Col-0) were used as a wild-type control in all experiments. Previously published mutants and marker lines used in this study include *seu-3* and *seu-4* (Pfluger and Zambryski, 2004), *ga1* (Sun and Kamiya, 1994), *scr-3*, *shr-2*, and *SHR*::*SHR-GFP* (Nakajima et al., 2001), *SCR*::*H2B-YFP* (Heidstra et al., 2004), *scl3*, *SCL3*::*GUS*, and *SCL3*::*SCL3-GFP* (Heo et al., 2011), *CYCD6;1*::*GFP-GUS* (Sozzani et al., 2010), and *SEU*::*SEU-GFP* (Azhakanandam et al., 2008). Primers used for mutant genotyping are listed in Supplemental Table S1. These marker lines were crossed to the indicated *seu* mutants to generate desired combinations. Seeds were germinated on vertically positioned plates (sealed with parafilm) containing half-strength Murashige and Skoog medium (Duchefa) supplemented with 1% (w/v) Suc and 1% (w/v) plant agar (Duchefa), and grown in a growth chamber at 22°C under long-day light conditions (16 h light/ 8 h dark).

Plasmid Construction and Plant Transformation

To generate *SEU*::*GUS* lines, a 3644 bp promoter region upstream of the *SEU* translation start codon was fused to the GUS coding region, followed by a nopaline synthase terminator engineered in pGreenII-0229 (www.pgreen.ac.uk). The resulting construct was then introduced into wild-type plants with *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998). To overexpress the *SEU* gene under the control of the cauliflower mosaic virus 35S promoter, the coding sequence of *SEU* was PCR amplified, verified by sequencing, and cloned into the pGreenII-0229 vector. Primers used for cloning and are listed in Supplemental Table S1.

Microscopy Analyses of Root Phenotypes

For Nomarski differential interference contrast (DIC) images, seedling roots at different developmental stages were mounted in clearing solution (chloral hydrate:water:glycerol, 8:2:1, w/v/v) on microscope slides and imaged using a Nikon Eclipse 80i microscope equipped with a Nikon DXM1200C digital camera and DIC optics. Confocal imaging of seedling roots was performed using the Leica TCS SP2 confocal laser scanning microscope and accompanying software. Propidium iodide (PI; Sigma-Aldrich; 10 μg/mL in distilled water) was used to outline the cell boundaries (red signal). Fluorescent signals from PI, GFP, and YFP were visualized with the following excitation/emission wavelengths: 488 nm/505 to 530 nm for GFP, 514 nm/530 to 560 nm for YFP, and 561 nm/591 to 635 nm for PI.

Histochemical Analysis of GUS Activity

GUS staining was performed as previously described (Sassi et al., 2012). Samples were incubated in assay buffer at 37°C until sufficient staining was observed in roots of *SCL3*::*GUS* (30 min of staining) and *SEU*::*GUS* (1 h of staining) seedlings. GUS staining images were captured using a Nikon Eclipse 80i microscope equipped with a Nikon DXM1200C digital camera and DIC optics.

Chemical Treatment

To examine the effects of GA and PAC on MC formation, 5-d-old seedlings were transferred from half-strength Murashige and Skoog medium to half-strength Murashige and Skoog medium supplemented with either 10 μM GA_3 or 10 μM PAC for 1 d before imaging. For mock treatments, half-strength Murashige and Skoog medium with dimethyl sulfoxide at the final concentration as for chemical treatments was used. On the day of analysis, seedling roots in a given population ($n > 30$) were analyzed for MC formation in the root endodermis.

qRT-PCR

RNA extraction was performed using plant RNA Trizol reagent (Ambion) according to the instruction manual. The reverse transcription of all RNA samples was carried out using PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara). qRT-PCR assay was performed using a StepOnePlus real-time PCR detection system (ABI). *GAPC* (*AT3G04120*) was chosen as reference gene using geNorm software (Vandesompele et al., 2002; Czechowski et al., 2005). The comparative ΔΔCT method was used to evaluate relative quantities of each amplified product in the samples. The threshold cycle (CT) was automatically determined for each reaction by the system set with default parameters. The specificity of the PCR reactions was determined by melt curve analysis of the amplified products using the standard method installed in the system. All experiments were performed with three independent biological replicates and three technical repetitions. The specific primers used are listed in Supplemental Table S1.

Yeast One-Hybrid Screening

Yeast one-hybrid assays were performed using the BD Matchmaker system (Clontech). The pJG4-5 vector was used for the generation of *SEU-GAL4-AD*, which consists of full-length SEU fused to the GAL4 activation domain (AD). The pLacZi vector was used for the cloning of cis-regulatory DNA fragments in promoter regions of *SHR*, *SCR*, and *SCL3*. The yeast strain EGY48 (Leu-, Trp-, Ade-, His-), which has chromosomally integrated reporter genes *lacZ* and *HIS* under the control of the *GAL1* promoter, was used for transformation. The PCR products were digested with *Xho*I and *Eco*RI and subcloned into the pJG4-5 and pLacZi vectors. Transformation of the EGY48 cells was performed according to the manufacturer's instructions. Colonies obtained were streaked on a medium without Ura and Trp. To confirm the results, β-galactosidase assays were carried out according to the system procedure.

ChIP Coupled to Detection by qRT-PCR (ChIP-qPCR)

ChIP experiments were performed as previously described (Yamaguchi et al., 2014) using Dynabeads Protein A (Life Technologies) and an anti-GFP polyclonal antibody (AB290; Abcam). ChIP experiments carried out using normal rabbit preimmune serum (AB7487; Abcam) were used as negative control. Col-0 or *SEU*::*SEU-GFP* plant materials (1.2 mg) previously dual cross-linked with 1.5 mM ethylene glycol bis(succinimidyl succinate) and 1% (w/v) formaldehyde were used for each biological replicate. Fragmented chromatin was split in three technical replicates and incubated with anti-GFP antibody or preimmune serum overnight at 4°C. QIAquick PCR purification columns (Qiagen) were used for purification of the DNA fragments. Enrichment of specific DNA fragment was validated by qPCR using SensiFAST SYBR Hi-Rox One-Step kit (Bioline). Two or three biological replicates for each experiment were performed. For promoter scanning of the *SHR*, *SCR*, and *SCL3* genes, different regions of each promoter were examined by ChIP-qPCR assay. Primers used for the assay are listed in Supplemental Table S1.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *SEU*, At1g43850; *SHR*, At4g37650; *SCR*, At3g54220; *SCL3*, At1g50420; *GA1*, At4g02780; *CYCD6;1*, At4g03270; *RGA*, At2g01570; *RGL1*, At1g66350; *RGL2*, At3g03450; *RGL3*, At5g17490; *GAI*, At1g14920; and *GAPC*, At3g04120.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of *SEU* in different genetic backgrounds as indicated.

Supplemental Figure S2. MC formation in *seu-4*.

Supplemental Figure S3. Expression of *SCR*, *SHR*, and *SCL3* in different genetic backgrounds as indicated.

Supplemental Figure S4. MC formation in different genetic backgrounds as indicated.

Supplemental Figure S5. SEU mediates the regulation of *SCL3* by GA signaling.

Supplemental Figure S6. Expression of DELLA protein-encoding genes in different genetic backgrounds as indicated.

Supplemental Table S1. Primers used in this study.

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