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Spatial coordination between stem cell activity and cell differentiation in the root meristem

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SUMMARY

A critical issue in development is coordination of the activity of stem cell niches with differentiation of their progeny to ensure coherent organ growth. In the plant root, these processes take place at opposite ends of the meristem and must be coordinated with each other at a distance. Here we show that in *Arabidopsis* the gene *SCR* presides over this spatial coordination. In the organizing centre of the root stem cell niche, *SCR* directly represses the expression of the cytokinin-response transcription factor *ARR1*, which promotes cell differentiation, controlling auxin production via the *ASB1* gene and sustaining stem cell activity. This allows *SCR* to regulate, via auxin, the level of *ARR1* expression in the transition zone where the stem cell progeny leave

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SUPPLEMENTAL INVENTORY

Supplementary Information contains: Supplementary Figures and Legends 1-5; Supplementary Methods and additional References. Supplementary Figure 1 provides additional data on the genetic relationship between, and the tissue specificity of, *SCR* and the cytokinin signaling pathway. It is related to main Figure 1 because it provides further evidence that *SCR* suppresses *AHK3*-mediated differentiation input in the QC cells.

Supplementary Figure 2 provides additional data on the genetic relationship between *SCR* and the cytokinin-response transcription factor *ARR1*. It is related to main Figure 2 because it provides further evidence that *SCR* specifically suppresses *ARR1*-mediated differentiation input indicating that ectopic expression of *ARR1* in the *scr* QC induces cell differentiation in the SCN via a mechanism different from that in the TZ.

Supplementary Figure 3 provides additional data on the molecular relationship between *SCR* and *ARR1*. It is related to main Figure 3 because it provides further evidence that *SCR* directly suppresses *ARR1* in the QC cells.

Supplementary Figure 4 shows how *SCR* maintains SCN activity. It is related to main Figure 4 since it further confirms that SCN and meristem defects of *scr* mutant are dependent on the *ARR1*-mediated control of *ASB1* expression.

Supplementary Figure 5 shows that both *SCR* and *ARR1* are auxin inducible. It is related to main Figure 5 because it provides further evidence that *SCR* controls *ARR1* at the TZ from the QC, via auxin.

the meristem, thus controlling the rate of differentiation. In this way, SCR simultaneously controls stem cell division and differentiation ensuring coherent root growth.

INTRODUCTION

In multicellular organisms, stem cell division and differentiation of the progeny cells must be coordinated to ensure coherent growth. In the Arabidopsis root, stem cells reside in the apical region of the meristem where they surround a small group of organizer cells. Together, they form a stem cell niche (SCN) (van den Berg et al., 1997; Scheres, 2007) (Figure 1A). As in the animal stem cell niche, the organizer cells, called the quiescent center (QC), maintain the divisional activity of the surrounding stem cells by means of unknown short range signals (van den Berg et al., 1997; Scheres, 2007). Stem cells generate transit-amplifying cells, which undergo additional divisions then differentiate in the transition zone (TZ, Figure 1A) (Moubayidin et al., 2009; Perilli et al., 2010). For meristem maintenance, and therefore continuous root growth, the rate of differentiation of transit-amplifying cells must be equal to the rate of generation of new cells. We have shown that, in the TZ, this balance is ensured by the interaction between two hormones: cytokinin, which promotes cell differentiation (Dello Ioio et al., 2007), and auxin, which promotes cell division (Blilou et al., 2005). In particular, cytokinins are perceived at the TZ by the ARABIDOPSIS HISTIDINE KINASE 3 (AHK3) cytokinin receptor, which transfers the signal, via a phosphorelay, into the nucleus and activates two cytokinin primary response transcription factors, ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) and 12 (ARR12) (Hwang et al., 2012). These genes activate the transcription of the *SHORT HYPOCOTYL 2* gene (*SHY2*), an inhibitor of auxin signalling (Tian et al., 2002; Dello Ioio et al., 2008). *SHY2*, in turn, negatively regulates the expression of several *PIN-FORMED (PIN)* genes encoding auxin transport facilitators (Friml, 2010), thus limiting auxin transport and distribution and allowing cell differentiation (Dello Ioio et al., 2008; Moubayidin et al., 2010).

Root SCN maintenance requires the activity of SCR, a member of the GRAS family of transcription factors (Di Laurenzio et al., 1996; Pysh et al., 1999; Lee et al., 2008; Sabatini et al., 2003). SCR was originally identified as a key factor regulating the asymmetric cell division producing the cortex and endodermis (Di Laurenzio et al., 1996; Heidstra et al., 2004; Cui et al., 2007). More recently, it has also been shown to be involved in the specification of xylem cell types within the vascular tissue (Carlsbecker et al., 2010) and in controlling ground tissue stem cell asymmetric division (Sozzani et al., 2010; Cruz-Ramírez et al., 2012). In addition, SCR has been shown to be necessary and sufficient, when acting in the QC cells, to sustain QC functions and, as a consequence, the surrounding stem cells (Sabatini et al., 2003), but the molecular mechanism for this activity remains unknown.

While molecular mechanisms regulating stem cell division (Spradling et al., 2001; Scheres, 2007; Sablowski, 2011; Wolpert and Tickle, 2011) and transit-amplifying cell differentiation (Wolpert and Tickle, 2011; Dello Ioio et al., 2008; Tsukagoshi et al., 2010) have been described, little is known as to how these two events are coordinated (Mondal et al., 2011).

Here, we show that SCR acts in the QC to control, at the same time, stem cell activity and the differentiation of their progeny by cell-autonomously preventing expression of the

cytokinin response regulator *ARR1* in the QC, and non-cell autonomously controlling *ARR1* expression in the TZ via auxin.

RESULTS

SCR sustains stem cell and meristem activity by suppressing cytokinin perception

We hypothesized that the coordination between stem cell activity in the SCN and cell differentiation in the TZ might be effected by a genetic interaction between key molecular components directly regulating each zone of the root meristem. We therefore asked whether a genetic interaction may exist between SCR, which is involved in QC and stem cell maintenance (Sabatini et al., 2003) and the cytokinin receptor AHK3, which mediates cell differentiation in the TZ (Dello Ioio et al., 2007). Mutation in the *SCR* gene results in meristem consumption and arrested root growth a few days after germination (Figure 1B), due to defective QC and stem cell activities (Figures 1C–1E) (Sabatini et al., 2003). Mutation in the *AHK3* gene results in an enlarged meristem and a longer root, due to delayed cell differentiation in the TZ (Figure 1B) (Dello Ioio et al., 2007). Interestingly, in *ahk3-3;scr-1* double mutants, meristem size and root growth are maintained over time (Figures 1B, S1A and S1D). In the *scr-1* mutant, the QC markers, *QC25* and *QC46*, are absent due to loss of QC identity, while cells immediately below the QC, at the position of columella stem cells, acquire differentiation markers such as amyloplasts and the *Q1630* marker (Figures 1C–1E) (Sabatini et al., 2003). By contrast, in the *ahk3-3;scr-1* double mutant, *QC25* and *QC46* expression is restored, and no amyloplasts or *Q1630* expression are detected within the columella stem cell layer (Figures 1C–1E). In addition, root hairs and xylem strands, characteristic of fully differentiated epidermal and vascular cells, are present in *scr-1* meristems (Figure S1D) (Sabatini et al., 2003) but are absent from the *ahk3-3;scr-1* root meristem (Figure S1D). These data indicate that in *ahk3-3;scr-1* roots stem cell maintenance is restored, leading to meristem maintenance and root growth. Thus, this suggests that SCR sustains stem cell and meristem activity by suppressing cytokinin perception. It is important to note that the *ahk3-3;scr-1* double mutant still displays an undivided ground tissue layer (Figure 1F) (Di Laurenzio et al., 1996), and a lack of protoxylem cells characteristic of the *scr-1* mutant (Figure S1B) (Carlsbecker et al., 2010), suggesting that its role in stem cell regulation is independent of its developmental role in these tissues.

SCR suppresses cytokinin activity in the QC

Comparison of the expression patterns of *SCR* (Wysocka-Diller et al., 2000) and *AHK3* (Dello Ioio et al., 2007) (Figure S1C) suggested that these genes might genetically interact either in the QC, where SCR maintains QC identity (Sabatini et al., 2003), or in the endodermis of the TZ, where AHK3 mediates cell differentiation (Dello Ioio et al., 2007). To determine if and where the interaction occurs, we exploited a tissue-specific complementation approach to restore AHK3 activity (and thus cytokinin perception) either, specifically in the QC, or in the TZ of the *ahk3-3;scr-1* double mutant. To accomplish this, we drove *AHK3* expression with the *WUS-RELATED HOMEBOX 5* (*WOX5*) promoter, which confers expression in the QC, (Sakar et al., 2007) and the *ROOT CLAVATA HOMOLOG 2* (*RCH2*) promoter, which confers expression in the TZ (Dello Ioio et al.,

2007) (Figure S1C). In *ahk3-3;scr-1/pRCH2::AHK3* plants, both meristem size and root growth are indistinguishable from that of *ahk3-3;scr-1* (Figures 1B and S1A and S1D). By contrast, the meristem of *ahk3-3;scr-1/pWOX5::AHK3* plants is consumed and root growth arrested as in the *scr-1* mutant (Figures 1B and S1A and S1D). These results indicate that AHK3 and SCR interact in the QC, thus suggesting that SCR maintains the SCN and meristem activity by suppressing cytokinin signalling in these cells.

SCR prevents SCN differentiation and maintains meristem activity by suppressing *ARR1*

To understand how SCR represses cytokinin-mediated cell differentiation, we examined *AHK3* expression in the *scr-1* mutant by means of qRT-PCR analysis and a *pAHK3::AHK3:GUS* translational fusion (Dello Ioio et al., 2007). Interestingly, neither the expression nor the accumulation of AHK3 (Figures S2A and S2B) is altered in the *scr-1* mutant. We have previously shown that AHK3 controls the rate of cell differentiation in the TZ via the activation of two primary cytokinin response transcription factors: *ARR1* and *ARR12* (Dello Ioio et al., 2007; Moubayidin et al., 2010). We therefore analysed the expression of *ARR1* and *ARR12* in *scr-1* utilizing *pARR1::ARR1:GUS* and *pARR12::ARR12:GUS* translational fusions (Dello Ioio et al., 2007; Moubayidin et al., 2010), as well as by qRT-PCR. Neither *ARR12* protein (Figure S2A) nor mRNA (Figure S2B) levels are altered, while the levels of both mRNA and protein of *ARR1* are upregulated in both *scr-1* and *scr-4* mutant backgrounds (Figures 2A, 2B -*scr-1* and 3A, 3B -*scr-4*). The *ARR1* protein, which is normally expressed in all tissues of the TZ, was ectopically expressed in the proximal meristem, including the SCN, of *scr* plants (Figures 2B, 3A and 3B). This suggests that SCR maintains the activities of both QC and stem cells by suppressing *ARR1* transcription, and therefore cytokinin signaling, in the QC. To further test this hypothesis, we analysed the root phenotype of plants carrying a double mutation in both *SCR* and *ARR1* genes. Similar to the *ahk3-3;scr-1* double mutant, the *arr1-4;scr-1* double mutant displays sustained meristem and indeterminate root growth (Figures 2C, S2C and S2D) due to an active SCN, as visualized by the presence of QC markers absent in the *scr-1* mutant (Figures 2D and S2E). In addition, amyloplasts were absent from the columella stem cells (Figures 2D and S2E). By contrast, the root phenotype of the *arr12-1;scr-1* double mutant was indistinguishable from that of *scr-1* (Figures 2C, S2C and S2D), consistent with the hypothesis that SCR maintains the stem cell niche and root growth by specifically suppressing *ARR1* expression.

SCR directly suppresses *ARR1* in the QC and acts non-cell autonomously to control *ARR1* at the TZ

The fact that *SCR* is expressed exclusively in the QC and endodermal lineage implies that SCR regulates *ARR1* cell autonomously in the QC but that outside the QC its regulation is non-cell autonomous.

To analyse SCR activity in the QC, we employed the GAL4VP16/UAS transactivation system (Brand and Perrimon, 1993; Sabatini et al., 2003). This allowed us to express an inducible version of the SCR protein fused to the glucocorticoid receptor (GR) in the *scr-4* mutant background and under control of the *WOX5* promoter, and simultaneously mark the SCR:GR expressing cells with GFP (*scr-4/pWOX5>>SCR:GR>>GFP* plants) (Figure S3A).

Four hours of SCR:GR activation with dexamethasone (Dex) were sufficient to downregulate *ARR1* mRNA specifically in the QC region, as shown by qRT-PCR experiments on sorted QC cells (using fluorescence activated cell sorting) (Figure 3C). However, there was no change in expression in the rest of the root meristem, as visualized by *pARR1::ARR1:GUS* expression (Figures 3A and 3B, compare 0h Dex with 4h Dex). *ARR1* down-regulation was followed by recovery of stem cell activity but not meristem size, after 8 hours of Dex treatment (Figures 3A, 3B and 3D, compare 0h Dex with 8h Dex). This was coincident with the time when *ARR1* expression was repositioned at the TZ (Figure 3A, 8h Dex). Root meristem size recovery occurred after 24 hours of Dex treatment (Figures 3A and 3D, 24h Dex). These results support the hypothesis that SCR suppresses *ARR1* expression in the QC to maintain stem cell activity. Importantly, these results also provide evidence that SCR acts on *ARR1* non-cell autonomously to position it at the TZ thereby controlling meristem size.

To determine if SCR binds directly to the *ARR1* promoter, we performed chromatin immunoprecipitation (ChIP) followed by quantitative PCR (ChIP-qPCR) on plants containing a complementing *SCR:GFP* chimeric protein under the control of its own promoter in the *scr-4* background. Initially, we used whole root meristems but found no enrichment for binding to the *ARR1* promoter region with the exception of region #3, where enrichment was not statistically significant (Figure S3B). If SCR binds the *ARR1* promoter specifically in the QC, we reasoned that we may have been diluting the ChIP signal when we used the entire meristem. Therefore, we next performed ChIP on extracts from dissected longitudinal sections (3–5 cell lengths) that encompassed the QC (Brady et al., 2007). In this QC-enriched material we found that binding to region #3 in the *ARR1* promoter was significantly enriched (Figure 3E), providing evidence that SCR directly binds to the *ARR1* promoter in the QC.

Suppression of *ARR1* expression in the QC titrates auxin production

We have previously shown that *ARR1* promotes cell differentiation in the TZ by directly activating the *SHY2* gene (Dello Ioio et al., 2008). Therefore, we hypothesized that in the QC of *scr-1* roots, *SHY2* is activated by *ARR1*, which triggers the onset of differentiation. However, we did not detect ectopic expression of *SHY2* in the *scr-1* QC (data not shown), and elimination of *SHY2* activity in the *scr-1* mutant did not restore root growth and meristem size (Figures S2C and D). This suggests that ectopic expression of *ARR1* in the *scr* QC induces stem cell differentiation via a different mechanism.

It was recently shown that cytokinin can induce auxin biosynthesis in the *Arabidopsis* root apex (Jones et al., 2010) and that high levels of auxin in the SCN result in stem cell differentiation (Ding and Friml, 2010). We observed abnormally high levels of auxin in *scr-1* root meristems, as visualized by *DR5::GFP* expression (Sabatini et al., 1999; Ottenschläger et al., 2003) and as measured by mass spectrometry (Figures 4A and 4B). *DR5::GFP* localization reverted to normal in the *arr1-4;scr-1* double mutant (Figure 4A), suggesting that excess auxin biosynthesis in *scr-1* is induced by ectopic expression of *ARR1* in the QC. Among the auxin biosynthesis genes induced by cytokinin (Jones et al., 2010) and expressed in the QC (Sun et al., 2008; Bartel and Fink, 1994; <http://www.arexdb.org>), *NITRILASE 3*

(*NIT3*), *NITRILASE 4* (*NIT4*) and *YUCCA6* (*YUC6*) are over-expressed in the *scr-1* mutant and revert to wild type level in the *arr1-4;scr-1* (Figure S4A). Since these genes control different branches of the tryptophan-dependent auxin biosynthesis pathway (Stepanova et al., 2011), we decided to prevent enzymatic redundant activities by focusing on the *ANTHRANILATE SYNTHASE BETA SUBUNIT 1* (*ASB1*) gene, which is expressed in the SCN and catalyzes a rate-limiting step of tryptophan biosynthesis (Stepanova et al., 2005). *ASB1* is induced by cytokinin, as visualized by the *pASB1::GUS* transcriptional fusion and confirmed by qRT-PCR (Figures S4B and S4C). Moreover, its induction is dependent on ARR1 function, since cytokinin treatment of the *arr1-4* mutant had no effect on *ASB1* levels (Figures S4B and S4C). In addition, a very brief staining of this transcriptional fusion shows that the expression of *ASB1* is highly upregulated in the QC of the *scr-1* mutant and reverted to wild-type levels in the *arr1-4;scr-1* double mutant (Figures 4C and S4D). This suggests that overexpression of *ASB1* in the *scr-1* mutant background depends on ARR1. To exclude the possibility that *ASB1* transcriptional activation is under the direct controls of SCR, we performed ChIP-qPCR analysis as described above for *ARR1*. Our ChIP-qPCR results suggest that SCR does not directly regulate *ASB1* (Figure S3C). Thus, in the QC of *scr-1*, loss of repression of *ARR1* leads to over expression of *ASB1* and consequently over-accumulation of auxin in the root meristem.

To determine if over-accumulation of auxin is responsible for stem cell differentiation in *scr-1*, we introduced the *ASB1* loss-of-function mutation *wei7-4* (*WEAK ETHYLENE INSENSITIVE 7*) (Stepanova et al., 2005) into the *scr-1* background. Consistent with our hypothesis, the root of *wei7-4;scr-1* displays a stable root meristem and indeterminate growth (Figures 4D and S4E) due to a functional SCN (Figures 4E and S4F). This phenotype is similar to the one of *arr1-4;scr-1* previously described (Figures 2C and S2D). In addition, *DR5::GFP* expression in the root tip of *wei7-4;scr-1* reverted to lower levels compared to *scr-1* (Figure 4A), further supporting the hypothesis that *ASB1* upregulation in the *scr-1* QC is responsible for the abnormal accumulation of auxin in this mutant.

Therefore, our working model is that in *scr-1*, lack of *ARR1* repression in the QC cells leads to *ASB1*-dependent auxin over-accumulation responsible for stem cell inactivation, meristem consumption and determinate root growth (Figure S4G). At the same time, we are showing that *SCR* can be induced by auxin (Figure S5A), thus generating a local feedback loop involved in homeostasis of auxin levels responsible for SCN activity.

SCR regulates ARR1 non-cell autonomously through auxin

We next asked if auxin could mediate the non-cell autonomous effect of SCR on *ARR1* expression at the TZ (Figure 3A). We found that exogenous auxin application induces *ARR1* expression in the TZ (Figures S5B and S5C). To further test this hypothesis, we employed a construct expressing the *IAAH* gene, the bacterial auxin biosynthetic enzyme, driven by the *WOX5* promoter (*pWOX5::IAAH*) (Figure S5D) (Blilou et al., 2005). In *pWOX5::IAAH* plants auxin biosynthesis can be induced specifically in the QC after indole-3-acetamide (IAM) auxin precursor treatment (Blilou et al., 2005). We observed that, as early as at 3 hours of IAM treatment, a QC-specific increase of auxin levels was sufficient to induce *ARR1* expression in the TZ but not in the QC (Figures 5A and 5B). To verify that auxin

produced in the QC was transported in the TZ and that this resulted in *ARR1* transcriptional activation, we treated plants in the presence of N-naphthylphthalamic acid (NPA), an inhibitor of auxin transport (Jacobs and Rubery, 1988). Production of auxin in the QC, via the *pWOX5::IAAH/IAM* system, resulted in accumulation of the auxin sensor *DR5::GFP* at the TZ. We could show that this accumulation is prevented via NPA treatment (Blilou et al 2005) (Figure S5D). Interestingly lack of *DR5::GFP* accumulation at the TZ upon NPA treatment was accompanied by lack of *ARR1* induction at the TZ (Figure 5A), strongly suggesting that auxin is the non-cell autonomous factor that controls *ARR1* activity in the TZ from the QC.

To determine if ASB1-dependent auxin production in the QC could control *ARR1* activity in the TZ, we analyzed *ARR1* expression in the *wei7-4* mutant. We observed lower *ARR1* mRNA and protein levels and an enlarged root meristem (Figures 5A, 5C and 5D). Interestingly, *ARR1* protein levels reverted to wild type after 3 hours of exogenous auxin application (Figure 5A), followed by root meristem size complementation after 16 hours of auxin application (Figure 5D). We then asked if auxin produced in the QC is sufficient to induce cell differentiation in the TZ and if so, if it acts through *ARR1*. Consistent with this hypothesis, we observed that root meristem size of wild-type plants carrying a *pWOX5::IAAH* construct was reduced after 24 hours of IAM supplying, and thus of auxin production, while no meristem size reduction was observed when the same construct was introduced into the *arr1-4* background (Figure 5E). Taken together, these results indicate that auxin is the non-cell autonomous signal, which originates in the QC and controls *ARR1* activity in the TZ (Figure 6). We hypothesize that heightened levels of auxin induce cell differentiation in the TZ via the cytokinin signalling pathway.

DISCUSSION

In both plants and animals, organ morphogenesis depends on the coordination between the activities of cells belonging to different tissues and at different developmental stages. In particular, SCN activity and differentiation of their progeny must be coordinated to ensure coherent growth. As observed in animal organogenesis (e.g. growth of mammalian bones or the gut crypt) (Spradling et al., 2001; Wolpert and Tickle, 2011), stem cell division and terminal differentiation of the progeny in the plant root take place in physically distinct zones separated by a group of transit-amplifying cells (Scheres, 2007; Sablowski, 2011). Here we show that in *Arabidopsis*, the *SCR* gene acts from the QC to coordinate stem cell division and cell differentiation (model in Figure 6). It had been previously shown that *SCR* is necessary and sufficient to maintain the SCN (Sabatini et al., 2003) but the molecular mechanisms through which it sustains QC and stem cell activity are unknown. We show that *SCR* directly represses *ARR1* specifically in the QC thereby repressing cytokinin dependent cell differentiation and sustaining SCN activity (model in Figure 6). This is in line with what is already observed in animal systems where master regulatory transcription factors mediate stem cell maintenance through direct transcriptional repression of factors that in turn would promote differentiation (Bernstein et al., 2006; Mikkelsen et al., 2007). Ectopic expression of *ARR1* in the *scr-1* QC induces stem cell differentiation via a mechanism different from the one acting in the TZ, which relies on the transcriptional activation of the *SHY2* gene (Dello Ioio et al., 2008). In the QC, *ARR1* regulates auxin production by modulating

expression of the auxin biosynthesis gene *ASB1*. *ASB1* controls a rate-limiting step in auxin biosynthesis and is specifically expressed in the SCN, being most active in the QC. Interestingly, other genes involved in auxin biosynthesis and acting downstream of the *ASB1* pathway, such as TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (*TAA1*) (Stepanova et al., 2008), *NIT3* (Sun et al., 2009), CYTOCHROME P450 FAMILY 79 SUBFAMILY B POLYPEPTIDE 2 (*CYP79B2*) and *CYP79B3* (Ljung et al., 2005) and *YUCCA2* (*YUC2*) (Sun et al., 2009), are specifically expressed in the QC, thus suggesting that the QC is the primary source of auxin in the root meristem. A future challenge will be to understand how auxin produced in the QC controls the activity of the surrounding stem cells. Recently, it has been shown that *SCR* in the cortex/endodermis stem cell is part of a complex specifically involved in ground tissue stem cell asymmetric division (Cruz-Ramírez et al., 2012). The activity of this complex depends on auxin levels (Cruz-Ramírez et al., 2012) leading to the intriguing hypothesis that *SCR*, by controlling auxin biosynthesis in the QC, controls division of the other stem cell types modulating the activities of similar complexes. On the other hand, it is well known that titration of auxin levels in the SCN is critical to the maintenance of stem cell activity since it has been shown that high levels of auxin promote stem cell differentiation (Ding and Friml, 2010).

By controlling the level of auxin production in the QC, *SCR* also exerts a long-distance control on *ARR1* in the TZ. Auxin produced by *ASB1* in the QC, distributed in the meristem via the auxin transport facilitators (*PIN*, *PGP* genes etc.) (Friml, 2010), is sufficient to activate expression of *ARR1* in the TZ, where *ARR1* controls *SHY2* expression. *SHY2* negatively controls *PIN*s activities and auxin transport enabling cytokinin to trigger differentiation of transit-amplifying cells (model in Figure 6) (Dello Ioio et al., 2008). It is worth mentioning that *SCR*, by controlling activity of the *PHABULOSA* gene (Carlsbecker et al., 2010) also controls *ARR1* protein activity by regulating cytokinin biosynthesis in the vascular tissues (Dello Ioio et al., 2012).

In conclusion, we propose a model in which the SCN organizer not only controls division of the surrounding stem cells (van den Berg et al., 1997), but also regulates the differentiation of the transit-amplifying cells in the TZ. QC activity depends on *SCR* (Sabatini et al., 2003), which suppresses *ARR1* thus titrating auxin production via the *ASB1* gene. Auxin produced in the QC not only controls stem cell division activity by an as yet unknown mechanism, but at the same time acts as a long distance signal to fine tune the level of *ARR1* transcription in the TZ, thus controlling the rate of differentiation. In this way a single gene, *SCR*, regulates the spatial coordination between stem cell division and differentiation ensuring coherent root growth.

EXPERIMENTAL PROCEDURES

Plant materials

The *Arabidopsis thaliana* ecotypes Columbia (Col-0), Wassilewskija (Ws) and Landsberg *erecta* (Ler) were used. *ahk3-3*, *arr1-4*, *arr1-3*, *arr12-1*, *wei7-4* and *wei7-1* mutants are in Col-0 background (Dello Ioio et al., 2007; Dello Ioio et al., 2008; Stepanova et al., 2005), *shy2-31* mutant is in Ler background (Dello Ioio et al., 2008), *scr-1* and *scr-4* mutants are in Ws background (Fukaki et al., 1998; Sabatini et al., 2003). *pAHK3::AHK3::GUS*,

pARR12::ARR12:GUS, *pARR1::ARR1:GUS*, *pWOX5::GFP*, *QC25*, *QC46*, *DR5::GFP*, *pSCR::SCR:GFP*, *pWOX5:IAAH/DR5::GFP* and *pASB1::GUS* transgenic plants have been described previously (Dello Ioio et al., 2007; Dello Ioio et al., 2008; Blilou et al., 2005; Sabatini et al., 2003; Stepanova et al., 2005; Ottenschläger et al., 2003). *Q1630* and *J0571* lines belong to Haseloff collection (<http://www.plantsci.cam.ac.uk/Haseloff/assembly/page167/index.html>).

Plant growth conditions

Seeds were surface sterilized using 50% bleach for 10 minutes and then rinsed four times with sterile water. After 5 days of cold treatment, *A. thaliana* seeds were plated and grown, in a near vertical position, at 22°C in long-day conditions (16 hours light/8 hours dark cycle) on 1/2 MS (Murashige & Skoog - Duchefa) supplemented with sucrose as previously described (Perilli and Sabatini, 2010). Plants for IAA measurement were grown as previously described (Ljung et al., 2005).

Root length and meristem size analysis

For root length measurements, plates were photographed and the resulting images were analyzed using the ImageJ software available on-line (<http://rsbweb.nih.gov/ij/>). For meristem size analysis, roots were prepared in a glass slide with chloralhydrate as described in Perilli and Sabatini, 2010, then observed using an Axio Imager.A2 (Zeiss) light microscopy. To measure root meristem size, the number of cortical cells along a cell file (from the QC to the first elongated cell) were counted. To measure root development over time, root length and meristem size were analyzed at different days post germination (dpg): 3 (dpg), 5dpg, 7dpg, 9dpg, 12dpg and 15dpg. For each experiment, at least 90 roots were analyzed, mean and standard deviation were calculated.

Hormonal treatments

5-day old seedlings were transferred to solid 1/2 MS medium containing mock conditions or a suitable concentration of hormone. For auxin treatment, we used indole-3-acetic acid (IAA, Duchefa) at a final concentration of 5 µM, prepared from a 10 mM stock in ethanol. Plants were treated for 2, 3 and 16 hours. For cytokinin treatment, we used trans-zeatin (tZ, Duchefa) at a final concentration of 5 µM, prepared from a 30 mM stock in 1N NaOH. Plants were treated for 4 hours. For auxin induction specifically within QC cells, plants carrying the *pWOX5:IAAH* construct, in wild type and in *arr1-4* mutant background, were treated for 3 hours with 10 µM indole-3-acetamide (IAM, Sigma-Aldrich), a precursor of IAA, prepared from a 10 mM stock in DMSO. To inhibit auxin transport, plants were pretreated 1 hour with 50 µM N-naphthylphthalamic acid (NPA, Duchefa), and then treated for 3 hours with both 10 µM IAM and 50 µM NPA together. For each experiment, at least 90 roots were analyzed.

GUS histochemical assay

To visualize *QC25*, *QC46*, *pASB1::GUS*, *pAHK3::AHK3:GUS* *pARR12::ARR12:GUS* and *pARR1::ARR1:GUS* lines, GUS histochemical assay was performed using the β-glucuronidase substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide, Duchefa)

dissolved in N-N-dimethyl-formamide. X-gluc solution, 100 mM Na₂HPO₄, 100 mM NaH₂PO₄, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X100 and 1 mg/ml X-gluc, was prepared as previously described (Perilli and Sabatini, 2010). 5-day old seedlings were incubated for 16 hours at 37°C in the dark and imaged using the Axio Imager.A2 (Zeiss) microscopy. *pASB1::GUS* in Figure 4C was stained for 30 minutes only. For each experiment, at least 90 roots were analyzed.

Lugol staining

Starch granules in the root tips were stained with Lugol's solution (Carlo Erba Reagenti) for few seconds and then immediately observed and photographed at microscopy as previously described (van den Berg et al., 1997; Sabatini et al., 2003).

Confocal image processing

Confocal images of median longitudinal sections of 5-day old roots were taken using a ZEISS LSM 780 microscope. A 10 µg/ml Propidium Iodide (Sigma) solution was used to visualize the cell wall.

Analysis of ARR1 expression *in vivo* using GUS histochemical assay

In vivo analysis of ARR1 expression in SCR:GR plants after Dex treatment were performed as follows: *scr-4/pWOX5*»*SCR:GR*»*GFP;pARR1::ARR1:GUS* were treated for 4, 8 and 24 hours with 2 µM Dex or an equivalent amount of DMSO as mock treatment. After Dex treatment, plants were stained 16 hours for the β-glucuronidase activity of *pARR1::ARR1:GUS* transgene and visualized as described above. Starch granules were visualized and meristem cell number was counted as described above (Dello Ioio et al., 2007; Sabatini et al., 2003; Perilli and Sabatini, 2010). To analyze long distance effect of both auxin and polar auxin transport on *ARR1*, 5-day old *pWOX5::IAAH;pARR1::ARR1:GUS* plants were treated for 3 hours with 10 µM IAM and with both 10 µM IAM and 50 µM NPA as described above. After treatment, plants were stained for 16 hours for the β-glucuronidase activity of *pARR1::ARR1:GUS* transgene and visualized as described previously (Dello Ioio et al., 2007; Perilli and Sabatini, 2010). Meristem cell numbers were counted as described above. For exogenous auxin application plants were treated for 3 hours with 5 µM IAA and stained as described above. For each experiment, at least 90 roots were analyzed.

FACS and qRT-PCR experiments

scr-4/pWOX5»*SCR:GR*»*GFP* seeds were plated, on top of nylon mesh (Nitex 03-100/44, Sefar America), on ½ MS agar (+ 1% sucrose) plates at a density of approximately 500 seeds per row 5-day old seedlings were transferred onto plates containing 10 µM dexamethasone (Dex, Sigma-Aldrich), prepared from a 10 mM stock in dimethylsulfoxid (DMSO), or an equivalent amount of DMSO, as mock treatment. Root tips (3–4 mm) were collected 4 hours after Dex treatment and protoplasted. QC cells expressing GFP were isolated on a fluorescence activated cell sorter (Becton Dickinson FACSARIAII) as described (Nawy et al., 2005). Sorted cells were collected directly into a RNA lysis buffer (Qiagen RLT buffer, RNeasy Micro kit), mixed and immediately frozen at –80°C. Experiments were carried out

in two consecutive days, obtaining two biological replicates for each treatment (Mock and Dex). mRNA was later isolated and treated with DNase using TURBO DNA-free™ Kit. Each RNA sample was reverse transcribed using the SuperScript™ III first-strand Synthesis system (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed in triplicates from each RNA sample and repeated twice using SYBR Green PCR Master mix (Applied Biosystems) with ViiA™ 7 Real-Time PCR system (Applied Biosystems). Expression levels were calculated relative to *ACTIN2* using the $2^{-\text{ct}}$ method. Statistical analysis was done in MS Excel (ANOVA: Single Factor) using $p < 0.05$. Primers were designed according to the recommendations of Applied Biosystems. Quantitative RT-PCR (qRT-PCR) analysis was conducted using the gene-specific primers listed below:

ARR1 FW: TTGAAGAAACCGCGTGTCTCT and ARR1 RV:
CCTTCTCAACGCCGAGCTGATTAA for *ARR1*

ACT FW: CCTTCTCAACGCCGAGCTGATTAA and ACT RV:
GTGGATTCCAGCAGCTTCCAT for *ACTIN2*

Chromatin immunoprecipitation followed by gene-specific quantitative real-time PCR (ChIP-qRT-PCR)

ChIP was conducted as in Sozzani et al., 2010 on either Col-0, as control, or *pSCR::SCR:GFP/scr-4* 5 days old dissected roots which included longitudinal sections comprised of 3–5 cell lengths from the QC as described in Brady et al., 2007. Immunoprecipitation was done using a rabbit polyclonal antibody to GFP (ab290; Abcam Ltd., Cambridge, United Kingdom). DNA from the ChIP, for both Col-0 and *pSCR::SCR:GFP* experiments, was individually amplified using a random-primer based genome amplification method according to the Agilent Mammalian ChIP-on-chip protocol with minor modifications. Briefly, chromatin from the Col-0 and *pSCR::SCR:GFP* was blunted using T4 DNA polymerase, then linkers were added using T4 DNA ligase (oligo JW102 5'-GCGGTGACCCGGGAGATCTGAATTC-3' and oligo JW103 5'-GAATTCAGATC-3'). The IP was then amplified with 15 PCR cycles (1st LM-PCR) and then the reaction was diluted and used as template for a second round of 20 cycles (2nd LM-PCR). The DNA was cleaned up each time using the MiniElute Reaction Cleanup kit (Qiagen). Enrichment of the *ARR1* putative target promoter-region DNA was determined using RT-qPCR. A qPCR efficiency of 2-fold amplifications per cycle was assumed, and sequences from ubiquitin 10 (UBQ10-F: GGCCTTGTATAATCCCTGATGAATAAG; UBQ10-R: AAAGAGATAACAGGAACGGAAACATAGT) were used to normalize the results between samples. Tiling along the *At3G16857 ARR1* was done using the following sets of adjacent specific amplified regions along the putative 2186 kb region of the *ARR1* promoter. In ascending order upstream from the *ARR1* ATG:

100 - 0 bp: ARR1#6-F: TCGAATAAAACTCGCGTCAA; ARR1#6-R:
ACCTCTCTCTATGTAGCTCGAACC

578 – 396 bp: ARR1#5-F: GCAACATCCAAAATGTTCTTCT; ARR1#5-R:
GCTGACATGTCCTTTATTTTCATC

687 - 580 bp: ARR1#4-F: CCCACTTTCTTCCCAATTAT; ARR1#4-R:
TGAGCTCAAATTCTGGGTTA

1120 - 1049 bp: ARR1#3-F: GTAGGTGATGATGGGAAATG; ARR1#3-R:
TACGTCTACGCACATTCAAG

1568 - 1497 bp: ARR1#2-F: CATTGAGATGAACCATTTGC; ARR1#2-R:
TGTGCGCTTCTTCTCTTAAA

2186 - 2100 bp: ARR1#1-F: GATAGATGGAGAGGTCGATG; ARR1#1-R:
GCTGAGAAGCTTCCCTATT

As negative control we used the following primers:

(+) 595 – 696 bp: ARR1#7-F: TCATGAGGTCCTTGTTGTCAG; ARR1#7-R:
TGCATTTTCGTTACTGCATGTC

(+) 2990 - 3061 bp: ARR1#8-F: CTTTGACGCATACTCCATCG; ARR1#8-R:
GTAGTCCCTCCGGTTTATCG

Detailed descriptions of the transgenic lines construction and analysis, IAA measurement, ChIP techniques and transcript level analysis are provided in the Supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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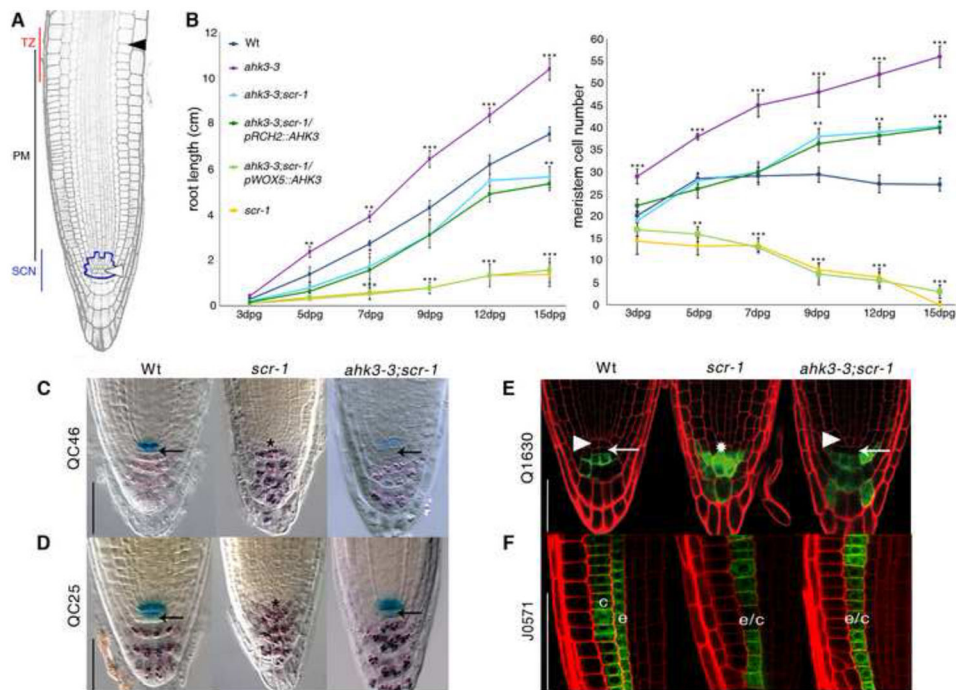


Figure 1. SCR maintains SCN activity by suppressing AHK3-mediated differentiation input in the QC cells

(A) Longitudinal section of the Arabidopsis root meristem. SCN stem cell niche (highlight in blue), PM proximal meristem, TZ transition zone. The white and black arrowheads include meristematic cortex file, indicating QC and the last meristematic cortical cell, respectively.

(B) From left to right, root length and meristem cell number measured over time of wild-type (Wt), *scr-1*, *ahk3-3*, *ahk3-3;scr-1*, *ahk3-3;scr-1/pRCH2::AHK3* and *ahk3-3;scr-1/pWOX5::AHK3*. dp: days post germination. Error bars, SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t-test.

(C-E) Elimination of AHK3 activity rescues SCN defect of *scr-1* mutant. Expression of *QC46* and *QC25* (C and D) and *Q1630* markers

(E) in Wt, *scr-1* and *ahk3-3;scr-1* root tips. (C and D) Double labeling of QC and differentiated columella cells visualized by QC specific markers and amyloplast staining, respectively. Note that black arrows indicate restoration of columella stem cells activity in the *ahk3-3;scr-1* double mutant (C). Lack of *Q1630* expression in the columella stem cells (white arrows) indicates that columella stem cell activity is restored in the *ahk3-3;scr-1* double mutant. White arrowheads indicate QC position in Wt and *ahk3-3;scr-1*. Asterisk indicates the presumptive position of QC cells in *scr-1* mutant.

(F) SCR-mediated cytokinin signalling suppression is not necessary for endodermis activity. *J0571* expression in Wt, *scr-1* and *ahk3-3;scr-1*. Note the lack of asymmetric cell division necessary to generate properly cortex (c) and endodermis (e) tissues in *scr-1* (Di Lorenzo et al., 1996) and in the *ahk3-3;scr-1* double mutant. The resulting monolayer is indicated by c/e. Scale bar represents 50 μm in all panels. See also Figure S1.

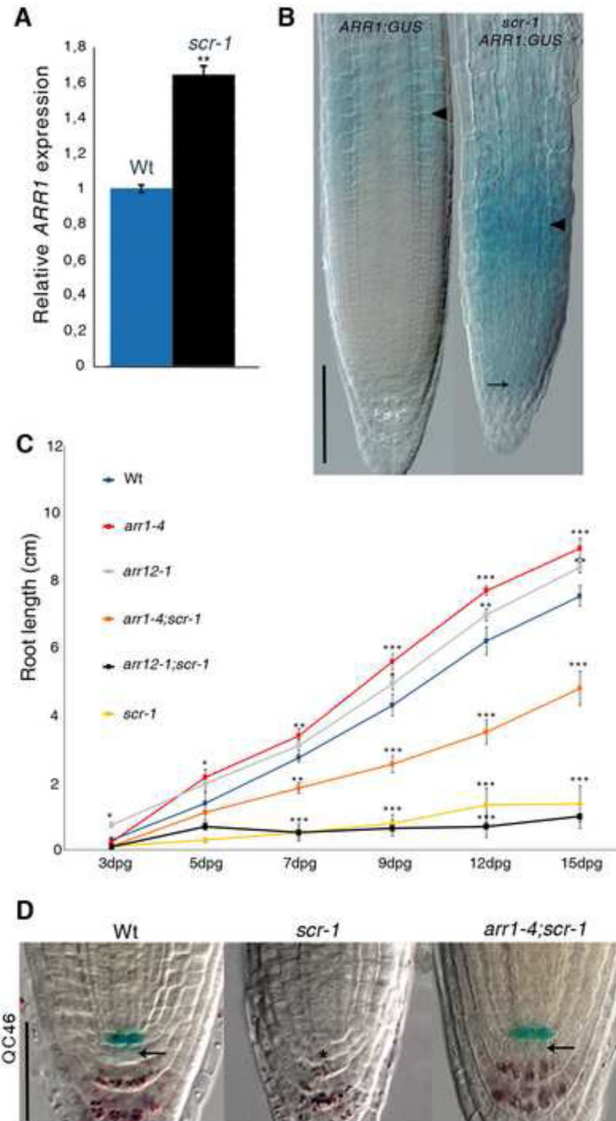


Figure 2. SCR maintains SCN activity by suppressing ARR1

(A) qRT-PCR showing low level of *ARR1* transcript in Wt compared to *scr-1* roots. Relative expression is normalized to *ACTIN2*. Error bars, SD; ** $p < 0.01$ Student's t-test.

(B) Expression of *pARR1::ARR1::GUS* construct in Wt roots and in *scr-1* mutant. Black arrowheads indicate the position of transition zone; arrow indicates *ARR1* ectopic expression in the *scr-1* QC. Scale bar represents 100 μm .

(C) Root length measured over time for Wt, *scr-1*, *arr1-4*, *arr12-1*, *arr1-4;scr-1*, *arr12-1;scr-1*. dpg: days post germination. Error bars, SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t-test.

(D) *QC46* expression in, from left to right, Wt, *scr-1* and *arr1-4;scr-1*. Double labeling of QC and differentiated columella cells visualized by *QC46* and amyloplast staining, respectively. Note that black arrows indicate restoration of columella stem cells activity in the *arr1-4;scr-1* double mutant. Asterisk indicates position of QC cells in the *scr-1* mutant.

The same results have been obtained with the *arr1-3;scr-1*, *arr1-3;scr-4* and *arr1-4;scr-4* double mutant combinations (data not shown). Scale bar represents 50 μm . See also Figure S2.

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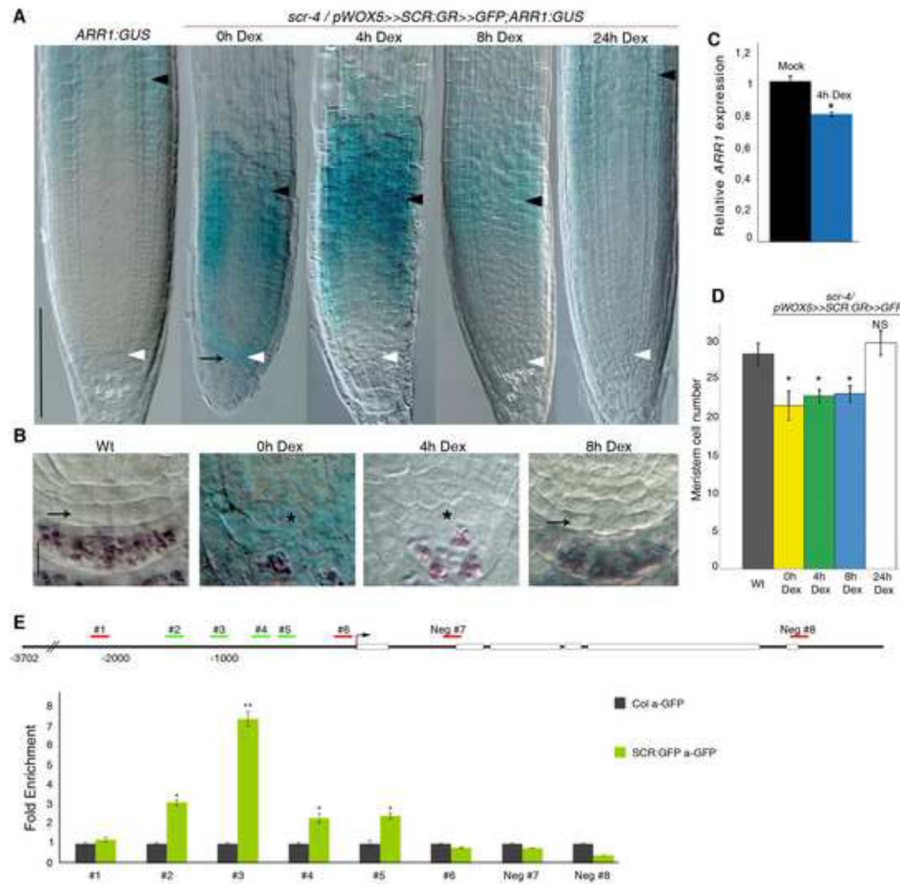


Figure 3. SCR controls stem cell activity by suppressing directly ARR1 in the QC

(A) Expression analysis of the *ARR1:GUS* translational fusion in, from left to right, Wt and *scr-4/pWOX5>>SCR:GR>>GFP* roots untreated (0h Dex) or treated with Dex for 4, 8 and 24 hours. Black arrow indicates ectopic ARR1 expression in the QC. Black and white arrowheads indicate, respectively, the cortex TZ and the QC. Scale bar represents 100 μ m.

(B) Stem cell niche of, from left to right, *ARR1:GUS* and *scr-4/pWOX5>>SCR:GR>>GFP;ARR1:GUS* untreated (0h Dex) or treated with Dex for 4 and 8 hours. Black arrow indicates active stem cells in Wt and *scr-4* roots after 8 hours of Dex treatment. Asterisk indicates the presumptive position of QC cells in *scr-4* untreated and treated with Dex for 4 hours. Scale bar represents 10 μ m.

(C) qRT-PCR performed on sorted QC cells of *scr-4* roots carrying the *pWOX5>>SCR:GR>>GFP* construct, showing significant down regulation of *ARR1* transcription upon 4 hours of Dex treatment. Relative expression is normalized to *ACTIN2*. Error bars, SD; * $p < 0.05$; Student's t-test.

(D) Meristem cell number of, from left to right, Wt and *scr-4/pWOX5>>SCR:GR>>GFP* roots untreated (0h Dex) or treated with Dex for 4, 8 and 24 hours. Error bars, SD; * $p < 0.05$, NS, not significant; Student's t-test.

(E) ChIP-RT qPCR of the *ARR1* promoter using 5 days old Col-0 (grey bars) and *pSCR::SCR:GFP scr-4* (green bars) plants. ChIP samples were prepared from QC-enriched material. RT-qPCR results are shown as fold enrichment compared to Col-0. We found

significant enrichment for the several fragments (#2, #3, #4 and #5) bound by SCR by scanning the 2186 Kb of sequence upstream of *ARR1*. The data shown are representative of three independent biological experiments with similar results. Error bars show the standard deviations of the ChIP-qPCR reactions performed in triplicate. * $p < 0.05$, ** $p < 0.01$; Student's t-test. See also Figure S3.

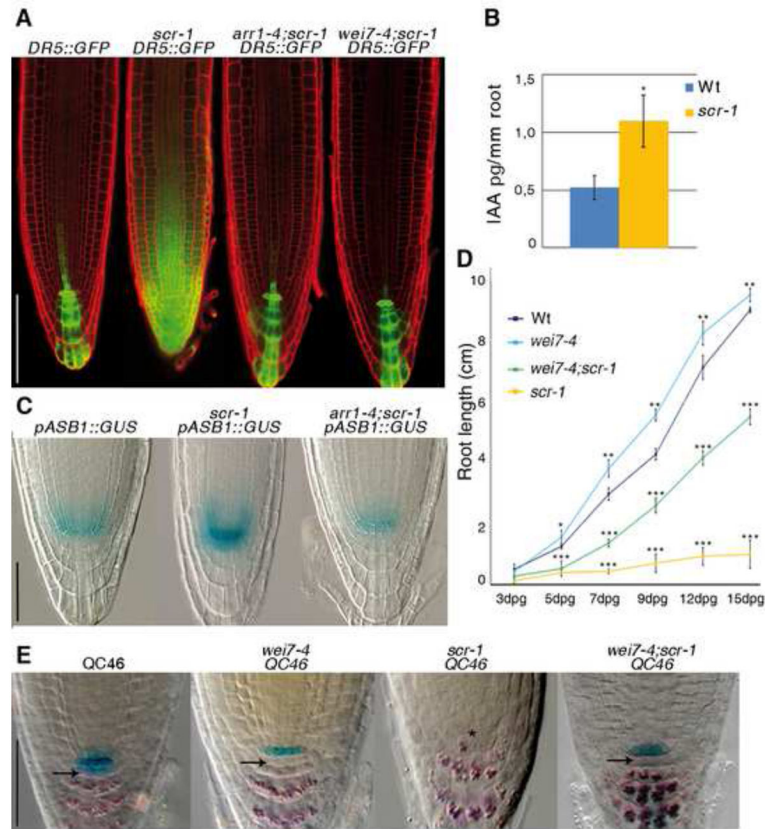


Figure 4. SCR controls auxin biosynthesis in the QC via ARR1

(A) *DR5::GFP* expression in, from left to right, Wt, *scr-1*, *arr1-4;scr-1* and *wei7-4;scr-1* 5-day old root meristems. Scale bar represents 100 μ m.

(B) Concentration of free IAA in root tips of Wt and *scr-1*. Error bars, SD; * $p < 0.05$; Student's t-test.

(C) Expression of the *pASB1::GUS* construct in, from left to right, Wt, *scr-1* and *arr1-4;scr-1*. Note that plants were stained for only 30 minutes. Scale bar represents 50 μ m.

(D) Root length measured over time of Wt, *scr-1*, *wei7-4*, and *wei7-4;scr-1*. dpg: days post germination. Error bars, SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's t-test.

(E) *QC46* expression and lugol staining of Wt, *wei7-4*, *scr-1* and *wei7-4;scr-1*. Black arrows indicate stem cells activity in Wt, *wei7-4* and *wei7-4;scr-1*. Asterisk indicate the presumptive position of QC cells in *scr-1*. The same result has been obtained with the *wei7-1;scr-1* double mutant combination. Scale bar represents 50 μ m. See also Figure S4.

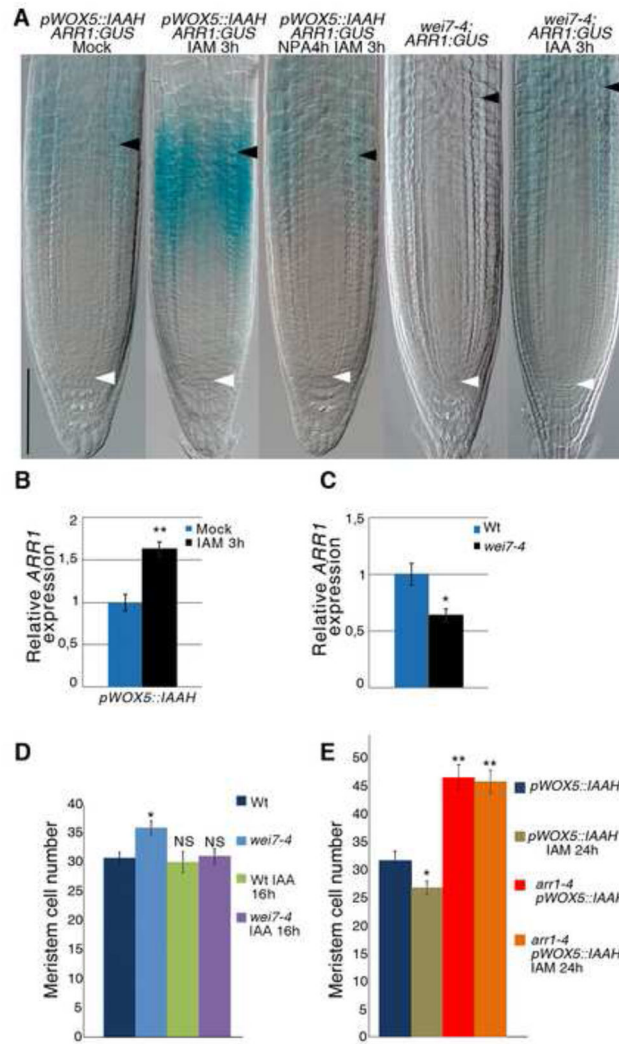


Figure 5. SCR controls *ARR1* activity at the TZ via auxin

(A) Expression of the *ARR1::GUS* translational fusion in, from left to right, mock-treated *pWOX5::IAAH* root, *pWOX5::IAAH* root treated with IAM, *pWOX5::IAAH* root treated with IAM and NPA, *wei7-4* roots untreated or treated with IAA. Black and white arrowheads indicate, respectively, the cortex TZ and the QC. Roots were analyzed 5dpg. Scale bar represents 100 μ m.

(B) qRT-PCR showing upregulation of *ARR1* transcription in *pWOX5::IAAH* roots upon 3 hours of IAM treatment. Error bars, SD; ** $p < 0.01$; Student's t-test.

(C) qRT-PCR showing low *ARR1* transcript levels in *wei7-4*. Error bars, SD; * $p < 0.05$; Student's t-test.

(D) Root meristem cell number in, from left to right, Wt, *wei7-4*, Wt upon 16 hours of IAA treatment, *wei7-4* upon 16 hours of IAA treatment. Error bars, SD; * $p < 0.05$, NS, not significant; Student's t-test. Roots were analyzed 5dpg.

(E) Root meristem cell number in, from left to right, mock-treated *pWOX5::IAAH* root, *pWOX5::IAAH* treated 24 hours with IAM, mock-treated *arr1-4*; *pWOX5::IAAH* and

arr1-4;pWOX5::IAAH treated 24 hours with IAM. Error bars, SD; * $p < 0.05$, ** $p < 0.01$; Student's t-test. Roots were analyzed 5dpg. See also Figure S5.

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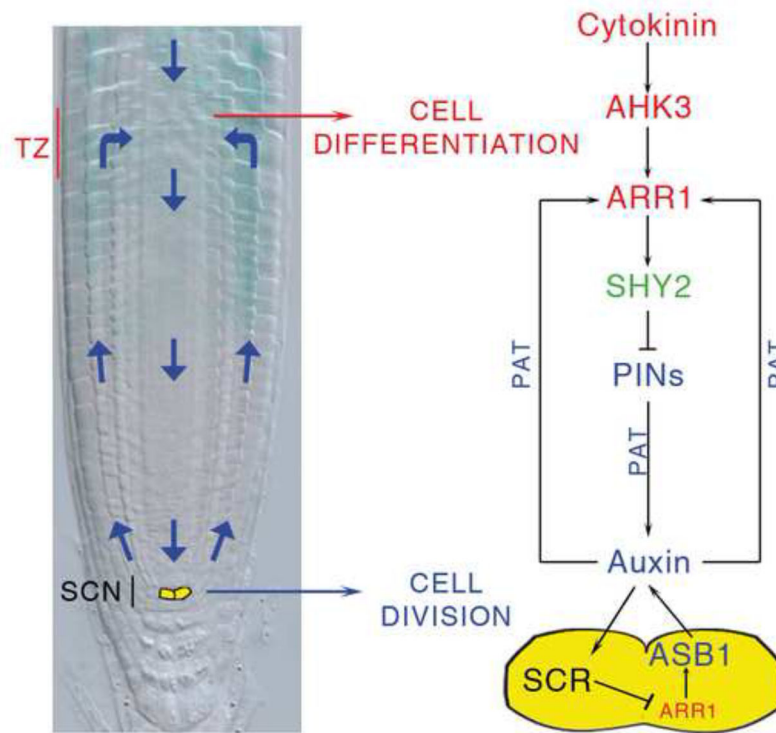


Figure 6. Model for the spatial coordination between SCN and TZ

Model showing how SCR presides over the spatial coordination between stem cell niche (SCN) and transition zone (TZ) activity. In the QC (yellow), SCR represses *ARR1*, which in turn controls auxin production via *ASB1*, thus enabling cell division along the trans-amplifying zone. SCR also exerts, via polar auxin transport (PAT), a long distance control on *ARR1* at the TZ, enabling cytokinin to sustain cell differentiation via *SHY2* (Dello Ioio et al., 2008).