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Interplay between SCARECROW, GA and LIKE HETEROCHROMATIN PROTEIN 1 in ground tissue patterning in the Arabidopsis root

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SUMMARY

Regulated cell division is critical for the development of multi-cellular organisms. In the Arabidopsis root, SCARECROW (SCR) is required for the first cell division, but represses the subsequent, longitudinal asymmetric cell divisions that generate the two cell types of the ground tissue – cortex and endodermis. To elucidate the molecular basis of the role of SCR in ground tissue patterning, we screened for SCR-interacting proteins using the yeast two-hybrid method. A number of putative SCR-interacting proteins were identified, among them LIKE HETEROCHROMATIN PROTEIN 1 (LHP1). In *Ihp1* mutants, a second longitudinal asymmetric cell division occurs in the ground tissue earlier than in wild-type plants. Similar to the *scr* mutant, this premature middle cortex phenotype is suppressed by the phytohormone gibberellin (GA). We provide evidence that the N-terminal domain of SCR is required for the interaction between SCR and LHP1 as well as with other interacting partners, and that this domain is essential for repression of asymmetric cell divisions. Consistent with a role for GA in cortex proliferation, mutants of key GA signaling components produce a middle cortex precociously. Intriguingly, we found that the *spindly* (spy) mutant has a similar middle cortex phenotype. As SPY homologs in animals physically interact with histone deacetylase, we examined the role of histone deacetylation in middle cortex formation. We show that inhibition of histone deacetylase activity causes premature middle cortex formation in wild-type roots. Together, these results suggest that epigenetic regulation is probably the common basis for SCR and GA activity in cortex cell proliferation.

Keywords

SCARECROW; LIKE HETEROCHROMATIN PROTEIN 1; SPINDLY; phytohormone gibberellin; cortex; epigenetic

INTRODUCTION

In plants, the number of cell divisions and the orientation of division planes must be strictly regulated, because together they dictate cell patterning, organ shape and size. The mechanisms that regulate these processes are remarkably precise, as the number of cells of a particular cell type or even for the whole organism remains largely constant for each species, although it may vary dramatically between organisms. However, little is known about the mechanisms that regulate this precise control of cell division during development.

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The Arabidopsis root is a tractable system for the study of these processes because it has a simple organization of well-defined cell types that are generated through stereotyped cell divisions. Epidermis, cortex and endodermis surround the central stele, which consists of the pericycle and vascular tissue (Figure 1a) (Dolan *et al.*, 1993). This radial tissue pattern is perpetuated through iterative divisions of their respective initial cells, which, together with the quiescent center (QC), form the stem cell niche at the root tip. For example, the cortex and endodermis, collectively called the ground tissue, are derived from the cortex/endodermis initial (CEI) cell through two consecutive asymmetric cell divisions. The CEI cell first divides transversely, giving rise to two daughter cells with different fates: the cell adjacent to the QC remains a stem cell, whereas the other, named the CEI daughter (CEID), divides longitudinally to produce the first cells of the two lineages. In the primary root, the endodermis and cortex each initially exist as a single layer consisting of eight cell files (Figure 1b).

SHORT-ROOT (SHR) and SCARECROW (SCR) are members of the plant-specific GRAS family of transcription factors (Pysh *et al*., 1999), and are key regulators of the asymmetric cell divisions that pattern the ground tissue. In *shr* and *scr* mutants, the asymmetric cell divisions that generate the two cell types do not occur, resulting in a single layer of ground tissue (Di Laurenzio *et al*., 1996; Helariutta *et al*., 2000). Expressed in the stele, the SHR protein moves into the adjacent cell layer where the CEI cells reside, and activates the programs for asymmetric cell division and endodermis specification (Nakajima *et al*., 2001). Acting downstream of SHR, SCR appears to be required primarily for longitudinal asymmetric cell division (Di Laurenzio *et al*., 1996; Heidstra *et al*., 2004). SCARECROW also plays an important role in restricting SHR movement to the inner layer of the ground tissue that eventually becomes the endodermis (Cui *et al*., 2007). Intriguingly, although both SCR and SHR are present in the endodermis, no further asymmetric cell divisions occur, and, as a consequence, the root maintains a two-layer ground tissue for a long period of time. This is in sharp contrast to other plant species, such as rice and maize, which produce multiple layers of cortex and a single layer of endodermis as a result of multiple rounds of asymmetric cell divisions of the CEID (Kamiya *et al*., 2003; Cui *et al*., 2007).

The endodermis in Arabidopsis root does undergo more asymmetric cell divisions, but this usually happens at later developmental stages (more than 2 weeks after germination) (Baum *et al*., 2002; Paquette and Benfey, 2005). Three layers of cortex have been observed (Baum *et al*., 2002), but more often only one additional asymmetric cell division occurs, generating a second layer of cortex, termed the middle cortex (Baum *et al*., 2002; Paquette and Benfey, 2005). Both SCR and the phytohormone gibberellin (GA) have been shown to play a key role in middle cortex formation. In the *scr* mutant, the single ground tissue layer occasionally divides, producing a cortex layer that is equivalent to the middle cortex, suggesting that SCR inhibits extra asymmetric cell divisions in the endodermis (Paquette and Benfey, 2005). The premature middle cortex phenotype in the *scr* mutant is suppressed by GA but is enhanced by paclobutrazol (Pac), a GA biosynthesis inhibitor (Paquette and Benfey, 2005). This study raised questions as to how SCR promotes the first but inhibits subsequent asymmetric cell divisions of the CEID, and how GA controls the increase in the numbers of cortex cell layers.

In addition to its role in cortex cell proliferation and restricting SHR movement, SCR is also a key player in stem cell renewal in the root (Sabatini *et al*., 2003) and gravitropic response in the shoot (Tasaka *et al*., 1999). The SCR protein appears to undergo post-translational modification, as indicated by the presence of multiple bands on Western blots (Cui *et al*., 2007). It is likely that SCR interacts with other proteins to perform various functions. We therefore screened a root-specific cDNA library for SCR-interacting proteins using the yeast two-hybrid system. Our analysis of the putative SCR-interacting partners suggests that epigenetic regulation is probably the common basis for SCR and GA activity in cortex cell

proliferation. We also found that the N-terminal domain of SCR plays an important role in protein complex assembly, SCR nuclear localization and ground tissue patterning.

RESULTS

Distinct roles of SCR domains in ground tissue patterning

Using the yeast two-hybrid method with an Arabidopsis root-specific cDNA library, we identified a number of putative SCR-interacting proteins (Table 1). Previously we showed that SCR interacts with SHR through its central domain (CD), which spans the VHIID motif and the two flanking leucine-rich heptad repeats (Cui *et al*., 2007) (Figure 1c). We therefore examined the interaction with various domains of SCR. Interestingly, we found that, in yeast cells, all the putative SCR-interacting proteins interact specifically with the N-terminal domain (ND) of SCR. This observation suggests that the ND may be important for SCR function.

Recently we have demonstrated that nuclear localization is critical for the restriction of SHR movement by SCR, resulting in a single layer of endodermis (Cui *et al*., 2007). We therefore examined the subcellular localization of the SCR domains, first using a transient assay (see Experimental procedures). As shown in Figure 1(d), the GFP–ND fusion protein was detected only in the nucleus, whereas the GFP–CD protein was present in both the nucleus and cytoplasm, similar to the freely diffusible GFP protein expressed from *35S::GFP*. In contrast, a GFP fusion protein with the C-terminal domain (GFP–PS) appeared to be excluded from the nucleus. These results confirm predictions by previous domain-swap experiments (Gallagher and Benfey, 2009). As the fusion proteins have similar sizes (57, 52 and 46 kDa, respectively), their distinct subcellular localization probably reflects the intrinsic properties of the various SCR domains. To assess their *in vivo* function, we then expressed the fusion proteins under the control of the *SCR* promoter in plants. The subcellular localization of the ND and PS domains was the same as observed in the transient assays, but the GFP–CD fusion protein in most transgenic lines was mostly localized in the nucleus (Figure 1e). Interestingly, we found that some transgenic lines expressing the GFP–ND fusion protein (3 of 10 examined) produced an extra ground tissue layer within 1 week of germination. The extra cell layer in GFP–ND-expressing plants appears to be middle cortex, as indicated by the reduction of GFP expression from the *SCR* promoter (Figure 1e). Premature middle cortex formation was not observed in any plants expressing the CD or PS domain.

As the transgenic plants described above are in the wild-type background, the premature middle cortex phenotype could arise as a consequence of the ND activating cell division or alternatively interfering with the full-length SCR protein. To distinguish between these possibilities, we next over-expressed the GFP–ND fusion protein in the *scr4* mutant. The *scr* mutant phenotype was not rescued, suggesting that the ND itself does not have the ability to activate cell division. To further examine the function of the ND, we deleted it from SCR and expressed the truncated protein as a GFP fusion under the control of the *SCR* promoter. Interestingly, there was a normal radial pattern in both the wild-type background and the *scr* mutant. Some plants (6 of 20) in the *scr* background also produced a middle cortex layer within the first week of germination (Figure 1e, pSCR::GFP-SCRΔND/scr-4). This is significant, because wild-type roots do not produce middle cortex at this stage of development ($P = 0.02$, Fisher's exact test). We next performed a time-course study and found that these plants produced a middle cortex at 3 days after germination, but not at 2 days. This suggests that the Arabidopsis root has the potential to produce middle cortex during early seedling growth. These results indicate that the C-terminal region of SCR is sufficient to activate asymmetric cell divisions of the CEID, but the ND is required to repress further cell divisions.

LHP1 acts together with SCR to suppress premature middle cortex formation

To determine how the ND of SCR regulates middle cortex formation, we next analyzed the function of putative SCR-interacting proteins. Preliminary observations indicated that a T-DNA insertional mutation in most of the putative interactor genes did not result in obvious radial patterning defects under normal growth conditions. Interestingly, SALK_011762 (Alonso *et al*., 2003), which has a T-DNA insert in the LIKE HETEROCHROMATIN PROTEIN 1 (*LHP1*) gene, not only shows severe growth defects in the shoot and an early flowering time as previously described (Gaudin *et al*., 2001), but also has a premature middle cortex phenotype (Figure 2a). This phenotype was also observed in the well-characterized *lhp1–4* mutant (Sung *et al*., 2006), indicating that LHP1, like SCR, has a role in the timing of middle cortex formation. Consistent with this hypothesized function, we found that *LHP1* is highly expressed in the meristem and elongation zone of the Arabidopsis root, as indicated by the expression of a genomic fusion between the *LHP1* gene and the coding sequence for GFP (Figure 2b). The premature middle cortex phenotype in the *lhp1* mutant is unlikely to be due to poor growth, as the *lhp1* mutant seedlings have nearly normal root length and premature middle cortex formation was not seen in the *sly1–10* mutant or wild-type seedlings treated with ABA, cytokinin or 5′-aza-cytidine (5-aza-dC), all of which severely retard root growth (see below).

The observation that *lhp1* and *scr* mutants have a similar middle cortex phenotype is consistent with the notion that LHP1 and SCR physically interact with each other. If LHP1 and SCR act together in the control of cortex cell proliferation, they are expected to share a common set of target genes, especially those involved in cell division. To test this hypothesis, we performed ChIP-PCR with LHP1 and SCR to determine whether they bind to the promoter of *MGP*, a previously confirmed SCR target (Cui *et al*., 2007) that appears to have a role in ground tissue patterning (Welch *et al*., 2007). As shown in Figure 2(c), LHP1 binds as well as SCR does to the *MGP* promoter. The binding profiles for LHP1 and SCR overlap considerably. Next we performed quantitative RT-PCR and found that the expression levels of *MGP* and *SCR* are increased in the *lhp1* mutant (Figure 2d), suggesting that LHP1 normally represses *MGP* and *SCR* transcription. These results together lend support to the conclusion that LHP1 and *SCR* act together to suppress premature middle cortex formation.

Interplay between LHP1, GA and the epigenetic machinery in middle cortex formation

In subsequent experiments, we found that roots from freshly harvested *lhp1* seeds have a normal radial pattern. As fresh seeds are known to have a high level of GA (Jones and Varner, 1967) and middle cortex formation can be suppressed by GA (Paquette and Benfey, 2005), this observation raised the possibility that the *lhp1* mutants might have reduced GA production or signaling. To investigate this possibility, we compared the radial pattern in *lhp1* and wild-type roots after treatment with GA or with the GA biosynthesis inhibitor paclobutrazol (Pac). We found that middle cortex formation in the *lhp1* mutants was completely suppressed by GA at 50 μM, and that Pac at 10 μM caused middle cortex formation in nearly all *lhp1* mutant roots (19 of 20 examined) but in none of the wild-type roots ($P = 3e^{-10}$, Fisher's exact test). These results suggest that LHP1 and *SCR* might suppress premature cortex proliferation by a similar mechanism in which GA has a dominant role.

As the phytohormones GA and ABA have antagonistic roles in many aspects of plant growth and development (Hoffmann-Benning and Kende, 1992), we asked whether ABA treatment could cause premature middle cortex formation. Precocious middle cortex formation was not observed in ABA treatment studies, even at a concentration of 10 μM when root growth was seriously compromised. In contrast, ABA appears to suppress middle cortex formation, because after 2 weeks of germination, when nearly all untreated plants have produced a middle cortex, few seedlings (1 of 10) treated with 1 μM ABA have a middle cortex layer ($P = 7e^{-7}$,

Fisher's exact test). These results suggest that GA has an ABA-independent role at least in the promotion of middle cortex formation.

Molecular basis of GA signaling in cortex cell proliferation

To understand the molecular basis of GA signaling in cortex cell proliferation, we analyzed the role of GA signaling components in root radial patterning. As expected, the GA receptor mutant *gid1a gid1b gid1c* (*gid1*), which cannot transduce GA signals (Ueguchi-Tanaka *et al.*, 2005) and the GA-insensitive mutant *rgaΔ17*, which blocks GA signaling transduction (Dill *et al.*, 2001), both show a premature middle cortex phenotype, even in seedlings from freshly harvested seeds (Figure 3a,b). Surprisingly, we found that the *sly1-l0* mutant, in which the DELLA protein RGA as well as other DELLA proteins cannot be degraded (McGinnis *et al.*, 2003), has a normal ground tissue pattern, even at 12 days after germination (data not shown). According to an Arabidopsis root gene expression map (Brady *et al.*, 2007), *SLY1* is preferentially expressed in the stele, hence it is possible that other SLY homologs are responsible for DELLA protein degradation in the ground tissue. Interestingly, the closest *SLY* homolog, *SNE* (Strader *et al.*, 2004), is preferentially expressed in the endodermis and the QC, and we therefore examined its role in middle cortex formation. As no *SNE* mutant has been characterized so far, we first obtained a T-DNA insertional line, FLAG_461E03 (Samson *et al.*, 2002), which harbors a T-DNA insert within the only exon of the gene. Compared to wild-type plants, FLAG_461E03 seedlings have slightly shorter roots on normal MS medium, but this phenotype is enhanced when the water content of the medium is reduced (Figure S2). We named this T-DNA line *sne-1*. The *sne-1* mutant seeds are also hypersensitive to Pac, as they do not germinate on MS medium containing 1 μM Pac (Figure S2), indicating GA signaling defects. Nevertheless, we did not observe any obvious radial patterning defects in the mutant root (data not shown).

Intriguingly, premature middle cortex formation was observed in the *spy* mutant (Figure 3c). All mutant alleles examined, including *spy-3, spy-4* and *spy-8* (Jacobsen and Olszewski, 1993; Silverstone *et al.*, 2007), showed a similar middle cortex phenotype. This result is unexpected, because the *spy* mutant is believed to have an elevated level of GA signaling (Jacobsen and Olszewski, 1993; Swain *et al.*, 2002). The premature middle cortex phenotype in the *spy* mutant was not rescued by exogenous application of Pac or GA. Because cytokinin signaling is compromised in the *spy* mutant (Greenboim-Wainberg *et al.*, 2005), we next treated *spy* mutants with cytokinins, but were unable to rescue the middle cortex phenotype. We did not detect any radial patterning defects in the *sec* mutants, indicating that *SECRET AGENT* (*SEC*), the closest *SPY* homolog in the Arabidopsis genome (Hartweck *et al.*, 2006), does not play a major role in ground tissue patterning.

As *SPY* encodes an O-GlcNAc transferase, it may affect ground tissue patterning by modulating the expression patterns or activities of key cell-fate determinants such as SHR and SCR, either through its effect on GA signaling or through post-translational modifications. To test this hypothesis, we first examined the expression of pSCR::GFP and pSHR::SHR-GFP (Cui *et al.*, 2007) in the *spy-3* background. Neither the expression pattern nor the subcellular localization of SHR and SCR appeared to be altered in the *spy* mutant (Figure 3d–f). We also examined protein–protein interactions using the yeast two-hybrid method, but did not detect any interaction between SPY and SHR or SCR, suggesting that SHR and SCR are unlikely to be SPY substrates.

In animals, the SPY homolog is present in a Sin3 repressor complex and physically interacts with histone deacetylases (HDACs) (Yang *et al.*, 2002). This observation raises the possibility that SPY may also interact with HDACs in plants and regulate middle cortex formation through an epigenetic regulatory mechanism. As LHP1 is known to repress gene expression through modification of chromatin structure (Sung *et al.*, 2006; Turck *et al.*, 2007), the physical

association between SCR and LHP1, together with the similar middle cortex phenotypes in *scr* and *lhp1*, lends support to this hypothesis. To test the role of histone deacetylation in cortex proliferation, we treated wild-type roots with trichostatin A (TSA), a specific HDAC inhibitor. At a concentration of 0.5 μg ml^{-1}, TSA caused a third layer of ground tissue in some of the roots examined (5 of 15), and 1 μg ml^{-1} TSA caused an extra layer of ground tissue in all 20 roots examined (Figure 4a). The number of endodermal cell files that generated an extra cell layer was also dramatically increased by 1 μg ml^{-1} TSA, in some cases reaching six (Figure 4b). While the concentration of TSA required to completely inhibit HDAC activity in the ground tissue appears high, it is consistent with that used in a recent report showing root hair growth from non-root hair cells after TSA treatment (Xu *et al.*, 2005). To determine the identity of this extra cell layer, we examined expression of the cortex and endodermis markers, pCO:HYFP and pEn7:HYFP, after TSA treatment. This analysis clearly showed that the extra cell layer is cortex (Figure S3).

However, TSA treatment also caused abnormal cell divisions in the QC and surrounding stem cells (Figure 4a), as well as decreased root growth. To determine whether the middle cortex induced by TSA is due to a disorganized QC, we conducted a time-course study of middle cortex formation after transfer of 3-day-old seedlings grown on regular MS medium to MS medium containing 1 μg ml^{-1} TSA. Although the QC and initial cells appeared normal even 48 h after TSA treatment, a middle cortex layer was already apparent after 24 h on TSA (Figure 4). Notably, middle cortex cells were produced randomly along the endodermis cell file, suggesting that the effects of TSA on the QC and middle cortex are uncoupled.

To determine whether TSA causes premature middle cortex formation by reducing the level of GA, we treated roots simultaneously with 1 μg ml^{-1} TSA and 100 μM GA. The presence of GA did not alleviate the effect of TSA on either middle cortex formation or root growth. We also treated roots with 5-aza-dC, which inhibits DNA methylation (5-methylcytosine). Although 1 μM 5-aza-dC inhibited root length to a greater extent than 1 μg ml^{-1} TSA did, it did not alter the radial pattern. These results indicate that reduced root growth is not the cause of premature middle cortex formation after TSA treatment; instead, it suggests a key role of histone deacetylation in this process.

DISCUSSION

SCR is a key regulator of ground tissue patterning in the Arabidopsis root. Previous studies have shown that while it is required for the first asymmetric division of the CEID, it represses subsequent divisions, resulting in the characteristic two cell-layer ground tissue. In this study, we present evidence that these two opposing functions of SCR are executed by different parts of the protein through interaction with various partners: asymmetric cell division is activated by the C-terminal region interacting with SHR, but is repressed by the ND through physical interaction with the LHP1 protein. Extending the previous findings that GA plays an important role in cortex cell proliferation (Paquette and Benfey, 2005), we found that mutations in key GA signaling components cause an increase in cortex layers. We further found that precocious middle cortex formation occurs when HDAC activity is chemically inhibited. Together our results suggest that epigenetic regulation is probably the common basis for SCR and GA signaling in ground tissue patterning in the root, as discussed below.

The N-terminal domain of SCR functions in ground tissue patterning

SCR is one of the founding members of the GRAS family of transcriptional regulators (Pysh *et al.*, 1999), which are characterized by the presence of highly conserved sequence signatures in the C-terminal portion of the proteins. Although structurally similar, the GRAS family of proteins has demonstrated a wide array of function, from photomorphogenesis to GA signaling and root radial patterning (Bolle, 2004), and the functional specificity appears to be largely

determined by their N-terminal sequences. For example, the GA responsiveness of the DELLA subgroup of proteins has been shown to be determined by the N-terminus (Willige *et al.*, 2007). Like other GRAS family members, SCR has a highly variable N-terminus (ND) as well as a highly conserved C-terminal region, which includes the CD and PS domains. Although a SCR-specific motif has been identified in the N-terminus (Tian *et al.*, 2004), raising the possibility that the ND may be a functional domain, so far no function has been assigned to it.

Using both transient and transgenic assays, we have shown that the ND has a role in root radial patterning: it appears to suppress ectopic asymmetric cell divisions and thus restricts the number of cortex cell layers in the ground tissue. Two lines of evidence support this conclusion. First, transgenic plants expressing a truncated SCR protein without the ND in the *scr* mutant background produce a middle cortex layer precociously, suggesting that the C-terminal region of SCR is able to activate asymmetric cell divisions in the CEID but cannot suppress further divisions in the endodermis. Second, when over-expressed, the ND-GFP fusion protein causes a middle cortex phenotype in the wild-type background, but does not rescue the ground tissue patterning defect in the *scr* mutant. This result suggests that the ND does not promote cell divisions but rather has a role in suppressing extra cell divisions. The ability of the ND to suppress cell division is probably achieved at two levels. First, as the ND has a very strong nuclear localization signal, enhancing SCR nuclear localization would ensure strict regulation of SHR movement and thus correct radial patterning (Cui *et al.*, 2007). Second, the ND could repress cell division genes through its physical interaction with the LHP1 protein, which appears to be a transcriptional repressor (Sung *et al.*, 2006; Turck *et al.*, 2007; our quantitative RT–PCR data on *MGP* and *SCR* expression in the *lhp1* mutant). The observation that both *scr* and *lhp1* mutants show precocious middle cortex formation is consistent with this hypothesis.

Our results also indicated that the ND is probably a versatile protein–protein interaction domain. Unlike the CD domain, which appears to interact only with SHR, the ND is able to interact with multiple proteins including LHP1, at least in yeast cells. Consistent with this, we identified multiple copies of the SRC homology (SH3) motif in the N-terminus (Figure S1, http://scansite.mit.edu/motifscan_seq.phtml), which is known to function in protein–protein interactions (Agarwal and Kishan, 2002). The N-terminus of SCR also contains homopolymeric repeats of tyrosine and serine, which are a feature of eukaryotic transcription factors that have multiple interacting partners (Faux *et al.*, 2005). One interpretation is that ND might be a scaffold for the assembly of multiple protein complexes that execute distinct functions at various developmental stages.

Sequential activation and repression by SCR of asymmetric cell divisions

Recently, we showed that SCR acts as a transcriptional activator of a number of target genes (Cui *et al.*, 2007). Our present finding that SCR interacts with LHP1 suggests that SCR may have dual activities in both transcriptional activation and repression. Several proteins with such dual transcriptional activities have been reported, such as YY1 and TnpA (Cui and Fedoroff, 2002; Gordon *et al.*, 2006). Like the TCF transcription factor (Roose and Clevers, 1999), SCR could activate or repress gene expression depending on the partners with which it interacts. This dual activity of SCR suggests a potential mechanism for its ability to activate the first cell division and then suppress subsequent asymmetric cell divisions. As depicted in Figure 5, one model would involve SCR first interacting with SHR through its CD domain, followed by activation of cell division genes in the CEI cells. This activity is then rapidly quenched as a consequence of interaction with LHP1 via the ND and local chromatin remodeling. Consistent with this model, known SHR/SCR target genes such as *MGP*, *NUC* and *SCL3* are predominantly expressed in cells close to the QC and CEI cell (Pysh *et al.*, 1999; Levesque *et al.*, 2006). Although *SCR* is expressed in the endodermis at later development stages, we have

shown that this is partly due to a SHR/SCR-independent mechanism (Helariutta *et al.*, 2000; Cui *et al.*, 2007). As LHP1 does not appear to change histone modifications, other mechanisms must also be involved to reverse the epigenetic markers in chromatin that is initially active in transcription.

This model can account for the ground tissue pattern in the Arabidopsis root, but appears to be at odds with observations in other plant species. In plants with a 'closed' meristem (i.e. clear cell lineages can be discerned and each lineage can be traced back to an initial cell; Baum *et al.*, 2002), such as rice and maize, the endodermis and cortex are derived from the same CEI cell, but the CEID undergoes a series of cell divisions, giving rise to multiple cortex layers (Dolan *et al.*, 1993; Kamiya *et al.*, 2003). In these plants, however, both *SHR* and *SCR* have duplicated, which could dramatically modify the SCR regulatory module in ground tissue patterning. A more complicated interplay between SCR, LHP1 and GA could lead to the production of multiple cortex layers.

LHP1 and related proteins in ground tissue patterning

Although the *lhp1* mutant has a premature middle cortex phenotype, the phenotype is much weaker compared to that in wild-type roots treated with TSA or in the *spy-3* mutants. One possibility is that LHP1 doe not cause gene silencing but rather enhances the silencing effect that is initiated by other mechanisms. It is possible that LHP1-like factors are also involved in middle cortex formation. Although identified as the closest homolog in plants to the animal HETEROCHROMATIN PROTEIN 1 (HP1), based on the presence of both a chromo domain and a chromo-shadow domain, LHP1 behaves quite differently from its animal counterpart. Unlike HP1, which binds to histone H3 trimethylated at lysine 9 (H3K9m3) in heterochromatin, LHP1 targets both heterochromatin and euchromatin (Nakahigashi *et al.*, 2005; Zemach *et al.*, 2006) and binds to H3 histones that are trimethylated at lysine 27 (H3K27m3) (Turck *et al.*, 2007; Zhang *et al.*, 2007). In addition, LHP1 also shows cell type-specific subcellular localization, suggesting that it might regulate different sets of genes in different cell types (Zemach *et al.*, 2006). It is also highly possible that it interacts with different proteins in different cell types to perform distinct functions. In the Arabidopsis genome, there are 13 genes encoding proteins with a bromo domain, and 10 genes whose protein products contain a chromo shadow domain (http://www.chromdb.org), which could partially complement the *lhp1* mutant phenotype. In the future, it will be interesting to determine the function of these genes in root radial patterning.

A common basis for SCR and GA signaling in cortex cell proliferation

Previously, a key role for GA in middle cortex formation was identified (Paquette and Benfey, 2005). In agreement with this result, we found that GA signaling mutants, such as *gid1*, *rgaΔ17* and *spy* all form middle cortex precociously. However, our present studies also reveal important subtle differences in the manner in which individual GA signaling components interact in the developmental program. First, the precocious middle cortex phenotype was not observed in *sly* or *sne* mutants. Because *SLY* and *SNE* are expressed in different cell types, the lack of middle cortex phenotype in these mutants is probably not due to functional redundancy. Another unexpected observation is that middle cortex formation was not promoted, but instead was suppressed, by ABA.

The most surprising finding is that *spy* mutants also exhibit a premature middle cortex phenotype. Unlike the other GA signaling mutants examined, which have reduced GA signaling, the *spy* mutant appears to have increased GA signaling (Jacobsen and Olszewski, 1993). Although SPY also plays a role in the signaling pathways of other phytohormones (Greenboim-Wainberg *et al.*, 2005; Shimada *et al.*, 2006), the similar middle cortex phenotype

in all GA signaling mutants suggests that SPY most likely regulates cortex cell proliferation through GA signaling.

An important clue as to the molecular basis of SPY in ground tissue patterning comes from the observation that the animal SPY homolog is present in a Sin3 repressor complex containing HDAC1 and HDAC2 (Yang *et al.*, 2002). This suggests that SPY may regulate gene expression through epigenetic mechanisms. In support of this hypothesis, we find that TSA treatment causes a similar but more severe premature middle cortex phenotype. Despite its negative role in GA signaling, SPY may affect cell division genes in a similar manner to other GA signaling components. Although it is not yet clear how SCR and LHP1 interact with the GA signaling pathway, our results suggest that SCR, LHP1, SPY and the GA signaling pathway probably converge on a common epigenetic mechanism that involves histone deacetylation. Resolution of this issue requires the identification of cell division genes in the ground tissue and characterization of their relationship with SPY and the GA signaling pathway.

EXPERIMENTAL PROCEDURES

Plant growth and chemical treatments

Unless specified otherwise, all Arabidopsis plants used in this study are in the Columbia ecotype. After sterilization, seeds were sown and allowed to germinate on 1% w/v MS agar plates positioned vertically in an incubator set at 22°C with 16 h daily illumination. For GA, TSA and 5′-aza-dC treatments, seeds were sown on a piece of nylon mesh (NITEX, catalog number 03–100/47, Sefar Infiltration Inc., http://www.devicelink.com/company/ivdt/co/01/198.html) placed on a MS plate containing the chemical at the desired concentration. For paclobutrazol (Pac) treatment, seeds were first germinated for 4 days on regular MS plates, and the seedlings were allowed to grow for two more days after addition of 1 ml of 100 μM Pac to the plate or after transfer of the seedling together with the nylon mesh to a MS plate containing 10 μM Pac. ABA treatment was performed similarly by transferring seedlings to MS plates supplemented with 1,2,5 or 10 μM ABA. ABA, BA, GA, trichostatin A and 5′-aza-cytidine were purchased from Sigma (http://www.sigmaaldrich.com/), and paclobutrazol was purchased from PhytoTechnology Laboratories (http://www.phytotechlab.com). The transgenic lines carrying pSCR::GFP-SCR, pSCR::GFP and pSHR::SHR-GFP have been described previously (Cui *et al.*, 2007).

Yeast two-hybrid screening

An Arabidopsis root-specific cDNA library was screened using the MATCHMAKER two-hybrid system 3 (BD Biosciences, http://www.bdbiosciences.com). The SCR bait construct pGBKT7-SCR has been described previously (Cui *et al.*, 2007). Approximately 2 million colonies were screened, and positive clones were selected based on their growth on histidine drop-out medium and β-galactosidase activity. Approximately 400 putative clones were sequenced, and the list of putative SCR interactors shown in Table 1 was compiled based on the strength of interactions (reporter activity), the number of matches, as well as in-frame fusion between the corresponding cDNA sequence and the Gal4 activation domain sequence, with common false-positive proteins subtracted. Each putative interacting protein was individually confirmed using the same reporter assay. Plasmid purification from yeast cells and re-transformation were performed using standard protocols.

Molecular cloning and genotyping

To clone the constructs for the transient assay, the sequences encoding the ND, CD and PS domains of SCR were obtained as *Nde*I–*Sal*I fragments from the pGBKT7–ND, pGBKT7–CD and pGBKT7–PS plasmids (Cui *et al.*, 2007), and, together with an *Spe*I–*Ase*I fragment from pBS-10–3 containing the GFP coding sequence, were subcloned into the *Spe*I–*Sal*I site of the

pBC-SK vector, giving rise to pBC-GFP-ND, pBC–GFP–CD and pBC-GFP-PS plasmids, respectively. The GFP–ND and GFP–PS coding sequences were then subcloned as *Xba*I–*Sma*I fragments into pBI221 cut with *Xba*I and *Sac*I and blunt-ended using the Klenow enzyme, resulting in the final constructs pBI221-35S::GFP-ND and pBI221-35S::GFP-PS. Due to the presence of an *Xba*I site within the CD region, to produce the pBI221-35S::GFP-CD construct, the GFP–CD coding sequence was subcloned as an *Spe*I–*Sma*I fragment into the pBI221 vector.

To express the GFP fusion proteins in plants, their expression cassettes were cut as *Spe*I–*BamH*I fragments from pBI221-35::GFP-ND, pBI221-35S::GFP-CD and pBI221-35S::GFP-PS, with the *Spe*I site blunt-ended by the Klenow enzyme, and then subcloned into the *Sma*I–*BamH*I sites of the pSCR::NosT vector. The resulting plasmids were named pSCR::GFP-ND, pSCR::GFP-CD and pSCR::GFP-PS, respectively. To clone the pSCR::GFP-SCRΔND construct, the sequence coding the CD and PS domain plus part of the endogenous 3′ UTR sequence was PCR-amplified from the pBS-SCR-NF plasmid using Phusion high-fidelity DNA polymerase (NeBiolabs, http://www.neb.com) and the primers SCR-GRAS-FW (5′-CTAC-TAGTCATATGCAAGACGAAGAAGGATTACACC-3′) and SCR-PS3′ (5′-AAAGAATTCCAATCAGGTAGCCAATA-3′). After purification and restriction digestion by *Nde*I and *Eco*RI, the truncated *SCR* sequence (*SCRΔND*) was subcloned into the pBBKT7 vector at the same restriction sites, resulting in pGBKT7-GPS3′. Subsequent cloning of the GFP–SCR expression construct was performed using the same strategy as for the pSCR::GFP-CD plasmid.

The primers for genotyping T-DNA knockout lines were as follows: for Salk_011762, primers LHP1_LP (5′-ACGCGATTACCTTGATTCACC-3′) and LHP1_RP (5′-CTAAAGGCTCCCATGTGTTGG-3′) were used, and for FLAG_461E03, primers SLY2_LP (5′-AGAAACAAGAACCACCCAACC-3′) and SLY2_RP1 (5′-TCAATGAAAATTAGTGAAGGCC-3′) were used.

Other assays

The transient gene expression assay for subcellular localization of SCR domains was performed using with a biolistic delivery system (BioRad, http://www.biorad.com). After coating with plasmids pBI221-35S::GFP-ND, pBI221-35S::GFP-CD and pBI221-35S::GFP-PS, gold particles (1.0 μM gold microcarriers, Bio-Rad, http://www.bio-rad.com/; catalog number 165-2263) were bombarded onto fresh onion epidermal cells on a 1% MS agar plate using 1100 psi rupture disks (Bio-Rad, catalog number 165-2329). GFP fluorescence was visualized using a Leica epifluorescence microscope (http://www.leica.com) with a narrow-band GFP filter. A Zeiss 510 upright confocal microscope (http://www.zeiss.com/) was used to image root samples.

The ChIP-quantitative PCR assay as well as the transgenic lines pSCR::GFP-SCR (Cui *et al.*, 2007) and *gLHP1::GFP*(Sung *et al.*, 2006) used to determine SCR and LHP1 binding has been described previously (Cui *et al.*, 2007). After ChIP, various regions of the *MGP* promoter were PCR-amplified from both the mock (no antibody) and ChIP samples, and the fold enrichment was calculated as the ratio between the C_t values for each DNA fragment after normalization against the 18S signal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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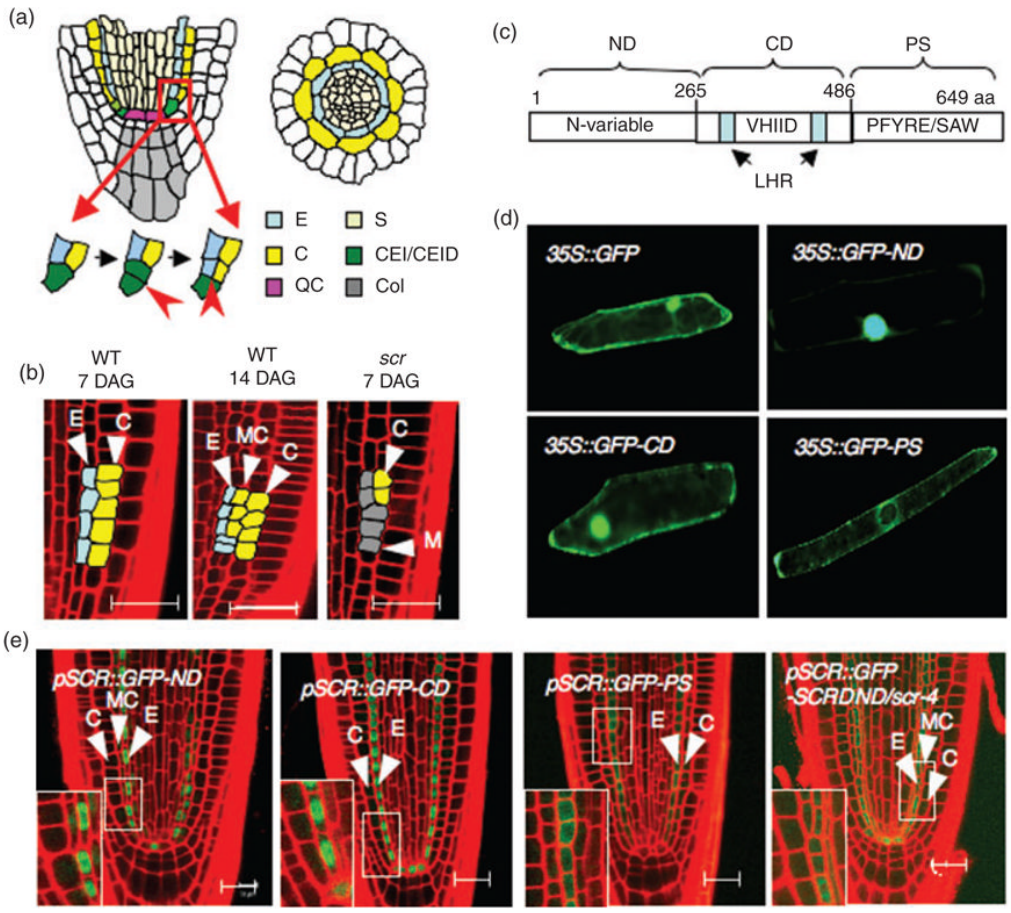

Figure 1. Functional analysis of SCR domains

(a) Schematic of the radial pattern in the Arabidopsis root, showing longitudinal (left) and transverse (right) sections of a primary root and the asymmetric cell divisions (framed area) that give rise to the endodermis and cortex. The outermost cell layer at the root tip is the lateral root cap, which protects the inner meristem.

(b) Confocal microscopy images showing the middle cortex in wild-type (WT) root after 14 days of germination (DAG) and sporadic cortex formation in the *scr-1* mutant at 7 DAG.

(c) Molecular structure of the SCR protein, and the various fragments used in the protein-protein interaction assay.

(d) GFP fluorescence detection showing subcellular localization of the fusion proteins between GFP and SCR domains after transient expression in onion epidermal cells.

(e) Confocal microscopy images of transgenic Arabidopsis roots expressing GFP fusion proteins with SCR domains or ND-truncated SCR (pSCR::GFP-SCRΔND). The insets show parts of the images at higher magnification (3.5× higher). QC, quiescent center; E, endodermis; C, cortex; S, stele; Col, columella; CEI/CEID, cortex/endodermis initial or daughter cell; MC, middle cortex; M, mutant cell layer; ND, N-terminal variable domain; CD, the central domain, which spans the VHIID motif and the leucine heptad repeats (LHR); PS, the C-terminal domain containing the conserved PFYRE and SAW motifs. Scale bar = 20 μm.

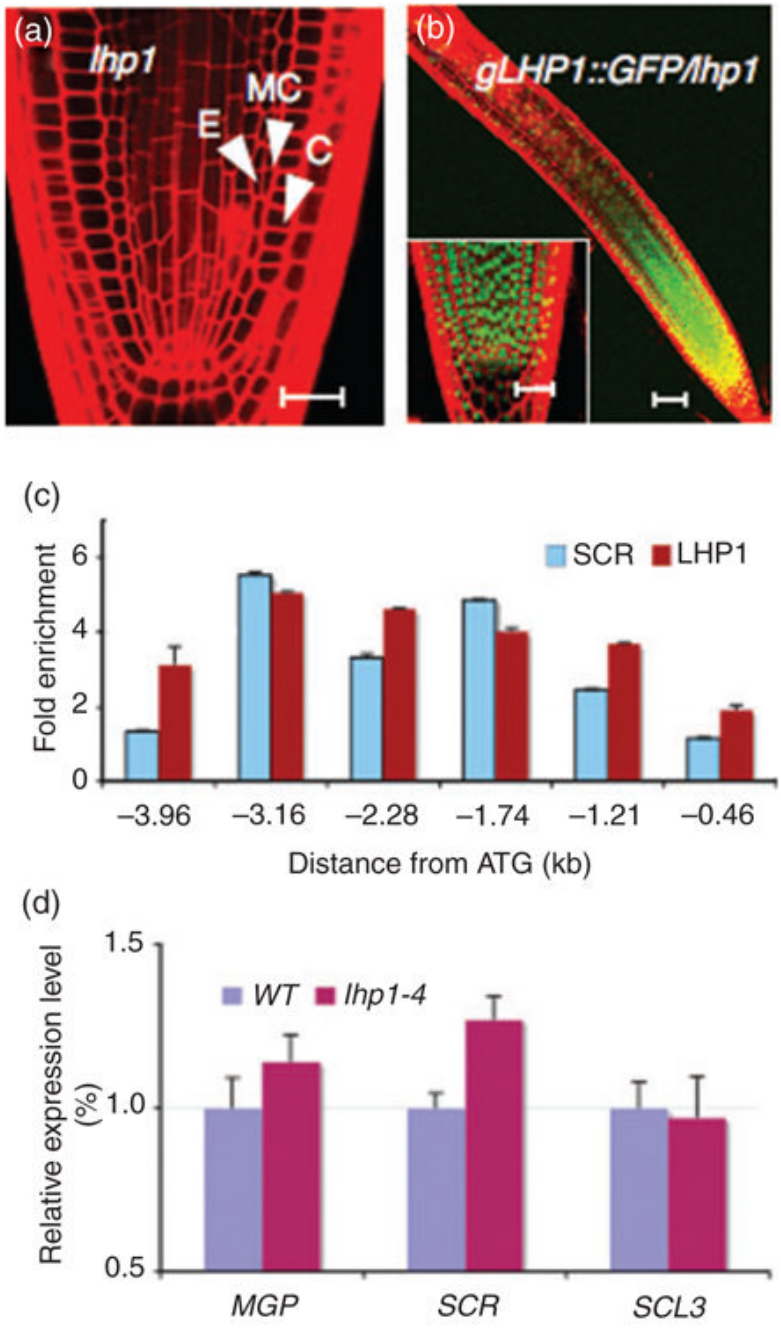

Figure 2. LHP1 acts together with SCR to regulate the timing of middle cortex formation

(a) Confocal microscope image of 1-week-old *lhp1* mutant root showing the presence of a middle cortex layer.

(b) GFP fluorescence detection in the *gLHP1::GFP* transgenic plants, showing the *LHP1* expression pattern in primary root. The meristematic region is shown in the inset at higher magnification (2.5× higher).

(c) ChIP-quantitative PCR assay for SCR and LHP1 binding to the *MGP* promoter.

(d) Quantitative RT-PCR assay of SCR target genes in the *lhp1–4* mutant and wild-type root. The error bars in (c) and (d) represent standard deviations between duplicate measurements. C, cortex; E, endodermis; MC, middle cortex. Scale bar = 20 μm.

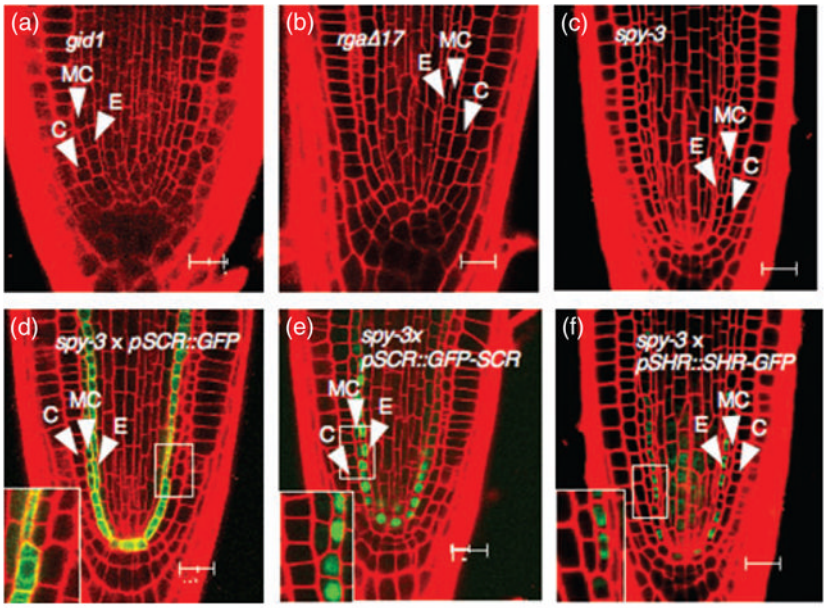

Figure 3. Confocal microscope images of primary roots of GA signaling mutants 1 week after germination

(a–c) *gid1*, *rgaΔ 17* and *spy-3* mutants, respectively.

(d–f) SCR and SHR expression and subcellular localization in the *spy-3* mutant, as visualized by expression of the pSCR::GFP, pSCR::GFP-SCR and pSHR::SHR-GFP constructs. The insets show the framed areas at higher magnification (2× higher). C, cortex; E, endodermis; MC, middle cortex. Scale bar = 20 μm.

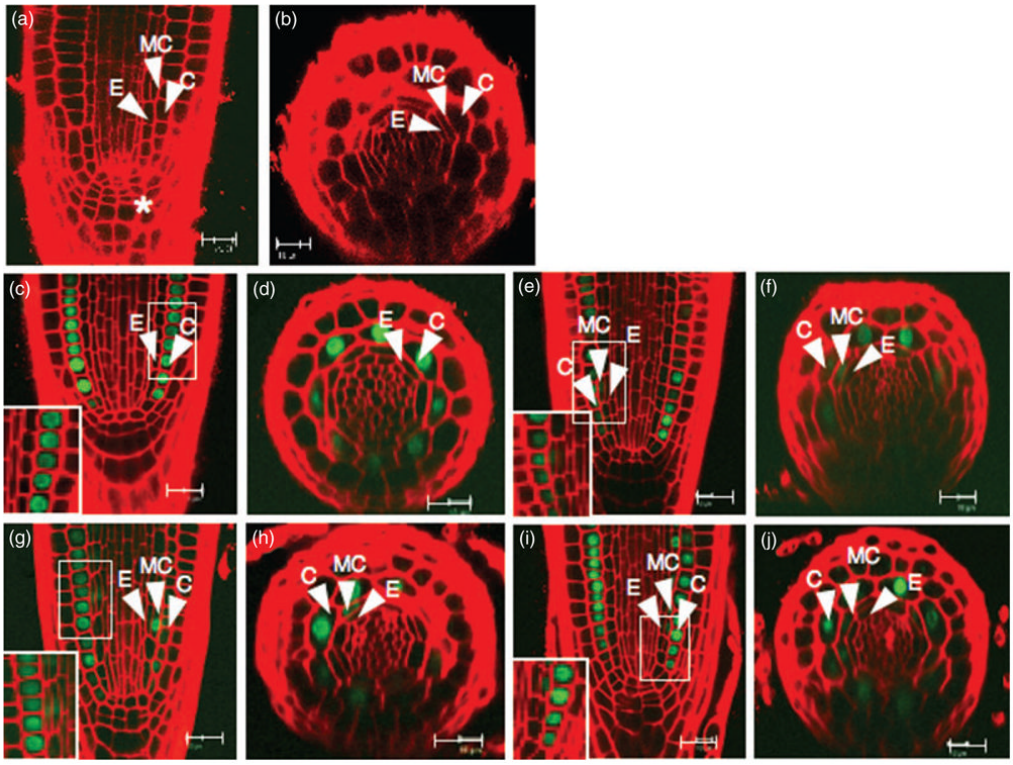

Figure 4. TSA causes premature middle cortex formation

(a,b) Confocal microscope images of wild-type roots 6 days after germination on MS medium containing 1 μg ml^{-1} TSA.

(c–j) Time-course study of middle cortex formation in wild-type roots that express the cortex marker pCO2:HYFP. Three-day-old seedlings were transferred onto MS medium containing 1 μg ml^{-1} TSA, and confocal microscope images were taken 8 h (c,d), 24 h (e,f), 48 h (g,h) and 72 h (i,j) after transfer. The framed areas in (a), (c), (e) and (f) are also shown at higher magnification (1.5× higher). C, cortex; E, endodermis; MC, middle cortex; QC, quiescent center. Scale bar = 20 μm.

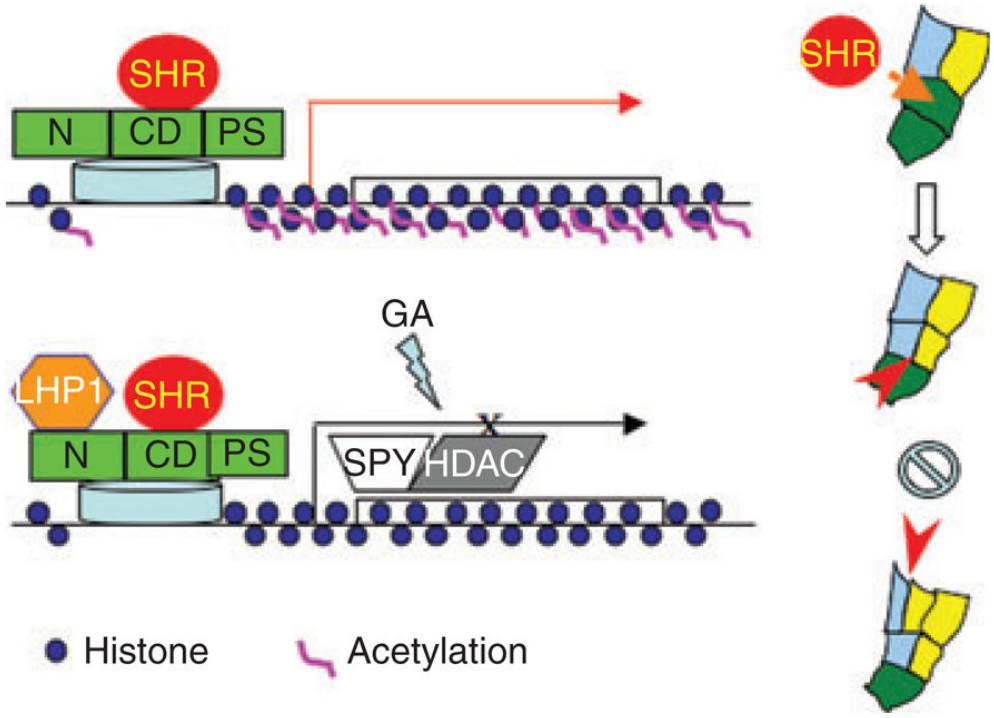

Figure 5. Model depicting the interplay between SCR, GA and the epigenetic machinery in cortex proliferation in the Arabidopsis root

Upon entry into the CEID, SHR together with SCR initially activates cell division genes, but the transcription is rapidly quenched by the interaction between SCR and LHP1, which may be preceded by changes in histone modifications due to other mechanisms. In parallel, GA also suppresses the cell division genes through SPY, which potentially interacts with and modulates the activity of associated HDACs.

Table 1

SCR-interacting proteins identified using the yeast two-hybrid method

AGI	Gene
At1g27050	Homeobox-leucine zipper family protein, with an RRM RNA binding domain
At3g18960	Transcriptional factor B3 family protein
At5g17690	Like heterochromatin protein (LHP1)
At1g72160	SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein
At2g01540	C2 domain-containing protein similar to zinc finger and C2 domain protein
At1g75750	Gibberellin-regulated protein 1 (GASA1)
At3g32980	PER32
At1g78570	NAD-dependent epimerase/dehydratase family protein
At1g07920	Transcription elongation factor 1a
At4g11880	MADS box protein (AGL14)
At2g45660	MADS box protein (AGL20)