Cytokinin Acts through the Auxin Influx Carrier AUX1 to Regulate Cell Elongation in the Root

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Cytokinin acts through the auxin influx carrier AUX1 to regulate cell elongation in the root

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ABSTRACT
Hormonal interactions are crucial for plant development. In Arabidopsis, cytokinins inhibit root growth through effects on cell proliferation and cell elongation. Here, we define key mechanistic elements in a regulatory network by which cytokinin inhibits root cell elongation in concert with the hormones auxin and ethylene. The auxin importer AUX1 functions as a positive regulator of cytokinin responses in the root; mutation of AUX1 specifically affects the ability of cytokinin to inhibit cell elongation but not cell proliferation. AUX1 is required for cytokinin-dependent changes of auxin activity in the lateral root cap associated with the control of cell elongation. Cytokinin regulates root cell elongation through ethylene-dependent and -independent mechanisms, both hormonal signals converging on AUX1 as a regulatory hub. An autoregulatory circuit is identified involving the control of ARR10 and AUX1 expression by cytokinin and auxin, this circuit potentially functioning as an oscillator to integrate the effects of these two hormones. Taken together, our results uncover several regulatory circuits controlling interactions of cytokinin with auxin and ethylene, and support a model in which cytokinin regulates shootward auxin transport to control cell elongation and root growth.

KEY WORDS: Arabidopsis, Cytokinin, Ethylene, Auxin, Root Development, AUX1

INTRODUCTION
The root systems of plants are essential for survival, performing such functions as absorbing water and nutrients from the soil, storing food and nutrients, and providing anchorage (Giehl et al., 2014; Jones and Ljung, 2012). The architecture of the root system is developmentally plastic and responds to its environment by modifying such characteristics as primary root growth, lateral root density and lateral root growth, with root growth itself being dependent on both cell proliferation and cell elongation (Giehl et al., 2014; Jones and Ljung, 2012). Cell proliferation occurs at the root apical meristem (RAM), the stem cells of the RAM being organized around a mitotically inactive quiescent center (QC). The stem cells and their derived cells divide, each division shifting the cells further away from the QC (Perilli et al., 2012; Scheres et al., 2002). The meristematic cells eventually cease dividing and begin to elongate and differentiate (Bennett and Scheres, 2010). Not surprisingly, multiple phytohormone signaling pathways interact to control root growth, including the hormone cytokinin, which inhibits both cell proliferation and elongation of root cells (Beemster and Baskin, 2000; Hwang et al., 2012; Moubayidin et al., 2009; Schaller et al., 2015; Vanstraelen and Benkova, 2012; Werner et al., 2003).

The cytokinin signaling pathway of plants is similar to the two-component response systems of prokaryotes (Kieber and Schaller, 2014; Schaller et al., 2011; Werner and Schmülling, 2009). In Arabidopsis, the cytokinin signaling pathway incorporates histidine kinase receptors (AHKs), histidine-containing phosphotransfer proteins (AHPs), and response regulators (ARRs), all encoded by multi-gene families. Signaling is initiated by cytokinin binding to and inducing autophosphorylation of the AHKs, which are predominantly localized to membranes of the endoplasmic reticulum. Phosphates are transferred from the receptors to cytosolic AHPs which, after movement into the nucleus, phosphorylate the type-B ARR transcription factors. Three type-B ARRs (ARR1, ARR10 and ARR12) play the predominant role in cytokinin signaling, with higher order mutants curtailing the ability of cytokinin to induce changes in gene expression and rendering the plant cytokinin insensitive (Argyros et al., 2008; Ishida et al., 2008; Mason et al., 2005) Among the transcriptional targets induced by the type-B ARRs are a second class of response regulators termed the type-A ARRs, which negatively regulate cytokinin signaling (Bhargava et al., 2013; To et al., 2004).

Cytokinin coordinates root development in concert with the phytohormones auxin and ethylene. Auxin, like cytokinin, regulates both cell proliferation and elongation of the root. Mutants affecting auxin biosynthesis, transport and signaling affect cytokinin responses (De Rybel et al., 2014; Dello Ioio et al., 2008; El-Showk et al., 2013; Schaller et al., 2015; Timpte et al., 1995; Zhou et al., 2011), indicative of the interaction between these two hormones in the control of root growth. The primary role explored for cytokinin-auxin cross-talk has been in the control of cell proliferation at the RAM, cytokinin inhibiting and auxin stimulating the anticlinal cell divisions that regulate RAM size (Dello Ioio et al., 2008; Schaller et al., 2014). Cytokinin-mediated control of cell proliferation appears to involve both transcriptional and post-transcriptional regulation of a subset of PIN auxin efflux carriers (Marhavý et al., 2011; Ruzicka et al., 2009; Zhang et al., 2011). Cytokinin and auxin also cross-talk to regulate cell division in the quiescent center of the root, with cytokinin repressing expression of LAX2, thus modulating auxin transport in the root tip (Zhang et al., 2013). Ethylene also plays a substantive role in root growth owing to its ability to inhibit cell proliferation and elongation, the inhibition of cell elongation being dependent on an ethylene-induced redistribution of auxin (Ruzicka et al., 2009; Swarup et al., 2007;
Thomann et al., 2009). Cytokinin stimulates ethylene biosynthesis, and the increased ethylene concentration is proposed to play a role in cytokinin-dependent inhibition of root cell elongation (Chae et al., 2003; Hansen et al., 2009; Vogel et al., 1998b). Substantial progress has been made in our understanding of how cytokinin, auxin and ethylene coordinate root growth and development but, given the complexity of this process, new mechanisms underlying their interactions continue to be discovered.

We performed a forward genetic screen to uncover key regulators that function in conjunction with cytokinin to control root growth. Through this screen, we identified the gene encoding the auxin influx-carrier AUX1 as a positive regulator of the root growth response to cytokinin. AUX1 is one of a four-member Arabidopsis family of auxin importers, and is a primary mediator for shootward auxin transport in the root (Bennett et al., 1996; Péret et al., 2012; Swarup et al., 2008). Through characterization of the role of AUX1 in cytokinin signaling, we determined that: (1) AUX1 mediates the ability of cytokinin to inhibit root cell elongation but not root cell proliferation; (2) AUX1 is required for cytokinin-dependent changes in auxin activity; and (3) cytokinin regulates root cell elongation through both ethylene-dependent and -independent mechanisms. Our results uncover several regulatory circuits that control the interactions of cytokinin with auxin and ethylene, and support a model in which cytokinin regulates shootward auxin transport to control cell elongation and, ultimately, root growth.

RESULTS

A mutant screen identifies AUX1 as a positive regulator of cytokinin signaling in the root

ARR1, ARR10 and ARR12 are the type-B ARRs that contribute most to cytokinin-dependent root development (Argyros et al., 2008; Ishida et al., 2008; Mason et al., 2005). Although single mutants have a minimal effect on cytokinin sensitivity, higher-order mutants show pronounced phenotypes consistent with overlapping function. For example, an arr1 arr12 double mutant has a longer root than the arr1 or arr12 single mutants when grown on cytokinin. To identify other genes that contribute to cytokinin regulation of root development, we generated ethyl-methanesulfonate (EMS)-mutagenized populations of arr12 and arr1 as sensitized backgrounds, and assayed for seedlings with long roots in the presence of exogenous cytokinin [0.1 µM 6-benzyl-aminopurine (BA); Fig. 1A]. We screened 20,000 M2 seeds of each genotype, using a pooling strategy, and confirmed six candidate mutations in the M3 generation. We named these mutants enhancer of response

![Fig. 1. A genetic screen identifies AUX1 as a positive regulator of cytokinin signaling in the primary root.](image-url)

(A) Design of the mutant screen to identify enhancer of response regulator (err) mutants; these enhance the cytokinin insensitivity, determined by the root growth response, of type-B ARR single mutants. (B) Root growth response to cytokinin of wild type (WT), single mutants err3-1 and arr12, double mutants err3-1 arr12 and arr1 arr12, and triple mutant arr3-1 arr1 arr12. Seedlings were grown on vertical plates in the absence or presence of 1 µM BA (n≥20) for five days illuminated from above. (C) Agravitropic phenotypes of five-day-old light-grown err3-1 mutants. (D) A missense mutation in err3-1 results in a Pro371Leu change in AUX1. (E) Root growth response to 1 µM BA of WT compared with the AUX1/LAX auxin importer family single mutants lax1, lax2, lax3, aux1-21 and aux1-121 (quad), and the quadruple mutant lax1 lax2 lax3 aux1 (quad) (n≥20). In B and E, groups marked by different letters are significantly different (P<0.05). Error bars represent s.e.m.
regulator (err) and describe in this paper results obtained from allelic err3 mutants.

The err3-1 mutant was identified in the arr12 enhancer screen. To examine the relative contribution of err3-1 to root growth responses, we introduced err3-1 from the err3-1 arr12 background into wild-type and arr1 arr12 backgrounds by crossing. The err3-1 mutant exhibited partial cytokinin insensitivity by itself in a root growth assay, but also significantly enhanced the cytokinin insensitivity exhibited by arr12 and arr1 arr12 (Fig. 1B). We also observed that the err3-1 mutant exhibited an agravitropic phenotype regardless of background (Fig. 1C), suggesting it might be allelic to AUX1 (At2g38120), which encodes an auxin influx carrier and mutations in which also lead to an agravitropic phenotype (Bennett et al., 1996; Péret et al., 2012; Swarup et al., 2004). Indeed, we identified a Pro371Leu missense mutation in the coding sequence of AUX1 in the err3-1 line, suggesting that err3-1 is allelic to aux1 (Fig. 1D). Similarly, we identified a second allele of err3 (err3-2) from the arr1 enhancer screen and found it was the result of a Gly374Ser missense mutation in AUX1 (Fig. S1A,B), confirming the significance of AUX1 in mediating the cytokinin response. Both err3-1 and err3-2 alter amino-acid residues present in the extracellular loop between the ninth and tenth predicted transmembrane segments of AUX1 (Swarup et al., 2004). This region is highly conserved in the Arabidopsis amino acid auxin permease superfamily that contains AUX1 (Swarup et al., 2004). A previously identified null allele of AUX1 (aux1-21) exhibited the same level of cytokinin insensitivity as err3-1 (Fig. 1E) (Bennett et al., 1996), consistent with err3-1 being a complete loss-of-function allele of aux1. Because err3-1 and err3-2 are allelic to AUX1, we designated them as aux1-121 and aux1-122, respectively (Swarup et al., 2004), and use these designations for the remainder of the manuscript.

AUX1 is the founding member of the four-member AUX/LAX family of auxin-influx carriers in Arabidopsis (Péret et al., 2012). We examined the lax1, lax2 and lax3 mutants to determine whether they also contributed to the cytokinin root growth response. The three single lax mutants were indistinguishable from wild type in their response to 1 µM cytokinin (Fig. 1E). Furthermore, the lax mutants did not enhance the aux1 mutant phenotype, as the cytokinin response of aux1-21 lax1 lax2 lax3 quadruple mutant was not significantly different from that of the aux1 single mutant (Fig. 1E) (Ugartechea-Chirino et al., 2010). These data are consistent with the hypothesis that AUX1 is a positive regulator of cytokinin-mediated root growth and that it performs a function that does not overlap with other family members in this regard. Our identification of AUX1 mutant alleles through a forward genetic screen also corroborates an earlier finding that an aux1-7 loss-of-function allele exhibits reduced cytokinin sensitivity for root growth (Timppe et al., 1995).

The ability of cytokinin to inhibit cell elongation is dependent on AUX1

Cytokinins control root growth through effects on cell proliferation in the meristem as well as on cell expansion in the elongation zone (Beemster and Baskin, 2000; Dello Ioio et al., 2008; Marhavý et al., 2011; Ruzicka et al., 2009; Zhang et al., 2011), both of which decrease in wild type treated with exogenous cytokinin (Fig. 2). The single type-B mutants arr1, arr10 and arr12 all exhibit a small increase in meristem cell number (Hill et al., 2013; Dello Ioio et al., 2007), presumably owing to a reduction in endogenous cytokinin.
signaling. The arr1 arr12 double mutant, which shows pronounced cytokinin insensitivity (Mason et al., 2005), exhibits significant increases in both meristem cell number and cell length at the elongation zone (Fig. 2). Furthermore, the arr1 arr12 mutant is significantly resistant to the effects of exogenous cytokinin on both cell proliferation and elongation (Fig. 2).

AUX1 facilitates basipetal (shootward) auxin transport via the lateral root cap (LRC) and epidermal tissues as well as acropetal (rootward) transport via the phloem (Marchant et al., 2002; Swarup et al., 2001). Because AUX1 expression spans the meristem and elongation zone, it could theoretically affect cytokinin’s control of cell proliferation and/or expansion. We therefore examined the effects of aux1-121 alone as well as in combination with arr1 and arr1 arr12 to determine the role of AUX1 in cytokinin-regulated cell proliferation and expansion (Fig. 2). We observed no effect of aux1-121 on meristem size (Fig. 2A). For example, the meristem size of the aux1-121 single mutant was indistinguishable from wild type in the absence or presence of cytokinin (Fig. 2A). Similarly, the aux1-121 mutant had no additive effect on the meristem phenotype in combination with arr1 and arr1 (Fig. 2A), suggesting that these type-B ARRs, but not AUX1, play a role in regulating cell proliferation in the root apical meristem (Fig. 2A). In contrast to what we observed for meristem size, the aux1-121 mutation had a substantial effect on the ability of cytokinin to regulate cell expansion in the elongation zone (Fig. 2B). Cell length of the aux1 mutants aux1-121, aux1-121 arr12 and aux1-121 arr1 arr12 were indistinguishable from wild type in the absence of cytokinin, but were all insensitive to treatment with exogenous cytokinin. Like aux1-121, the independent aux1-21 allele affected the ability of cytokinin to inhibit cell expansion but not meristem size (Fig. S2C,D). Taken together, these data indicate that AUX1 plays a role in the ability of cytokinin to regulate cell elongation but not cell proliferation in the root. Consistent with this role for the shootward transport of auxin by AUX1 in mediating the cytokinin response, sensitivity for aux1 root growth to cytokinin was fully restored by expressing AUX1 in the mutant LRC and epidermal tissues (Fig. S1C) using a GAL4-based transactivation system (Swarup et al., 2005).

AUX1 was previously found to mediate the inhibitory effect of ethylene on root cell elongation (Růžička et al., 2007; Stepanova et al., 2005; Strader et al., 2010; Swarup et al., 2007). As cytokinin increases ethylene biosynthesis (Cary et al., 1995; Chae et al., 2003; Hansen et al., 2009; Vogel et al., 1998a), we examined whether the inhibitory effect of cytokinin on cell elongation was ethylene dependent. To examine the role of ethylene, we used the ethylene-insensitive mutant ein2-5 (Fig. 3A) (Alonso, 1999). As shown in Fig. 3B, both ein2-5 and aux1-7 affect the ability of cytokinin to inhibit root growth. The effect of ein2-5 on the cytokinin response is primarily due to the regulation of cell size, not cell proliferation (Fig. 3C,D), consistent with ethylene acting downstream of cytokinin to control cell elongation. However, whereas the aux1 mutant is completely insensitive to cytokinin, the ein2-5 mutant is only partially insensitive to cytokinin. These data indicate that cytokinin inhibits root cell elongation through ethylene-dependent as well as ethylene-independent mechanisms, with both mechanisms converging on AUX1 as a key mediator.

Expression of the type-B response regulator ARR10 is dependent on AUX1 and auxin

Because AUX1 genetically behaved as a positive regulator of cytokinin signaling, enhancing the type-B arr mutant phenotype, we hypothesized that the aux1 mutant might affect expression of type-B ARRs. We therefore examined expression of the seven subfamily-1 type-B ARRs in root tips by use of the NanoString nCounter system, a high-throughput, extremely sensitive and precise method of quantifying transcript abundance (Geiss et al., 2008; Malkov et al., 2009) and which is an effective means of following phytohormone responses (Bhargava et al., 2013; Tsai et al., 2012; Zhang et al., 2011). We performed NanoString analysis on wild type, aux1-21 and the arr1 arr12 cytokinin-insensitive mutant, with expression analyzed following 24-h growth on 1 mM BA or the DMSO vehicle control. We detected expression of ARR1, ARR2, ARR10 and ARR12 (Fig. 4A; Fig. S2A), consistent with these being the most abundantly expressed type-B ARRs in the root tip (Hill et al., 2013). Two independent factors influenced expression of ARR10. First, expression of ARR10 was significantly reduced in aux1 compared with wild type, being expressed at 64% of the wild-type level; this reduction in ARR10 expression was dependent on cytokinin signaling as no significant difference was observed in the arr1 arr12 background (Fig. 4A). In the aux1 background, ARR10 was as responsive to cytokinin as in wild type, indicating that these factors independently influence expression of ARR10. We confirmed these effects on ARR10 expression by qRT-PCR using independently isolated root-tip mRNA and our aux1-121 allele (Fig. 4B). The cytokinin-dependent suppression of ARR10 expression required both ARR1 and ARR12, based on single mutant analysis (Fig. S2B). We did not observe inhibition of ARR10 expression by cytokinin in
root tissue lacking the root tip (Fig. 5A; Fig. S2F). In contrast to the ARR10 expression changes in the root tip, expression of ARR1, ARR2 and ARR12 was not significantly affected by aux1 or by cytokinin (Fig. S2A).

The reduced expression of ARR10 observed in the aux1 mutant background suggests that mobilization of auxin is required for maintaining ARR10 expression. We therefore predicted that treatment of the aux1 mutant with exogenous auxin could rescue ARR10 expression back to its wild-type level. For this purpose, we treated seedlings with the natural auxin indole-3-acetic-acid (IAA) as well as with a membrane-permeable auxin naphthalene-acetic-acid (NAA) (Fig. 4C,D). We observed rescue of ARR10 expression in the root tips of aux1 seedlings treated with NAA but not with IAA. These data are consistent with rescue of the aux1 agravitropic phenotype by the membrane-permeable NAA (Marchant et al., 1999). Expression of the known auxin-regulated genes IAA19 and SHY2 was induced by IAA in aux1 (Fig. S2D,E), demonstrating that the IAA treatment was effective and that expression of ARR10 differs from these genes in its requirement for AUX1-mediated transport. Furthermore, auxin induced expression of ARR10 in the aux1 arr12 background (Fig. 4C,D), consistent with auxin being a positive regulator of ARR10 expression and counteracting the suppression by cytokinin.

Based on the reduced expression of ARR10 in aux1 mutants, we predicted there could be effects on the induction of the type-A ARRs, which are cytokinin primary-response genes (To et al., 2004). We therefore examined expression of the ten type-A ARRs by NanoString analysis with the same RNA samples (wild type, aux1 and aux1 arr12) used for Fig. 4A. Nine of the type-A ARRs were significantly induced by cytokinin in wild-type root tips (Fig. 5A; Fig. S2F). In the cytokinin-insensitive aux1 arr12 mutant, the expression of multiple type-A ARRs was reduced compared with wild type, this effect being particularly apparent in the cytokinin-treated samples (Fig. 5B,C; Fig. S2F), consistent with expression of type-A ARRs being dependent on transcriptional activity of type-B ARRs. In the aux1 mutant, expression of multiple type-A ARRs was reduced compared with wild type, but here the effect was most pronounced on the basal expression level absent of exogenous cytokinin (Fig. 5D,E; Fig. S2F). Taken together, our results support the hypothesis that AUX1 and shootward auxin transport act to maintain ARR10 transcript levels in root apices, thereby influencing the expression of cytokinin primary-response genes. Furthermore, based on the role of ARR10 in mediating cytokinin signaling, control of ARR10 expression represents one mechanism by which AUX1 might positively regulate cytokinin signaling in the root. As discussed later, the interaction of auxin and cytokinin in the regulation of ARR10 could form an autoregulatory circuit controlling sensitivity to these two hormones.

The type-B ARRs and AUX1 are similarly required for cytokinin-dependent regulation of auxin activity in the root

To examine the role of cytokinin in regulation of the root auxin response, we crossed the DR5::GFP auxin reporter into various type-B ARR mutant backgrounds. Treatment with 1 µM BA significantly induced DR5::GFP expression in the outer cell layer of the wild-type LRC, the increased level of auxin activity being observed following 24-h or constant cytokinin treatment (Fig. 6A-E). The region of increased DR5::GFP activity in the LRC extends from a location approximately parallel to the QC up to the transition zone. We also observed significant induction of the DR5::GFP signal in stele tissue but with longer-term kinetics of induction (Fig. 6A,C,E).

Induction of DR5::GFP in the outer cell layer and stele was eliminated in the cytokinin-insensitive arr1 arr12 mutant (Fig. 6A,C,E), consistent with it being dependent on the transcriptional activity of the type-B ARRs. Induction of DR5::GFP was also attenuated in the single type-B ARR mutants, indicating an overlapping function in the control of auxin activity (Fig. S3). Similar to arr1 arr12, induction of DR5::GFP in response
Changes in auxin activity are likely to involve multiple regulators, and so we also examined the root tip for additional cytokinin-dependent effects on gene expression, and these may thus contribute to changes in auxin activity.

Changes in auxin activity are likely to involve multiple regulators, and so we also examined the root tip for additional cytokinin-dependent changes in gene expression. NanoString analysis of the PIN family of auxin efflux carriers revealed that
cytokinin induced a modest decrease in PIN2 expression to 77% of the untreated control, and modest increases in PIN4 and PIN7 expression to 166% and 160% of the untreated control, respectively (Fig. S4B), consistent with previous reports (Ruzicka et al., 2009). The arr1 arr12 mutant eliminated or attenuated these effects of cytokinin on gene expression. Expression analysis also indicated that cytokinin stimulated the expression of ABCG36, which encodes an indole-3-butyric acid (IBA) efflux carrier, and TAR2, which encodes a tryptophan aminotransferase for auxin biosynthesis (Fig. S4C) (Stepanova et al., 2008; Strader and Bartel, 2009).
data indicate that cytokinin induces additional changes in gene expression that are predicted to affect both auxin transport and biosynthesis. Significantly, PIN2, tissue-specific expression of which overlaps with that of AUX1, exhibits a similar reduction in expression in response to cytokinin.

**DISCUSSION**

Cytokinin regulates primary root growth through inhibitory effects on both cell proliferation and elongation (Beemster and Baskin, 2000; Hwang et al., 2012; Kieber and Schaller, 2014; Moubayidin et al., 2009; Schaller et al., 2014). Here, we define regulatory elements specific to the mechanism by which cytokinin inhibits root cell elongation, these involving modulation of both auxin and ethylene activity. Our results establish a genetic circuit whereby the auxin-influx carrier AUX1 operates downstream of cytokinin perception to regulate shootward auxin transport, this involving both ethylene-dependent and -independent mechanisms (Fig. 7A).

Our results also provide insight into how cytokinin-dependent changes in transporter expression regulate auxin activity and, through a feedback circuit (Fig. 7B), have the capacity to establish oscillating patterns of gene expression. Our results complement the extensive literature on cytokinin-auxin interactions, and, more specifically, the significance of cytokinin control of auxin transport, which, within the root alone, regulates such diverse processes as vascular patterning, lateral root development and the control of meristem size (Bishopp et al., 2011; Schaller et al., 2015). Below, we discuss our results within the context of what is known about the interactions of cytokinin, auxin and ethylene in the control of root cell elongation and RAM size.

Our results define a genetic circuit whereby cytokinin inhibits cell elongation through AUX1-dependent changes in auxin activity at the root tip (Fig. 7A). A role for cytokinin in controlling shootward auxin transport is consistent with the distribution of cytokinin activity based on analysis of the Two-Component Signaling Sensor (TCS) reporter and measurement of cytokinin levels in cells of the root apex (Antoniadi et al., 2015). Cytokinin-dependent stimulation of auxin activity in outer cells of the LRC was previously observed (Ruzicka et al., 2009), consistent with what we observe. Our results expand on this prior observation by directly linking the effect of cytokinin on auxin activity in this region to the control of cell elongation, not cell proliferation. Furthermore, we find that cytokinin insensitivity (type-B ARR mutants) or loss of AUX1 result in an inability to induce this zone of auxin activity, as well as an inability to inhibit cell elongation. Because a primary function for AUX1 in these tissues is to mediate shootward auxin transport (Band et al., 2014; Marchant et al., 1999; Swarup et al., 2005), our results support a model in which modulation of shootward auxin flux acts as a key mechanism by which cytokinin controls cell elongation.

We find that AUX1 mediates the inhibitory effects of cytokinin on cell elongation, but is not essential for the inhibition of cell proliferation by cytokinin in the RAM. These results indicate that the regulatory effects of cytokinin on cell expansion and proliferation in the root are separable, such regulatory independence probably being facilitated by the spatial separation of the RAM and elongation zone, with shootward auxin flux controlling cell expansion and rootward auxin flux controlling cell proliferation (Fig. 7A) (Dello Ioio et al., 2008). It has been recently proposed that a shootward auxin flux may mediate communication between the QC and the transition zone to control cell proliferation at the RAM (Moubayidin et al., 2013). However, a prediction of that proposal is that loss of the shootward flux, such as that which occurs in an aux1 mutant, would result in an altered RAM size. We do not observe such an aux1-dependent effect on RAM size (i.e. the ability of cytokinin to inhibit cell proliferation of the RAM is unaffected by the aux1 mutation), indicating that alternative mechanisms exist to coordinate RAM behavior between the QC and the transition zone.

Ethylene also contributes to the inhibitory effects of cytokinin on root cell elongation based on genetic analysis (Fig. 7A). Ethylene-insensitive mutants were first demonstrated to be hypersensitive to cytokinin in regards to overall root growth over two decades ago (Su and Howell, 1992). Since then, a role for ethylene in mediating the effects of cytokinin specifically on root cell elongation has been proposed based on the ability of cytokinin to induce ethylene biosynthesis (Chae et al., 2003; Hansen et al., 2009; Vogel et al., 1998a) and the finding that ethylene inhibits root growth through effects on cell elongation (Bleecker et al., 1988; Kieber et al., 1993; Le et al., 2001). Furthermore, the effects of ethylene on root cell elongation, like cytokinin, require AUX1-dependent changes in auxin distribution (Ruzicka et al., 2007; Swarup et al., 2007). Our results confirm the proposed role for ethylene in the cytokinin response but also, in contrast to prior results, indicate that ethylene-independent mechanisms exist by which cytokinin controls auxin activity. Thus, cytokinin directly affects the AUX1-dependent auxin flux. An overestimation of the role played by ethylene in the cytokinin response may have arisen in part due to use of the ethylene biosynthesis inhibitor 2-aminoethoxyvinyl glycine (AVG) and signaling inhibitor silver ion (Ruzicka et al., 2009; Růžička et al., 2007), which both also affect auxin activity (Soeno et al., 2010; Strader et al., 2009), thereby confounding interpretation of some results. Additionally, ethylene inhibits cell proliferation at the RAM.

**Fig. 7. Model for cytokinin inhibition of root cell elongation and proliferation.** (A) Genetic model for control of root cell elongation and proliferation by cytokinin (CK). The cytokinin signal is transmitted through a two-component signaling pathway involving AHKs, AHPs and type-B ARRs. The auxin influx carrier AUX1 functions downstream of the cytokinin signaling pathway to mediate shootward auxin transport, leading to localized increases in auxin activity, and inhibition of cell elongation. The auxin signaling repressor SHY2 functions downstream of the cytokinin signaling pathway to inhibit rootward auxin transport, resulting in reduced cell proliferation of the RAM. The regulation of auxin activity by cytokinin involves ethylene-dependent and -independent mechanisms. The model is based on results from this study, as well as those of Růžička et al. (2007), Dello Ioio et al. (2008) and Street et al. (2015). (B) An autoregulatory circuit (oscillator) by which auxin and cytokinin regulate expression of ARR10 and AUX1.
and contributes to the cytokinin response here as well (Street et al., 2015) (Fig. 7A), which may also affect analysis of the role of cytokinin in regulating root growth.

Although AUX1-mediated transport of auxin is necessary for cytokinin-dependent control of cell elongation, we find that the expression of AUX1 is inhibited by cytokinin, a finding that might seem counterintuitive at first. AUX1 is a direct target for cytokinin regulation based on type-B ARR12 binding to intron 8, the repression of gene expression by transcription factor binding to introns being well-documented and serving, for example, to repress the expression of AGAMOUS (Sieburth and Meyerowitz, 1997; Dinh et al., 2012). The expression of PIN2, which encodes an auxin-efflux carrier involved in shootward auxin transport, is also reduced in response to cytokinin (Ruzicka et al., 2009). Cytokinin thus represses the expression of two key regulators that mediate the shootward auxin flux, this correlating with the increase in auxin activity in the LRC. We hypothesize that the transition zone is a ‘bottleneck’ for auxin transport, based on the lower level of AUX1 in the epidermal cells of the transition zone compared with the LRC, such that a decrease in AUX1 levels will reduce auxin transport out of this region and result in increased auxin activity and an inhibition of cell elongation. Thus, AUX1 is needed for shootward auxin transport to its site of action at the transition zone, the observed expression changes serving to concentrate auxin in this region. Consistent with this hypothesis, cytokinin reduces the shootward transport of IAA (Zhou et al., 2011). Cytokinin also induced expression of ABCG36, which encodes an IBA efflux carrier, as well as TAR2, which encodes a tryptophan aminotransferase, further emphasizing the importance of auxin transport and biosynthesis in controlling the cytokinin response at the root tip (Stepanova et al., 2008; Strader and Bartel, 2009).

We uncovered a feedback circuit involving expression of the type-B response regulator ARR10 and AUX1 (Fig. 7B), and it is likely that this circuit also plays a role in integrating the effects of cytokinin and auxin on cell elongation and differentiation. AUX1-dependent increases in auxin activity positively regulate expression of ARR10, which would increase cytokinin activity. However, cytokinin acting through the type-B ARRs negatively regulates expression of AUX1 as well as of ARR10, which would decrease cytokinin activity. Regulation of ARR10 expression, in comparison to other members of the type-B ARR family, may be of particular significance owing to its protein stability (Argyros et al., 2008; Hill et al., 2013; Kim et al., 2012, 2013) and enrichment in the epidermal layer where AUX1 is also most active (Argyros et al., 2008; Band et al., 2014), thereby placing ARR10 within a region particularly sensitive to changes in auxin flux. Such cytokinin-auxin antagonism is a common motif in hormonal regulation (Schaller et al., 2015) and here takes the form of a regulatory feedback circuit similar to that of circadian oscillators. Interestingly, the region bordering the RAM has been referred to as the ‘oscillation zone’ owing to its role in establishing oscillating patterns of gene expression in a manner that is partially dependent on root cap-derived auxin (Moreno-Risueno et al., 2010; Xuan et al., 2015). This is also the region in which type-B ARRs are maximally expressed and mediate effects of cytokinin on cell differentiation. Therefore, the auxin-cytokinin feedback circuit described here could establish oscillating patterns of gene expression and of shootward auxin flux through AUX1 in the root. Non-transcriptional mechanisms might also play a role in short-term regulation of auxin transport, such as phosphorylation and/or subcellular trafficking of the transporters (Kleine-Vehn et al., 2006; Rashotte et al., 2001; Rigó et al., 2013; Robert and Offringa, 2008; Tatipawatanakun and Murphy, 2009; Zourelidou et al., 2014), although cytokinin-dependent changes in membrane localization/endocytosis have not yet been observed for AUX1 or PIN2 (Marháv et al., 2011).

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type and mutant lines of Arabidopsis thaliana are Columbia ecotype. The type-B ARR, AUX/LAX and cin2-2 mutants have been previously described (Argyros et al., 2008; Mason et al., 2005; Ugartechea-Chirino et al., 2010; Vogel et al., 1998a). We used the arr12-1 and arr1-2 mutant alleles for these studies and refer to these as arr12 and arr1 for convenience. The AUX1:AUX1:YFP (Swapur et al., 2004) and DR5:GFP (Ulmov et al., 1997) reporter constructs were introduced into mutant backgrounds by crossing; genotyping primers are given in Argyros et al. (2008) and Table S1. The TCS:GFP reporter for cytokinin activity has been previously described (Zürcher et al., 2013). Seedlings for molecular and physiological assays were grown on medium containing 1× Murashige and Skoog (MS) salts with Gamborg’s vitamins and MES (Research Products International), 1% (w/v) sucrose and 0.9% (w/v) phytagar (Research Products International) under continuous white light (100 μE m⁻² s⁻¹) at 22°C, as previously described (Argyros et al., 2008). BA was included in the medium for exogenous cytokinin treatment, the control containing the DMSO vehicle.

Mutant screen

For mutagenesis, 5000 M0 generation arr12 or arr1 seeds were treated with 0.2% (v/v) EMS (Sigma), pooled (~100 seeds per pool), and the M1 generation seeds harvested from the plants and selfed. We screened 20,000 M2 seeds of each genotype, using a pooling strategy with approximately 500 seeds per pool for the identification of err mutants. The M2 seeds were sown on media containing 0.1 μM BA with wild-type and type-B arr mutant background controls. Five-day-old M2 seedlings with long roots relative to the arr12 or arr1 single mutants were selected. Cytokinin insensitivity was confirmed in the M3 generation for six of the candidate err mutants. err mutants were crossed into wild type and arr1 arr12 to assess the contribution of the type-B arr mutant background to the err cytokinin sensitivity. The primers used for genotyping err and arr mutant alleles are given in Table S1, with genotyping performed as previously described (Argyros et al., 2008). Mutant alleles used in this study are given in Table S2.

Root meristem size and cell length determination

Seedlings were cleared with chloral hydrate as described (Perilli and Sabatini, 2010). To determine the size of the root meristem, cells of the cortex layer were counted in a file extending from the QC to where the cell length exceeded its width using a Nikon Eclipse 90i optical microscope with Nomarski optics and the 20× objective as previously described (Perilli and Sabatini, 2010). Cell length was determined from images captured with a Cool Snap HQ2 digital camera (Photometrics) from trichoblast cells at the shootward end of the elongation zone, based on where root hair primordia emerge in wild-type plants (Le et al., 2001). Cell length was measured using ImageJ software (Abramoff et al., 2004).

RNA isolation and expression analysis

Seeds were plated on 20-μm nylon mesh (BioDesign). Fifty root tips (1 mm) from seven-day-old seedlings were harvested following 24-h treatment with 1 μM BA or a vehicle control. Root tips were placed immediately in RNAlater (Life Technologies); the RNAlater solution was removed within 24 h and the tissue frozen in liquid nitrogen. The tissue was ground using a Retesch TissueLyzer and RNA extracted using an RNeasy Plant RNA Isolation Kit (Qiagen). Reverse transcription was performed using Bio-Rad iScript cDNA Synthesis Kit.

NanoString gene expression analysis was performed using a custom probe set (NanoString Technologies) targeted against type-A and type-B ARRs, AUX1/LAX family members and PIN family members (Table S3). Three biological replicates, with 400 ng of total RNA from 1 mm root tips,
were visualized by staining with 10 µg/ml propidium iodide. To image and evaluate relative expression of fluorescent reporters, a Nikon A1 confocal microscope was used. At least five seedlings per line were imaged and ImageJ software was used to quantify fluorescence by the Nikon A1 confocal microscope was used. At least five seedlings per line normalized to the wild-type non-treated control. Primers are listed in Table S1.

ChIP DNA were examined by qRT-PCR using the primers listed in as described (Zhang et al., 2013). Immunoprecipitation was performed cross-linking, chromatin isolation, and immunoprecipitation performed by staining with 10 µg/ml propidium iodide.

Supplementary information available online at Supplementary information (R.S. and M.J.B.). Deposited in PMC for release after 6 months.

Accession numbers
ARR1 (At3g16857), ARR20 (At4g31920), ARR12 (At2g25180), AUX1 (At2g38120), LAX1 (At5g01240), LAX2 (At2g21050), LAX3 (At1g77690), EIN2 (At5g03280).

Table S1.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
I.H.S., J.J.K. and G.E.S. designed the study. I.H.S., with contributions from M.V.Y., R.T.J. and A.S., performed the experiments and analyzed the data. D.E.M., R.S. and M.J.B. generated materials. I.H.S. and G.E.S. wrote the manuscript with input from all authors.

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REFERENCES


DEVELOPMENT


