Current Biology 23, 1979–1989, October 21, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.08.008

Article

Cytokinin Induces Cell Division in the Quiescent Center of the *Arabidopsis* Root Apical Meristem

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Summary

Background: In the root apical meristem, which contains the stem cells that feed into root development, the phytohormones auxin and cytokinin play opposing roles, with auxin promoting cell division and cytokinin promoting cell differentiation. Cytokinin acts in the root tip in part by modulating auxin transport through regulation of the level of the PIN auxin efflux carriers. Auxin plays a key role in the specification of the quiescent center (QC), which is essential for maintaining the stem cell fate of the surrounding cells.

Results: We demonstrate that cytokinin promotes cell division in the QC, which is generally mitotically inactive. Cytokinin downregulates the expression of several key regulatory genes in the root tip, including *SCARECROW*, *WOX5*, and the auxin influx carriers *AUX1* and *LAX2*. The decrease in *LAX2* expression in response to cytokinin requires ARR1 and ARR12, two type B ARRs that mediate the primary transcriptional response to cytokinin. ARR1 was found to bind directly to the *LAX2* gene in vivo, which indicates that type B ARRs directly regulate genes that are repressed by cytokinin. Disruption of the *LAX2* gene results in a phenotype similar to that observed in response to cytokinin, including increased division of the cells in the QC and decreased expression of *WOX5* and the auxin response reporter DR5.

Conclusions: Cytokinin acts to regulate auxin distribution in the root apical meristem by regulating both the PINs and LAX2. This redistribution of auxin, potentially coupled with other auxin-independent effects of cytokinin, regulates the mitotic activity in the QC.

Introduction

The growth and development of roots in plants is maintained by a group of stem cells (stem cell niche; SCN) in the root apical meristem. These stem cells maintain a balance between division and differentiation, continually giving rise to the various cell files of the root and thus ensuring the continued growth and development of the root [1]. In the center of the SCN, there are four to eight mitotically inactive cells that form the quiescent center (QC), which is essential for the maintenance of the stem cell fate of the surrounding cells [2]. The *PLETHORA* (*PLT*) and *SCARECROW* (*SCR*) transcription factors are involved in QC specification [3, 4]; the homeodomain transcription factor *WOX5* is required in the QC to maintain the undifferentiated state of the surrounding stem cells [5]. The plant hormone auxin is an important regulator of QC specification [6]. The directed flow of auxin in the root tips generated by the combined action of multiple PIN auxin efflux carriers generates and stabilizes an auxin maximum that specifies the position of the QC [7, 8]. Studies of root regeneration have shown that after ablation of the QC, the auxin maximum shifts to respecify the QC. Furthermore, this shift results in a repatterning of the expression of the *PLT*s and *SCR*, which in turn regulates the expression and polar localization of PINs, thus stabilizing the reconstituted auxin distribution in the root tip [9].

The phytohormone cytokinin interacts with auxin to regulate various aspects of plant growth and development through the control of cell division and differentiation. Recently, several studies have shed light on the mechanism underlying the antagonistic interaction between cytokinin and auxin in the control of the root meristem activity [10–12]. In this model, cytokinin functions in the transition zone to induce the expression of a negative regulator of auxin responsiveness, the *AUX/IAA* gene *SHY2*, via *ARR1*, a type B *Arabidopsis* response regulator (ARR) that mediates the transcriptional response to cytokinin. The induction of *SHY2* in turn leads to a negative regulation of the *PIN* auxin efflux carriers. In the meristematic zone of the root, auxin acts to mediate the degradation of SHY2, thus maintaining the expression of *PIN*s in the meristem. This antagonistic regulation of *SHY2* by cytokinin and auxin restricts the expression of *SHY2* in the transition zone and determines the meristem size. The type A ARRs, which are negative regulators of cytokinin signaling, have been suggested to play important roles in the maintenance of both the root proximal meristem activity and the root stem cell niche function through posttranscriptional regulation of *PIN*s [13]. In addition, an incoherent regulatory loop formed by cytokinin and *PHABULOSA* (*PHB*), a HD-ZIPIII transcription factor, has been shown to determine the balance of cell division and differentiation in the root tip [14]. PHB induces cytokinin biosynthesis through direct activation of a cytokinin biosynthetic gene *ISOPENTENYL TRANSFERASE 7* (*IPT7*) in the proximal meristem, and cytokinin feeds back to repress the expression of both *PHB* and a negative regulator of *PHB*, *MIR165*, which establishes a regulatory circuitry to regulate root proximal meristem size. Despite the well-characterized role of cytokinin in regulating the proximal meristem, little is known about its role in the SCN and QC.

The cytokinin signaling pathway is similar to bacterial two-component phosphorelays [15]. In *Arabidopsis*, cytokinin is perceived by the *Arabidopsis* histidine kinase receptors (*AHK*s), which autophosphorylate and then transfer the phosphoryl group to *Arabidopsis* histidine phosphotransfer proteins (AHPs). Ultimately, the phosphoryl group is transferred from the AHPs to ARRs, which include the type A and type B ARRs. Genetic studies of type B ARRs indicate that they are positive elements in cytokinin signaling, acting as transcription factors to regulate the expression of cytokinin response genes [16]. Unlike the type B ARRs, the type A ARRs lack a DNA-binding domain, and their expression is rapidly induced by

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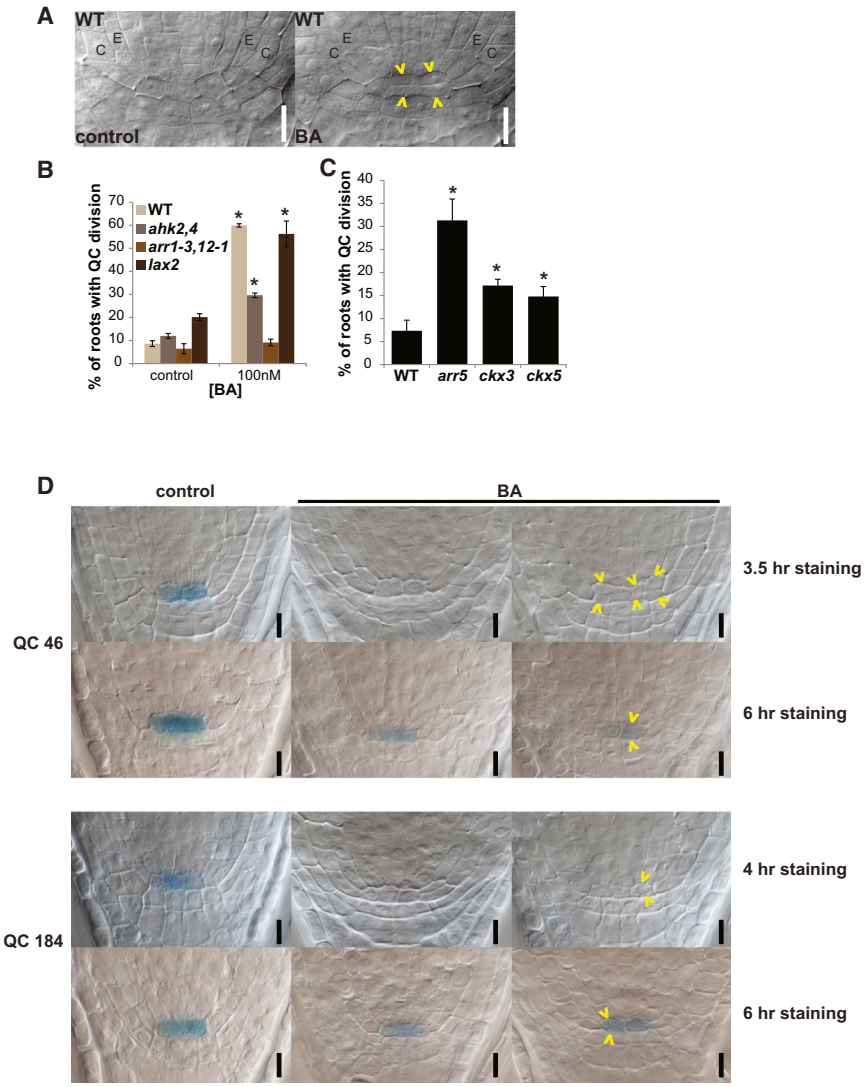

Figure 1. Cytokinin Induces QC Cell Divisions and Affects QC Cell Identity

(A) Root tips of 5-day-old seedlings grown on DMSO control and 100 nM 6-benzylaminopurine (BA) plates visualized by differential interference contrast (DIC) microscopy. Yellow arrowheads indicate the divided QC cells. E, endodermis; C, cortex. Scale bar represents 10 μm.

(B) Percentage of roots with QC divisions from wild-type, *ahk2 ahk4*, *arr1-3 arr12-1*, and *lax2* mutants grown on DMSO control and 100 nM BA plates. At least 50 roots were examined per genotype per experiment. Error bars represent SE from triplicate experiments. *p < 0.05, Student's t test, BA treatment compared to control treatment.

(C) Percentage of roots with QC divisions from wild-type, *arr5*, *ckx3*, and *ckx5* mutants. At least 50 roots were examined per genotype per experiment. Error bars represent SE from triplicate experiments. *p < 0.05, Student's t test.

(D) QC 46 and QC 184 expression with two durations of β-glucuronidase (GUS) staining. Scale bar represents 10 μm.

See also Figure S1.

cytokinin. Furthermore, the type A ARRs act as negative regulators of cytokinin signaling [17].

Auxin transport is mediated by auxin influx and efflux carriers. In *Arabidopsis*, auxin influx carriers are encoded by a small family comprised of four genes: *AUX1*, *LAX1*, *LAX2*, and *LAX3*. Auxin plays a critical role in the initiation of leaf primordium [18], and members of the *AUX1/LAX* gene family have been shown to be involved in this process. An analysis of *aux1/lax* mutants revealed that *AUX1/LAX* facilitates the coordinated polarization of PIN1 and the establishment of defined auxin maxima, which stabilizes the patterning of leaf primordium initiation [19]. Despite the functional conservation of the *AUX1/LAX* family and their redundant roles in maintaining phyllotactic patterning, in the root they display reasonably distinct expression patterns and are suggested to be involved in distinct developmental processes [20]. In the root, *AUX1* has been shown to play a role in gravitropism [21], and both *AUX1* and *LAX3* are shown to regulate lateral root development [22]. Although *LAX2* is strongly expressed in the root tip, its function there is not clear.

Here, we show that cytokinin negatively regulates QC specification and functions by modulating the auxin response in the QC through downregulation of *LAX2* and possibly also by repressing the expression of the transcription factor *SCR*. Furthermore, we demonstrate that the type B response regulator ARR1 directly represses the expression of *LAX2*. These studies, together with previous work on the effect of cytokinin on the PINs, indicate that a key aspect to cytokinin function is to regulate the distribution of auxin via the regulation of both the PINs and AUX/LAX transporters.

Results

Cytokinin Induces Division in QC Cells, Leading to Compromised QC Function

We examined the effect of exogenous cytokinin on QC function in *Arabidopsis* seedlings. In wild-type *Arabidopsis* roots, the QC cells are mitotically inactive, as it was shown previously that no active DNA synthesis is observed in QC cells [23]. Almost 60% of wild-type seedlings grown on plates supplemented with cytokinin displayed cell divisions in the QC (Figures 1A and 1B), consistent with previous studies that demonstrated increased divisions in QC cells in high-order type A *arr* mutants [13]. Additionally, loss of a single type A ARR, *ARR5*, was sufficient to cause the increased QC cell division phenotype (Figure 1C), suggesting that this isoform is predominantly responsible for the effect of type A ARRs on QC cell division. Elevating endogenous cytokinin via mutations in either one of two cytokinin oxidases (*CKX3* and *CKX5*), which encode enzymes that degrade cytokinin, also increased QC cell division as compared to wild-type (Figure 1C). Both of these *CKX* genes are expressed in root tips, and their disruption leads to an increase in endogenous active cytokinins [24]. The cytokinin-induced cell divisions in the QC are dependent on the known cytokinin response pathway, as this phenotype was markedly reduced in an *ahk2 ahk4* double mutant as compared to the wild-type. The type B ARRs are transcription factors that are the targets of the cytokinin-mediated phosphorelay. To explore whether ARR1 and ARR12, the two major type B ARRs

mediating cytokinin response in the root, are involved in this process, we examined the effect of cytokinin on QC cell division in an *arr1-3 arr12-1* double mutant. The cytokinin-induced QC divisions were nearly completely lost in this double mutant (Figure 1B), suggesting that this response requires *ARR1* and *ARR12*.

Previous studies found that ethylene is able to induce QC cell divisions [25]. As cytokinin increases the production of ethylene [26], we examined whether the cytokinin-induced QC cell division is dependent on ethylene. Blocking ethylene signaling through either treatment with 1-MCP, an inhibitor of ethylene binding, or the ethylene-insensitive mutation *ein2* did not compromise the ability of cytokinin to induce QC cell division in the root tips (see Figure S1 available online), indicating that cytokinin-induced QC division is independent of ethylene.

We next determined whether cytokinin affects QC cell identity by examining expression of two QC-specific markers (QC 46 and QC 184) [6]. Seedlings grown in the presence of cytokinin displayed a substantial reduction in the expression of both of these QC markers (Figure 1D), suggesting partial loss of QC identity. The reduction of expression of the markers was observed in both the cytokinin-treated seedlings displaying extra cell divisions in the QC and those with wild-type QC cell division patterns. These results suggest that cytokinin plays a negative role in the maintenance of QC cell identity.

The Expression of Two Transcription Factors, *SCR* and *WOX5*, Is Downregulated by Cytokinin in the QC

To further analyze the role of cytokinin in regulating the QC, we examined the expression of several transcription factors that are involved in QC specification and function in response to cytokinin treatment. Total RNA isolated from the last 0.5 mm of root tips, which includes cells from the root cap to the elongation zone, was used for gene expression analysis using the NanoString nCounter analysis system. The transcript level of *SCR* in the root tips was reduced approximately 17% and 30% after 8 and 24 hr of cytokinin treatment, respectively (Figure 2A). In contrast, the transcript level of *PLT1* or *PLT2* was not altered in response to cytokinin in these tissues (Figure 2B). In addition, the spatial pattern of *SCR* transcript in response to cytokinin was examined using a *pSCR::GFP* reporter line. *SCR* is expressed in the QC and endodermis in the root tips [27]. The reduction of *SCR* expression in response to cytokinin appeared more pronounced in the QC cells (Figure 2D). However, the reduction was not significant until 8 hr after treatment with cytokinin, suggesting that *SCR* might not be a direct target of regulation. Previous studies have shown that *SCR* expression in the QC is necessary and sufficient to establish QC identity [4]. *scr* mutants lack a functional QC, and the root meristem collapses after germination [4]. However, the QC phenotype observed in cytokinin-treated seedlings was not nearly as strong as in the *scr* mutants, which is likely the result of only a partial decrease in *SCR* expression in response to cytokinin. The weaker *scr-3* allele [28] displays a mixture of both QC phenotypes: 35.7% display inappropriate QC divisions, similar to those observed in cytokinin-treated seedlings; 36.7% of *scr-3* seedlings displayed the loss-of-QC phenotype (Figure S2). These results suggest that the downregulation of *SCR* by cytokinin may influence QC specification.

Next, we examined the expression of another QC-specific transcription factor, *WOX5*, in response to cytokinin treatment. *WOX5* transcript was substantially reduced within 8 hr of cytokinin treatment (Figure 2C). The high level of variation observed in the level of *WOX5* transcript measured by the NanoString technique is likely due to the low level of expression of *WOX5*, which was close to the detection limit of this system. In order to confirm the downregulation of *WOX5* by cytokinin, a *pWOX5::GFP* reporter was used. The level of GFP fluorescence was substantially reduced in this reporter line 8 hr after cytokinin treatment (Figure 2F). *WOX5* has been shown to be required for the QC activity to maintain the undifferentiated state of adjacent cells in the SCN, but not for QC specification [5]. *WOX5* expression is dependent on SCR [5], and thus its repression by cytokinin may occur through the downregulation of *SCR* by cytokinin. The downregulation of *WOX5* by cytokinin may contribute to the negative role of cytokinin in the maintenance of QC function.

We used a TaqMan quantitative RT-PCR (qRT-PCR) assay to examine the transcript level of *WOX5* and *SCR* in various mutants to explore the role of endogenous cytokinin on the expression of these two genes. The downregulation of both *SCR* and *WOX5* expression in the root tips in response to cytokinin requires *ARR1* and *ARR12* (type B ARRs), as no reduction was observed in an *arr1-3 arr12* double mutant (Figure 2G). Furthermore, the basal level of expression of *SCR* and *WOX5* is significantly reduced in *ckx5* root tips as compared to wild-type (Figure 2H), indicating that endogenous cytokinin represses the expression of these genes. Finally, disruption of *arr5*, which negatively regulates cytokinin signaling, leads to a decrease in the basal level of *SCR* (Figures 2E and 2H). Together, these results confirm that endogenous cytokinin negatively regulates *SCR* and *WOX5* expression in root tips.

Cytokinin Negatively Regulates the Expression of the *LAX2* Auxin Influx Carrier in the Root Tips

The auxin maximum in the QC is generated by a directed auxin flow and is essential for QC specification [6]. Several studies have shown that cytokinin can dampen the auxin response in the QC through the downregulation of auxin efflux carriers PINs [10, 12, 13]. However, little is known regarding the role of auxin influx carriers in this process and how cytokinin regulates them. To investigate the role of cytokinin in regulating auxin influx in the root tips, we examined the expression of the auxin influx carriers *AUX1*, *LAX2*, and *LAX3* in root tips using NanoString technology. As *LAX1* is not appreciably expressed in the root tip [20], it was not included in this study. *AUX1* and *LAX2* transcript levels were reduced ~13% and 60%, respectively, within 8 hr of cytokinin treatment, but *LAX3* expression was not responsive to cytokinin (Figure 3A). qRT-PCR analysis was used to confirm the response of *AUX1* and *LAX2* to cytokinin and to further refine the kinetics of these changes. Within 3 hr of cytokinin treatment, there was a substantial reduction in *LAX2* transcript, whereas *AUX1* transcript was not significantly reduced until 8 hr after treatment (Figure 3B). As *LAX2* transcript level responded to cytokinin more rapidly and with a greater fold change, we further analyzed its role in the cytokinin regulation of root meristem function.

LAX2 is expressed primarily in the provascular cells in the root tips, with relatively low expression in the QC and root cap [20]. To study the effect of cytokinin on the pattern of *LAX2* expression, we examined the expression of a *pLAX2::LAX2-VENUS* transgene generated using a recombineering-based gene tagging system [29]. After 24 hr of cytokinin treatment, the level of LAX2-VENUS in the provascular cells was

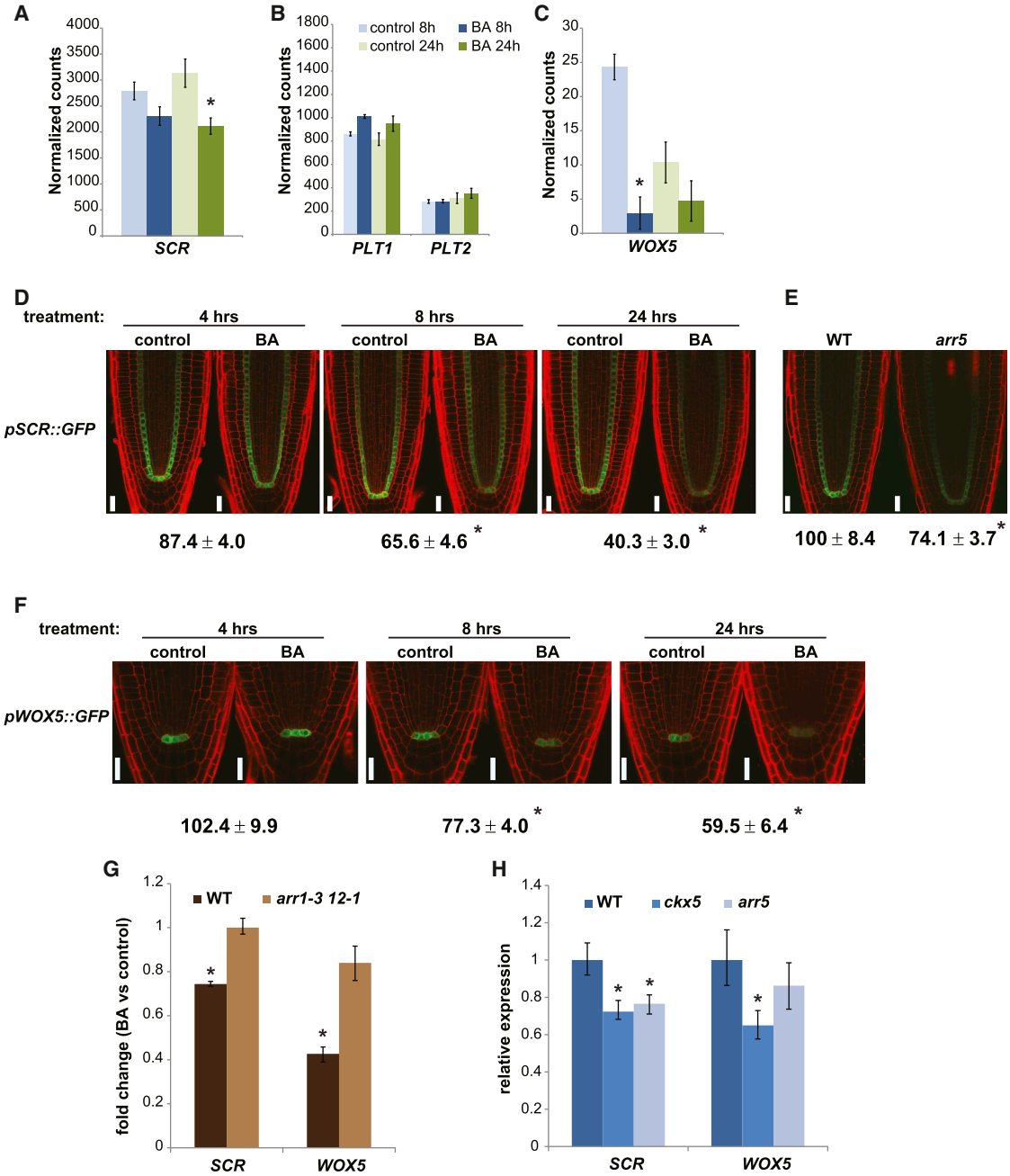

Figure 2. Effects of Cytokinin on the Expression of *SCR*, the *PLT*s, and *WOX5* in Root Tips

(A–C) NanoString nCounter gene expression analysis of *SCR*, *PLT1*, *PLT2*, and *WOX5* in wild-type root tips treated with DMOS or 5 μM BA for 8 or 24 hr. Error bars represent SE from three biological replicates.

(D–F) Confocal images of root tips expressing *pSCR::GFP* (D and E) or *pWOX5::GFP* (F) in wild-type seedlings treated with DMSO or 5 μM BA for specified durations (D and F) or in wild-type and *arr5* seedlings (E). The relative fluorescence in the QC presented in percentage (D and F, BA versus control) with SE was shown under the images. At least seven seedlings per treatment per time point were examined, and the experiment was conducted twice with similar results. Scale bar represents 20 μm.

(G and H) Gene expression analysis of *SCR* and *WOX5* as determined by a TaqMan RT-PCR assay in wild-type and *arr1-3 arr12-1* root tips treated with DMSO control or 5 μM BA for 24 hr (G) or in wild-type, *ckx5*, and *arr5* root tips (H). Error bars represent SE from three technical replicates, and the experiment was conducted twice using independent biological replicates with similar results.

*$p < 0.05$, Student's t test, BA treatment compared to control treatment or mutants compared to wild-type. See also Figure S2.

markedly reduced (Figure 3C), suggesting that the expression of *LAX2* in these cells is sensitive to cytokinin. We also examined the level of endogenous LAX2 protein in response to cytokinin using immunocytochemistry. Consistent with our analysis of the *LAX2* transcript, the level of endogenous LAX2 protein in the provascular cells in the root tips was substantially reduced after 24 hr of cytokinin treatment (Figure 3D). Taken together, these data suggest that cytokinin represses the expression of *LAX2* in the root tips, most notably in the provascular cells.

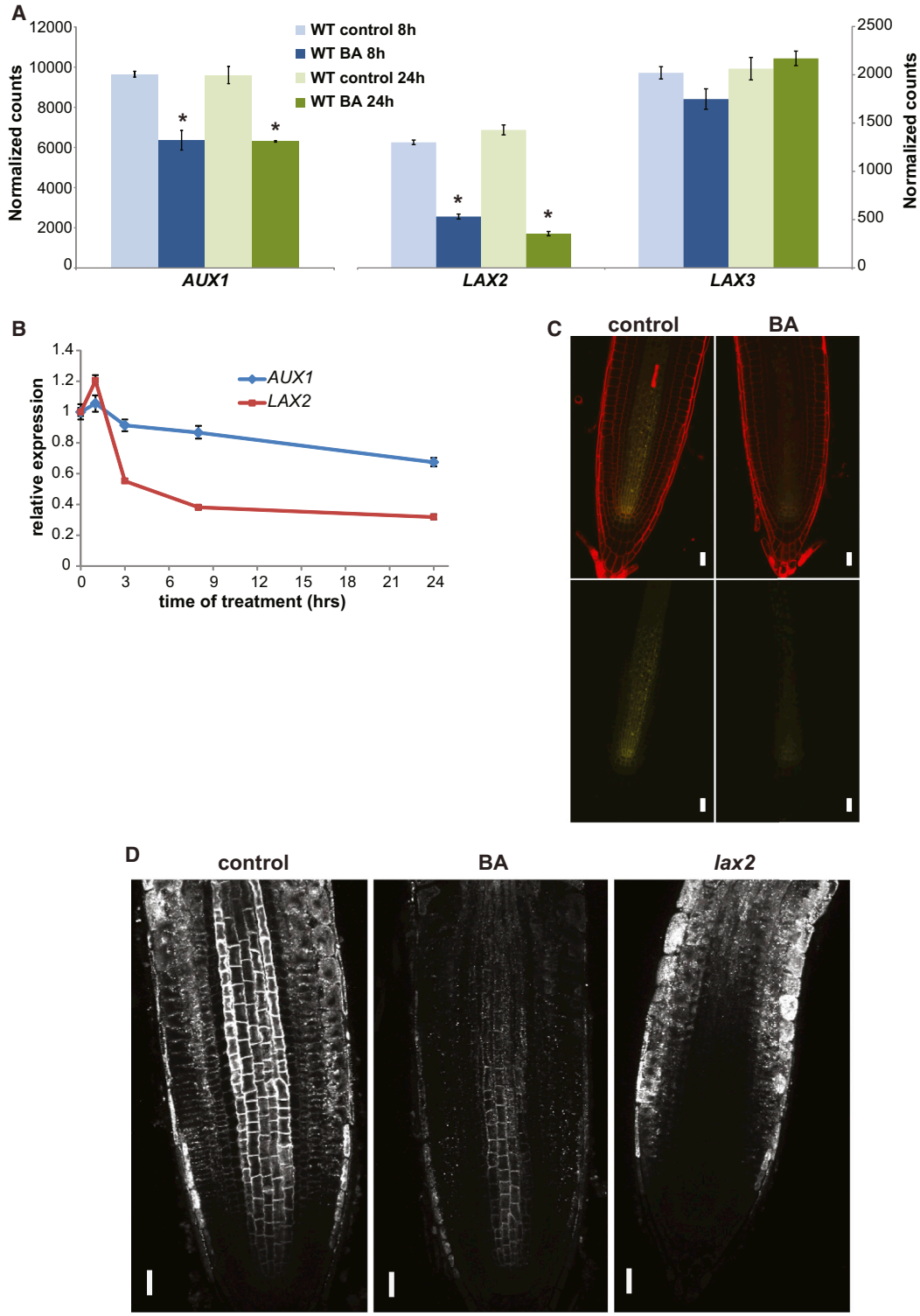

Figure 3. Cytokinin Downregulates Expression of the Auxin Influx Carrier *LAX2* in Root Tips

(A) NanoString nCounter gene expression analysis of *AUX1*, *LAX2*, and *LAX3* in wild-type root tips treated with DMSO control or 5 μM BA for 8 or 24 hr. Error bars represent SE from three biological replicates. *$p < 0.05$, Student's t test, BA treatment compared to control treatment.

(B) Quantitative RT-PCR expression analysis of *AUX1* and *LAX2* in wild-type root tips treated with 5 μM BA for specified durations, or with DMSO control for 24 hr. Error bars represent SE from three technical replicates, and the experiment was conducted three times using independent biological replicates with similar results.

(C) Confocal images of wild-type seedling roots expressing *pLAX2:: LAX2-VENUS* treated with DMSO control or 5 μM BA for 24 hr. Scale bar represents 20 μm.

(D) In situ immunolocalization of LAX2 protein in wild-type root tips treated with DMSO control or 5 μM BA for 24 hr, or *lax2* mutant roots (negative control). Scale bar represents 20 μm. See also Table S1.

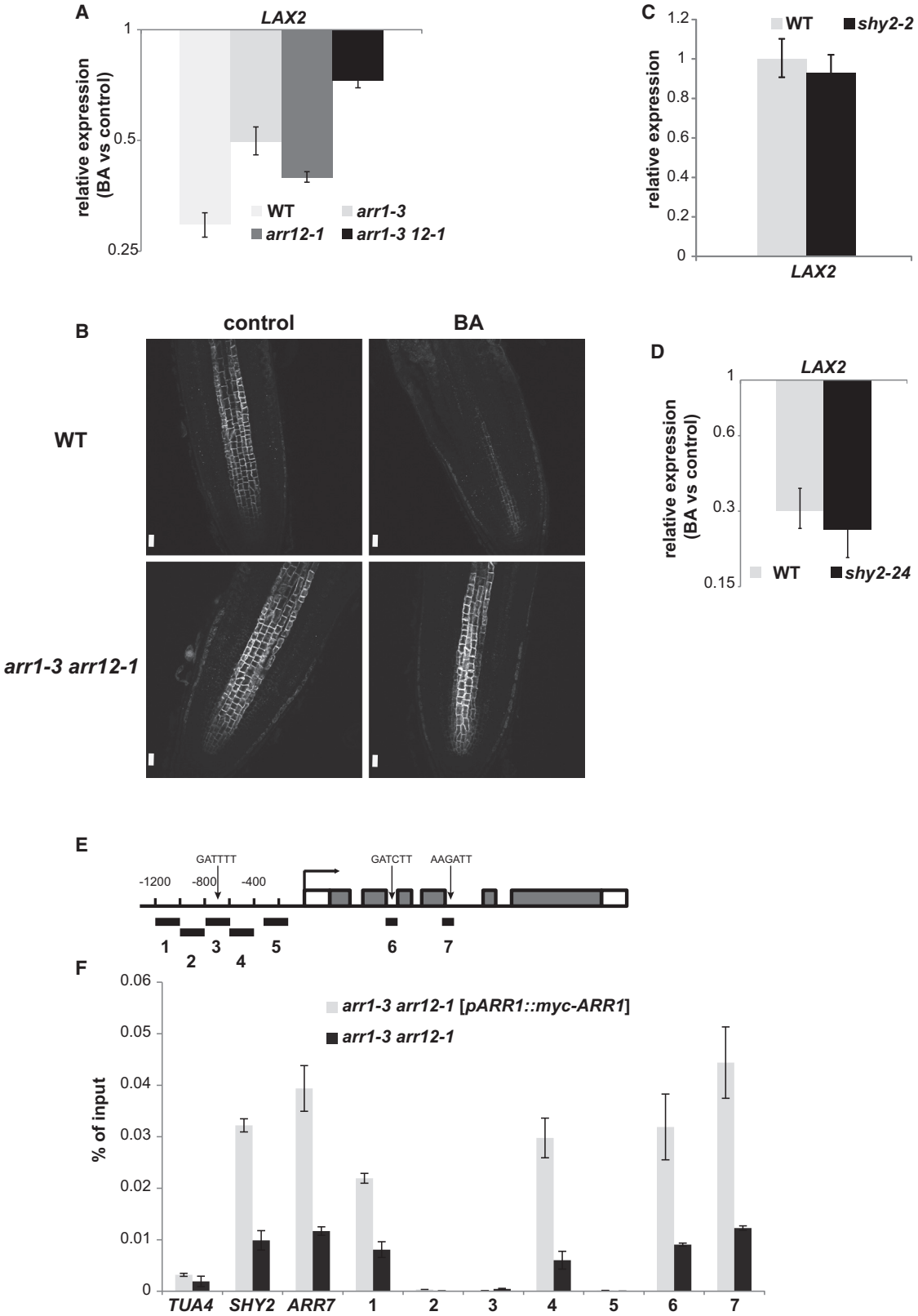

Figure 4. The Negative Regulation of *LAX2* by Cytokinin Is Dependent on the Type B ARRs *ARR1* and *ARR12*

(A) Quantitative RT-PCR analysis of the expression of *LAX2* in wild-type, *arr1-3*, *arr12-1*, and *arr1-3 arr12-1* root tips treated with DMSO control or 5 μM BA for 24 hr. Error bars represent SE from three technical replicates, and the experiment was conducted three times with similar results. The y axis is shown in a log2 scale.

(B) In situ immunodetection of LAX2 protein in wild-type and *arr1-3 arr12-1* root tips treated with DMSO control or 5 μM BA for 24 hr. Scale bar represents 20 μm.

(legend continued on next page)

The Type B Response Regulator ARR1 Binds Directly to the Promoter and Intron Regions of the *LAX2* Gene

The reduction of *LAX2* transcript was detectable substantially earlier in response to cytokinin than that of *SCR*, *AUX1*, or *WOX5*, suggesting that *LAX2* could be a direct target of cytokinin, and by inference of the type B ARRs. To determine which cytokinin signaling components are involved in the downregulation of *LAX2*, we examined *LAX2* expression in several cytokinin signaling mutants. The reduction of *LAX2* transcript in response to cytokinin was compromised in an *ahk2 ahk4* mutant (Figure S3), suggesting that these cytokinin receptors are necessary for the regulation of *LAX2*. The single *arr1-3* and *arr12-1* mutations display a slight effect on the repression of *LAX2* expression in response to cytokinin, but repression was nearly eliminated in an *arr1-3 arr12-1* double mutant (Figure 4A). Consistent with the analysis of the *LAX2* transcript, the decrease in LAX2 protein levels in response to cytokinin was substantially impaired in the *arr1-3 arr12-1* double mutants (Figure 4B). Taken together, these results suggest that *ARR1* and *ARR12* redundantly mediate the repression of *LAX2* by cytokinin.

Previous studies have found that cytokinin negatively regulates the expression of the PIN auxin efflux carriers in the root tip via increased expression of the *SHY2* AUX/IAA gene [10]. This induction of *SHY2* is mediated by *ARR1* and *ARR12* through direct binding to the regulatory regions of *SHY2* [10, 30]. Because the negative regulation of *LAX2* by cytokinin is also dependent on *ARR1* and *ARR12*, we examined whether this also occurs in a *SHY2*-dependent manner. The level of *LAX2* transcript in *shy2-2*, a gain-of-function allele, was similar to that observed in wild-type root tips (Figure 4C). Furthermore, the repression of *LAX2* by cytokinin in *shy2-24*, a loss-of-function allele, was comparable to that observed in wild-type root tips (Figure 4D), suggesting that the *LAX2* expression is independent of *SHY2.*

To assess whether *LAX2* is a direct target of ARR1, we first determined whether there are ARR1 binding motifs in the *LAX2* regulatory regions, focusing on an extended type B motif that has been found to be enriched upstream of cytokinin-regulated genes [31]. There is one potential ARR1 binding site within 1.2 kb upstream of the *LAX2* gene and two others in the second and fourth introns (Figure 4E). We examined whether ARR1 binds to *LAX2* genomic sequences using chromatin immunoprecipitation (ChIP)-qPCR from *arr1-3 arr12-1* seedlings expressing a *myc-ARR1* transgene under the control of the *ARR1* promoter. This transgene is functional, as it is capable of complementing the *arr1-3 arr12-1* mutations [32]. Two fragments in the *LAX2* upstream region and two fragments in *LAX2* introns were significantly enriched in ChIP from *pARR1::myc-ARR1* seedlings compared to control seedlings (Figure 4F), although the upstream fragments did not include the one with an extended canonical type B binding motif. The *ARR7* and *SHY2* genes served as positive controls [10, 33], and *TUA4* as a negative control in this analysis. These results suggest that ARR1 directly regulates *LAX2* repression by cytokinin.

lax2 Mutant Roots Display a Reduced Auxin Response and Abnormal Cell Divisions in QC Cells

Given the distinct expression pattern of *LAX2* in the SCN and QC as compared to the other *AUX/LAX* family members [20], we tested whether *LAX2* contributes to generating the auxin maximum that occurs in the QC using the auxin response reporter *DR5::GFP*. A sharp maximum of DR5 expression was observed in the QC cells of wild-type roots (Figure 5A), consistent with previous reports [7]. In *lax2* roots, there was a substantial reduction in the expression of the DR5::GFP reporter in the QC cells but no substantial change in the spatial pattern of expression, suggesting that the auxin levels in the QC cells are reduced in *lax2* roots (Figures 5A and 5B). Previous studies have suggested that auxin influx carriers in the shoot apical meristem are required for the coordinated PIN1 polarization and sharp peaks of auxin concentration, thus playing an important role in the maintenance of phyllotaxis [19]. As the *LAX2* expression pattern largely overlaps with several auxin efflux carriers, including *PIN1*, *PIN3*, *PIN4*, and *PIN7*, we examined whether the expression or localization of the auxin efflux carriers were altered in *lax2* roots. The transcript levels of *PIN1*, *PIN3*, *PIN4*, and *PIN7* and the polarization of PIN1 protein were similar in *lax2* root tips as compared to the wild-type (Figures S4A and S4B). These results suggest that *LAX2* plays a positive role in generating and/or stabilizing the auxin response maximum in the QC cells, though in contrast to its role in the SAM, the regulation of auxin maxima in the QC by *LAX2* in the RAM does not appear to involve the regulation of PIN1 polarization.

The auxin gradient in the root tip plays an important role in regulating the pattern of cell division and differentiation [6]. As the auxin response in the QC cells was significantly reduced in *lax2* roots, we examined the pattern of cell division in the QC of this mutant. A significant percentage of *lax2* roots (~25%) showed cell divisions in QC cells (Figures 5C and 5D). A comparable QC division phenotype was also seen in the independent *lax2-3* allele (Figure 5D), which appears to be a molecular null (Figures S4E and S4F). This is consistent with a previous study that demonstrated that disruption all four *AUX1 LAX* family members resulted in changes in the establishment of cell pattern in the QC in the embryo [34]. Next, we examined the expression of *WOX5* and *SCR* in the *lax2* roots. As revealed by the *pWOX5::GFP* reporter, the expression of *WOX5* was reduced by approximately 20% in *lax2* roots as compared to the expression in wild-type roots (Figures 5E and 5F), suggesting that the QC function is compromised in *lax2* roots. However, the expression of *SCR* in *lax2* did not seem to be different

(C) Quantitative RT-PCR expression analysis of *LAX2* in wild-type and *shy2-2* root tips. Error bars represent SE from three technical replicates, and the experiment was conducted three times with similar results. The y axis is shown in a log2 scale.
(D) Quantitative RT-PCR expression analysis of *LAX2* in wild-type and *shy2-24* root tips treated with DMSO control or 5 μM BA for 24 hr. Error bars represent SE from three technical replicates, and the experiment was conducted three times with similar results.
(E) Schematic representation of the *LAX2* genomic region. The bent arrow indicates the start site of transcription. The open and close boxes represent the UTR and exons, respectively. Three potential ARR1-binding sites are pointed out with straight arrows with respective sequences. Bars with numbers indicate DNA fragments used in CHIP-qPCR experiments.
(F) ChIP analysis from *pARR1::myc-ARR1* followed by qPCR of the *LAX2* promoter and intron regions illustrated in (E), and also *TUA4*, *SHY2*, and *ARR7* promoter regions, which served as negative and positive controls, respectively. Error bars represent SD from two technical replicates. This experiment was conducted three times with similar results.
See also Figure S3 and Table S1.

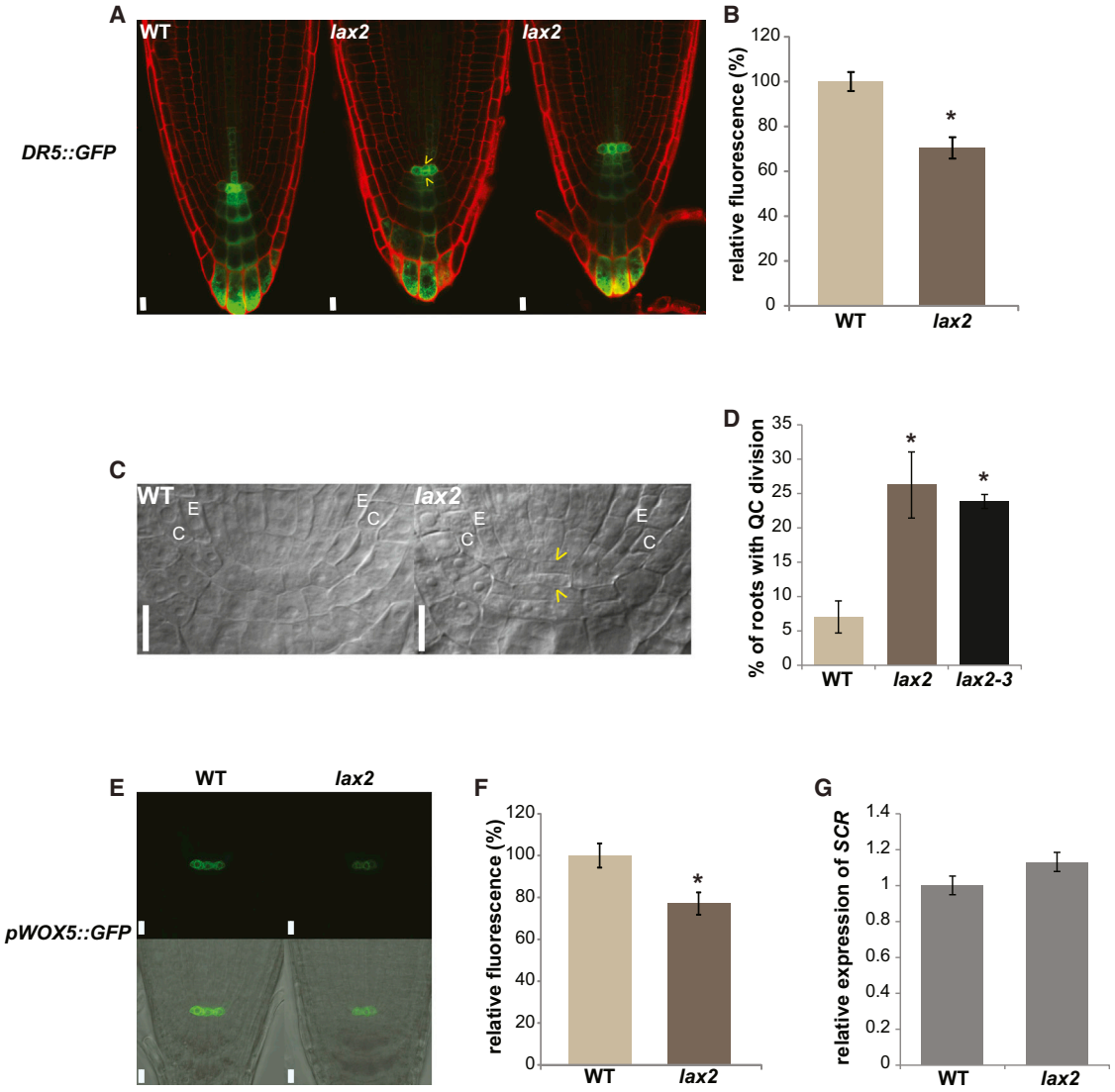

Figure 5. *lax2* Mutant Roots Display Attenuated Auxin Response in the QC and Divisions in QC Cells

(A and E) Confocal images of wild-type and *lax2* roots expressing the auxin response reporter *DR5::GFP* (A) or *pWOX5::GFP* (E). Outlines of cells were visualized by propidium iodide staining or by DIC.

(B and F) Quantification of GFP fluorescence in the QC in wild-type and *lax2* roots expressing *DR5::GFP* (A) or *pWOX5::GFP* (E). Error bars represents SE, n = 12. The experiment was conducted three times with similar results.

(C) Root tips of 5-day-old wild-type and *lax2* mutants visualized by DIC microscopy.

(D) Percentage of roots with dividing QC cells from wild-type, *lax2*, and *lax2-3* mutants. n > 50. Error bars represent SE from triplicate experiments.

(G) Analysis of *SCR* gene expression in wild-type and *lax2* root tips using TaqMan RT-PCR. Error bars represent SE from three technical replicates, and the experiment was conducted twice with similar results.

Yellow arrowheads indicate dividing QC cells. *p < 0.05, Student's t test. Scale bar represents 10 μm. See also Figure S4 and Table S1.

from that in wild-type (Figure 5G), suggesting that cytokinin regulates *SCR* and *LAX2* expression independently. The disruption of *LAX2* phenocopies the effects of elevated cytokinin on QC function, which, coupled with the observation that *LAX2* expression is decreased in response to cytokinin, suggests that the effect of cytokinin on QC function is mediated at least in part through downregulation of *LAX2* expression. However, *LAX2* is likely not the only component mediating the effect of cytokinin on QC function, as the *lax2* mutant still responded to cytokinin to induce QC cell division and to reduce the auxin response in the QC (Figures 1B and S4D). Likely candidates for these additional cytokinin targets regulating QC function are the PIN auxin efflux carriers.

Discussion

The QC, lying in the center of the SCN, sustains the indeterminate growth of the root by preventing the differentiation of the surrounding stem cells. Both auxin and a group of transcription factors including the *PLT*s, *SCR*, and *WOX5* are important inputs in QC specification and stem cell activity [1]. Studies of a local regeneration response in the root tip following the laser ablation of QC cells suggest that a shift in the auxin maximum first respecifies the position of the new QC; the resulting cell-fate changes require the *PLT*s and *SCR* transcription factors, which in turn regulate the expression and localization of *PIN*s and further stabilize the

auxin distribution in the root tips [9]. Although much progress has been made in the understanding of the interaction between auxin and these transcription factors in the regulation of the QC, little is known regarding the role of cytokinin in the QC and how cytokinin regulates these transcription factors.

Here, we demonstrate that cytokinin plays a negative role in QC specification through enhancing the mitotic activity of the QC cells. This likely occurs at least in part through the downregulation of *SCR* and the attenuation of the auxin response in the QC. Previously, it has been shown that cytokinin represses the expression of the *PIN* auxin efflux carriers in the root meristem and thus modulates the distribution of auxin [10–13]. The results presented here indicate that in addition to its effects on these auxin efflux carriers, cytokinin also regulates auxin influx through directly targeting the expression of the auxin influx carrier *LAX2* via ARR1 and ARR12. Previous studies have demonstrated that type B ARRs directly modulate genes whose expression is elevated in response to cytokinin, and the analysis of *LAX2* regulation here indicates that at least ARR1 and presumably ARR12 can also directly regulate genes repressed by cytokinin. Presumably these different outcomes on gene expression occur via interactions with distinct accessory proteins. In addition to its effects on transcription, cytokinin also has been shown to regulate the stability of the type A ARR proteins [35]. Thus, cytokinin may also affect meristem genes through a posttranscriptional mechanism.

The direct repression of *LAX2* expression at least partially accounts for the negative effect of cytokinin on the auxin maximum in the QC, and thus QC identity and function. The expression of *SCR* in the QC has been shown to be both necessary and sufficient for the specification of the QC and the maintenance of the stem cells [4]. A model has been proposed wherein *SCR* expression potentiates a group of cells for QC fate, and the auxin distribution then acts on a subset of these SCR-expressing cells to induce the QC cell fate [4]. Thus, cytokinin modulates these two essential cues of QC specification to negatively regulate QC.

The effect of cytokinin on QC mitotic activity is similar to that of the phytohormone ethylene, which also positively regulates QC cell divisions [25]. Although cytokinin does induce ethylene biosynthesis [26], we demonstrate that it acts independent of ethylene to elevate QC cell divisions. However, we cannot exclude the formal possibility that the role of ethylene in the QC division induced by exogenous cytokinin differs from the effect of endogenous cytokinin. Cytokinin appears to act in part through modulating the distribution of auxin in the root tip and by decreasing the expression of *SCR*. In contrast, Ortega-Martínez et al. concluded that ethylene acts independently of auxin in promoting QC division and does not alter *SCR* expression [25]. Thus, the two hormones could potentially additively influence cell division in the QC through different mechanisms. Previously, we have shown that disruption of multiple type A ARRs, which are negative regulators of cytokinin signaling, caused, in addition to increased mitotic activity of the QC cells, premature differentiation of the root columella initials [13]. In contrast, the increased mitotic activity of the QC cells in response to elevated cytokinin does not impair the ability of these cells to maintain the surrounding cells in an undifferentiated state. This difference between the effects of elevated cytokinin levels and disruption of the type A ARRs could result from a difference in the level of activation of cytokinin responses in the type A ARR mutants relative to that achieved via application of exogenous cytokinin or disruption of the *CKX* genes, or could potentially reflect a role of the type A ARRs that is independent of cytokinin signaling.

In addition to its critical role in QC specification, SCR together with SHR in the endodermis has also been shown to function together with a group of class III homeodomain-leucine zipper transcription factors, including *PHB*, in the stele to regulate the tissue patterning in the root [36]. SCR/SHR induces the expression of MIR165/6 in the endodermis, thus restricting *PHB* expression in the vascular cylinder to determine the xylem cell type. Here, we found that cytokinin downregulates *SCR* expression. Consistently, microRNA-insensitive gain-of-function *phb* mutants have been shown to display short roots and small root meristems, reminiscent of the cytokinin-treated roots [37]. Cytokinin also represses the expression of *MIR165/6* [14], and it is possible that this may occur via the downregulation of *SCR* expression in response to cytokinin.

The *PLT* transcription factors have been shown to be expressed in response to auxin accumulation and have been hypothesized to translate the auxin cue into positional information for the SCN [3, 38]. Although there is a reduced auxin response in the root tips in both cytokinin-treated seedlings and in *lax2* mutants, there was no accompanying change in *PLT1* or *PLT2* expression level (Figure S4A). It is possible that cytokinin-induced reduction in auxin response in the QC is not of sufficient magnitude to affect *PLT* expression, or there may be a change in the spatial pattern of *PLT* expression that is not revealed by analysis of the total transcript level.

The *WOX5* homeodomain transcription factor is required for QC function, preventing the distal stem cells (DSCs) from differentiation, and its expression is dependent on *SCR* [5]. However, the link between *WOX5* and auxin is not clear. One study has shown that applied auxin promotes the differentiation of the DSC through repression of *WOX5* expression [39]. However, this regulation of *WOX5* by auxin does not seem to be direct, given the slow kinetics of repression [39]. Here, we showed that *WOX5* expression is downregulated by cytokinin and surprisingly is also downregulated in *lax2* mutant roots, which displayed an attenuated auxin response in the QC. This suggests that perhaps a window of auxin levels in the QC is essential for proper *WOX5* expression; concentrations either above or below these thresholds reduce *WOX5* expression. The fact that *pin* mutants showed increased sensitivity to applied auxin in the differentiation of DSC is consistent with the notion that an appropriate auxin distribution, maintained by auxin influx and efflux carriers, is required for *WOX5* expression [39]. Thus, cytokinin represses *WOX5* expression in the QC probably through modulating auxin flow and thus auxin distribution in the root tips.

In the shoot apical meristem, WUSCHEL (WUS), a paralog of WOX5, plays an important role in the initiation and maintenance of stem cell fate. There is a positive feedback loop between WUS and cytokinin in the shoot apical meristem, with high levels of cytokinin inducing WUS expression and WUS acting as a direct negative regulator of expression of the type A ARRs [35, 40]. Thus, the role of cytokinin differs in this regard in the root and shoot, with cytokinin repressing *WOX5* expression in the root and promoting *WUS* expression in the shoot. This parallels distinct effects of cytokinin on the activity of the apical meristems in the root and shoot [24].

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.08.008.

Acknowledgments

We gratefully acknowledge Chia-Yi Cheng and Christian Burr for reading the manuscript. We also thank Yan Shi and Mike Topal at the UNC Genomics Core for assistance with NanoString analysis, Philip Benfey for *pSCF::GFP* and *scr-3* seeds, Jason Reed for *shy2-2* and *shy2-24* seeds, Jose Alonso for *pLAX2::LAX2-VENUS* lines, and Thomas Schmülling for *ckx3* and *ckx5* seeds. This project was supported by National Science Foundation grant IOS-1022053 to J.J.K. and G.E.S.

Received: June 5, 2013
Revised: July 12, 2013
Accepted: August 6, 2013
Published: October 10, 2013

References

1. Bennett, T., and Scheres, B. (2010). Root development-two meristems for the price of one? Curr. Top. Dev. Biol. *91*, 67–102.
2. van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P., and Scheres, B. (1997). Short-range control of cell differentiation in the Arabidopsis root meristem. Nature *390*, 287–289.
3. Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C., Nussaume, L., Noh, Y.S., Amasino, R., and Scheres, B. (2004). The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell *119*, 109–120.
4. Sabatini, S., Heidstra, R., Wildwater, M., and Scheres, B. (2003). SCARECROW is involved in positioning the stem cell niche in the Arabidopsis root meristem. Genes Dev. *17*, 354–358.
5. Sarkar, A.K., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R., and Laux, T. (2007). Conserved factors regulate signalling in *Arabidopsis thaliana* shoot and root stem cell organizers. Nature *446*, 811–814.
6. Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P., and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell *99*, 463–472.
7. Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature *433*, 39–44.
8. Grieneisen, V.A., Xu, J., Marée, A.F., Hogeweg, P., and Scheres, B. (2007). Auxin transport is sufficient to generate a maximum and gradient guiding root growth. Nature *449*, 1008–1013.
9. Xu, J., Hofhuis, H., Heidstra, R., Sauer, M., Friml, J., and Scheres, B. (2006). A molecular framework for plant regeneration. Science *311*, 385–388.
10. Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science *322*, 1380–1384.
11. Pernisová, M., Klíma, P., Horák, J., Válková, M., Malbeck, J., Soucek, P., Reichman, P., Hoyerová, K., Dubová, J., Friml, J., et al. (2009). Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. Proc. Natl. Acad. Sci. USA *106*, 3609–3614.
12. Ruzicka, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M.C.E., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc. Natl. Acad. Sci. USA *106*, 4284–4289.
13. Zhang, W., To, J.P.C., Cheng, C.-Y., Schaller, G.E., and Kieber, J.J. (2011). Type-A response regulators are required for proper root apical meristem function through post-transcriptional regulation of PIN auxin efflux carriers. Plant J. *68*, 1–10.
14. Dello Ioio, R., Galinha, C., Fletcher, A.G., Grigg, S.P., Molnar, A., Willemsen, V., Scheres, B., Sabatini, S., Baulcombe, D., Maini, P.K., and Tsiantis, M. (2012). A PHABULOSA/cytokinin feedback loop controls root growth in Arabidopsis. Curr. Biol. *22*, 1699–1704.
15. To, J.P., and Kieber, J.J. (2008). Cytokinin signaling: two-components and more. Trends Plant Sci. *13*, 85–92.
16. Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., Ecker, J.R., and Schaller, G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell *17*, 3007–3018.
17. To, J.P., Haberer, G., Ferreira, F.J., Deruère, J., Mason, M.G., Schaller, G.E., Alonso, J.M., Ecker, J.R., and Kieber, J.J. (2004). Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell *16*, 658–671.
18. Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. Nature *426*, 255–260.
19. Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., and Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. Genes Dev. *22*, 810–823.
20. Péret, B., Swarup, K., Ferguson, A., Seth, M., Yang, Y., Dhondt, S., James, N., Casimiro, I., Perry, P., Syed, A., et al. (2012). AUX/LAX genes encode a family of auxin influx transporters that perform distinct functions during Arabidopsis development. Plant Cell *24*, 2874–2885.
21. Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C., and Bennett, M.J. (1999). AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. EMBO J. *18*, 2066–2073.
22. Swarup, K., Benková, E., Swarup, R., Casimiro, I., Péret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. Nat. Cell Biol. *10*, 946–954.
23. Vanstraelen, M., Baloban, M., Da Ines, O., Cultrone, A., Lammens, T., Boudolf, V., Brown, S.C., De Veylder, L., Mergaert, P., and Kondorosi, E. (2009). APC/C-CCS52A complexes control meristem maintenance in the Arabidopsis root. Proc. Natl. Acad. Sci. USA *106*, 11806–11811.
24. Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell *15*, 2532–2550.
25. Ortega-Martínez, O., Pernas, M., Carol, R.J., and Dolan, L. (2007). Ethylene modulates stem cell division in the Arabidopsis thaliana root. Science *317*, 507–510.
26. Vogel, J.P., Woeste, K.E., Theologis, A., and Kieber, J.J. (1998). Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. Proc. Natl. Acad. Sci. USA *95*, 4766–4771.
27. Cui, H., Levesque, M.P., Vernoux, T., Jung, J.W., Paquette, A.J., Gallagher, K.L., Wang, J.Y., Blilou, I., Scheres, B., and Benfey, P.N. (2007). An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. Science *316*, 421–425.
28. Fukaki, H., Wysocka-Diller, J., Kato, T., Fujisawa, H., Benfey, P.N., and Tasaka, M. (1998). Genetic evidence that the endodermis is essential for shoot gravitropism in *Arabidopsis thaliana*. Plant J. *14*, 425–430.
29. Zhou, R., Benavente, L.M., Stepanova, A.N., and Alonso, J.M. (2011). A recombineering-based gene tagging system for Arabidopsis. Plant J. *66*, 712–723.
30. Moubayidin, L., Perilli, S., Dello Ioio, R., Di Mambro, R., Costantino, P., and Sabatini, S. (2010). The rate of cell differentiation controls the Arabidopsis root meristem growth phase. Curr. Biol. *20*, 1138–1143.
31. Bhargava, A., Clabaugh, I., To, J.P., Maxwell, B.B., Chiang, Y.H., Schaller, G.E., Loraine, A., and Kieber, J.J. (2013). Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq in Arabidopsis. Plant Physiol. *162*, 272–294.
32. Hill, K., Mathews, D.E., Kim, H.J., Street, I.H., Wildes, S.L., Chiang, Y.H., Mason, M.G., Alonso, J.M., Ecker, J.R., Kieber, J.J., and Schaller, G.E. (2013). Functional characterization of type-B response regulators in the Arabidopsis cytokinin response. Plant Physiol. *162*, 212–224.
33. Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. Science *294*, 1519–1521.
34. Ugartechea-Chirino, Y., Swarup, R., Swarup, K., Péret, B., Whitworth, M., Bennett, M., and Bougourd, S. (2010). The AUX1 LAX family of auxin influx carriers is required for the establishment of embryonic root cell organization in *Arabidopsis thaliana*. Ann. Bot. (Lond.) *105*, 277–289.
35. To, J.P., Deruère, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., Schaller, G.E., and Kieber, J.J. (2007). Cytokinin regulates type-A Arabidopsis Response Regulator activity and protein stability via two-component phosphorelay. Plant Cell *19*, 3901–3914.

36. Carlsbecker, A., Lee, J.-Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vatén, A., Thitamadee, S., et al. (2010). Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature *465*, 316–321.
37. Grigg, S.P., Galinha, C., Kornet, N., Canales, C., Scheres, B., and Tsiantis, M. (2009). Repression of apical homeobox genes is required for embryonic root development in Arabidopsis. Curr. Biol. *19*, 1485–1490.
38. Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R., and Scheres, B. (2007). PLETHORA proteins as dose-dependent master regulators of Arabidopsis root development. Nature *449*, 1053–1057.
39. Ding, Z., and Friml, J. (2010). Auxin regulates distal stem cell differentiation in Arabidopsis roots. Proc. Natl. Acad. Sci. USA *107*, 12046–12051.
40. Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J.J., and Lohmann, J.U. (2005). WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature *438*, 1172–1175.