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

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Summary Statement. A model is developed for how cytokinin inhibits root cell elongation in concert with the hormones auxin and ethylene.

ABSTRACT

Hormonal interactions are critical 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, *AUX1* mutants specifically affecting 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.

INTRODUCTION

The root systems of plants are critical to 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 a phosphorelay 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 these 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, 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 due 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 *ARR*s that contribute most to cytokinin-dependent root development (Argyros et al., 2008; Ishida et al., 2008; Mason et al., 2005). While 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 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 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 if they also contributed to the cytokinin root growth response. The three single *lax* mutants were indistinguishable from wild type in their response to 1μM 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 (Timpte 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; DelloIoio et al., 2008; Marhavý et al., 2011; Ruzicka et al., 2009; Zhang et al., 2011) both decreasing 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 due 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 *arr12* 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 *arr12* and *arr1* (Fig. 2A), suggesting that these type-B ARRs, but not AUX1 plays 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 cytokinin's ability 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 lateral root cap (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 (Růzicka 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 if 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, both mechanisms converging on AUX1 as a key mediator.

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

Since *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 *ARR*s. We therefore examined expression of the seven subfamily-

1 type-B *ARR*s in root tips by use of the NanoString nCounter system, a high-throughput, extremely sensitive and precise method to quantify transcript abundance (Geiss et al., 2008; Malkov et al., 2009) and which is an effective means to follow 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 µM 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 to wild type, being expressed at 64% of the wild-type level (Fig. 4A). Second, expression of *ARR10* was also influenced by cytokinin, cytokinin treatment resulting in a reduction in *ARR10* expression to 59% 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. S2C). 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 *arr1 arr12* background (Fig. 4C, D),

consistent with auxin being a positive regulator of *ARR10* expression and countering 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 *ARR*s, which are cytokinin primary response genes (To et al., 2004). We therefore examined expression of the ten type-A *ARR*s by NanoString analysis with the same RNA samples (wild type, *aux1*, and *arr1 arr12*) used for Fig. 4A. Nine of the type-A *ARR*s were significantly induced by cytokinin in wild-type root tips (Fig. 5A, Fig. S2F). In the cytokinin-insensitive *arr1 arr12* mutant, the expression of multiple type-A *ARR*s is reduced compared to 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 *ARR*s is reduced compared to wild type, but here the effect is 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* may positively regulate cytokinin signaling in the root. As discussed later, the interaction of auxin and cytokinin in the regulation of *ARR10* may 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-hr 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 its 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 to cytokinin in was also strongly attenuated in the *aux1* mutant (Fig. 6A, C, E). We conclude that the cytokinin induction of auxin activity in the root outer cell layer is dependent on both the type-B ARRs and AUX1, consistent with both acting as positive regulators of cytokinin responses in the root.

To determine if cytokinin regulates auxin activity by controlling expression of *AUX1/LAX* family genes, we performed NanoString analysis on its four members. Expression was analyzed in root tips in the presence or absence of 24-hr treatment with 1 μM BA. Consistent with previous work (Zhang et al., 2013), *LAX2* exhibited the most pronounced change, decreasing in response to exogenous cytokinin to 31% of the untreated control; *AUX1* exhibited a more modest decrease to 73% of the untreated control (Fig. 6F, Fig. S4B). The decrease in message levels of *AUX1* is reflected at the protein level based on fluorescence analysis of a *pAUX1:AUX1-YFP* translational fusion (Swarup et al., 2004), which decreases to 58% of the untreated control in response to growth on 1 μM BA (Fig. 6G). The BA-induced decrease in *pAUX1:AUX1-YFP* occurred similarly in both the LRC and in the epidermal cell layer just above it, although the LRC region exhibited 20-fold higher AUX1-reporter expression than the epidermal cell region. We noted that the *AUX1* gene was enriched for extended type-B ARR binding motifs in intron 8 (Fig. S4A) (Franco-Zorilla et al., 2014) and therefore hypothesized that *AUX1* might be a direct target of the type-B ARRs. ChIP-qPCR with a *CaMV35S:ARR12:MYC* construct revealed binding of ARR12 to intron 8 but not to intron 6 of *AUX1*, consistent with cytokinin suppressing *AUX1* expression through direct action of the type-B ARRs (Fig. 6H). Additionally, examination of cytokinin transcriptional activity using the *TCS:GFP* reporter (Zürcher et al., 2013) revealed that cytokinin activity overlaps the region of *AUX1* expression (Fig. 6G, I), consistent with cytokinin modulating auxin flux through action of AUX1. These data indicate that the expression level of *AUX1* is regulated in part through 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 IBA efflux carrier, and *TAR2,* which encodes a tryptophan aminotransferase for auxin biosynthesis (Fig. S4C) (Stepanova et al., 2008; Strader and Bartel, 2009). These data indicate that cytokinin induces additional changes in gene expression that are predicted to affect both auxin transport and biosynthesis. Significantly, *PIN2,* whose tissue-specific expression 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 yield 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 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 cytokinin's effect 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 likely 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 quiescent center (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 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 hyposensitive 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 (Růzicka 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 AVG and signaling inhibitor silver ion (Ruzicka et al., 2009; Růzicka 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 and contributes to the cytokinin response here as well (Street et al., 2015) (Fig. 7A), which may also affect analysis of cytokinin's role 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 a 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 to 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* (Figure 7B), and this circuit is likely to also play 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 due to its protein stability (Argyros et al., 2008; Hill et al., 2013; Kim et al., 2013; Kim et al., 2012) 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' due 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 that type-B ARRs are maximally expressed and mediate effects of cytokinin on cell differentiation. Therefore the auxin-cytokinin feedback circuit described here may establish oscillating patterns of gene expression and of shootward auxin flux through AUX1 in the root. Non-transcriptional mechanisms may also play a role in short-term regulation of auxin transport, such as phosphorylation and/or subcellular trafficking of the transporters (Klein-Vehn et al., 2006; Rashotte et al., 2001; Rigó et al., 2013; Robert and Offringa, 2008; Titapiwatanakun and Murphy, 2009; Zourelidou et al., 2014), although cytokinin-dependent changes in membrane localization/endocytosis have not yet been observed for AUX1 or PIN2 (Marhavý 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 *ein2-5* 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 *arr1-3* mutant alleles for these studies and refer to these as *arr12* and *arr1* for convenience. The *AUX1:AUX1:YFP* (Swarup et al., 2004) and *DR5:GFP* (Ulmasov 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 1x Murashige and Skoog (MS) salts with Gamborg's vitamins and MES (Research Products International, Mount Prospect, IL), 1% (w/v) sucrose, and 0.9% (w/v) phytoagar (Research Products International) under continuous white light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 22°C as 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, 5,000 M0 generation *arr12* or *arr1* seeds were treated with 0.2% (v/v) ethane-methyl sulfonate, 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 described (Argyros et al., 2008).

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 20X objective as described (Perilli and Sabatini, 2010). Cell length was determined from images captured with a Cool Snap HQ2 digital camera 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-micron nylon mesh (BioDesign NY, NY). 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 Retsch 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 submitted for nCounter analysis. Geometric mean and background subtraction were performed using nSolver software v1.1 (Geiss et al., 2008; Malkov et al., 2009). Counts were normalized using NanoString internal positive controls as well as three Arabidopsis control genes included in the probe set: At1g13320 (*Protein Phosphatase 2A*), At4g05320 (*Ubiquitin 10*), and At5g44340 (*Beta-Tubulin 4*). Normalized counts were averaged and analyzed by a two-way ANOVA.

qRT-PCR was performed using the Bio-Rad iTaq SYBR-green system, with three biological replicates and three technical replicates each per treatment. Relative expression was calculated using the ΔΔCt values and normalized to the wild-type non-treated control. Primers are listed in Table S1.

To image and evaluate relative expression of fluorescent reporters, a Nikon A1 confocal microscope was used. At least 5 seedlings/line were imaged and Image J software was used to quantify fluorescence by the Corrected Total Cell Fluorescence method (Burgess et al., 2010). Cell walls were visualized by staining with 10 µg/mL propidium iodide.

ChIP analysis

ChIP-qPCR was performed in biological triplicate with two independent lines of *35S:ARR12-Myc* expressed in wild-type background. The *ARR12* construct was generated by cloning a genomic DNA fragment of *ARR12* into the vector pEarlyGate 203 (Early *et al.,* 2006), using primers ATGACTGTTGAACAAAATTTAGAA and TATGCATGTTCTGAGTGAACT for initial amplification of *ARR12*. Three-week-old seedlings were treated with 5 µM BA in liquid MS media for 30 min, and tissue cross-linking, chromatin isolation, and immunoprecipitation performed as described (Zhang *et al*., 2013). Immunoprecipitation was performed using anti-Myc (ab9132, Abcam, Cambridge, UK) antibodies and MagnaChIP protein A+G Magnetic beads (EMD Millipore, Massachusetts, USA). Input and ChIP DNA were examined by qRT-PCR using the primers listed in Table S1.

Statistical analyses

ANOVA tables were generated using http://www.physics.csbsju.edu/stats/anova.html and multiple comparison tests done using http://graphpad.com/quickcalcs/posttest1/ quick calc web tool that uses the Bonferroni correction for post-test comparisons.

Accession numbers

ARR1 (At3g16857), *ARR10* (At4g31920), *ARR12* (At2g25180), *AUX1* (At2g38120), *LAX1* (At5g01240), *LAX2* (At2g21050), *LAX3* (At1g77690), *EIN2* (At5g03280)

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Competing Interests

No competing interests declared.

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Author Contributions

I.S., J.J.K., and G.E.S. designed the study. I.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.S. and G.E.S. wrote the manuscript with input from all authors.

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Figures

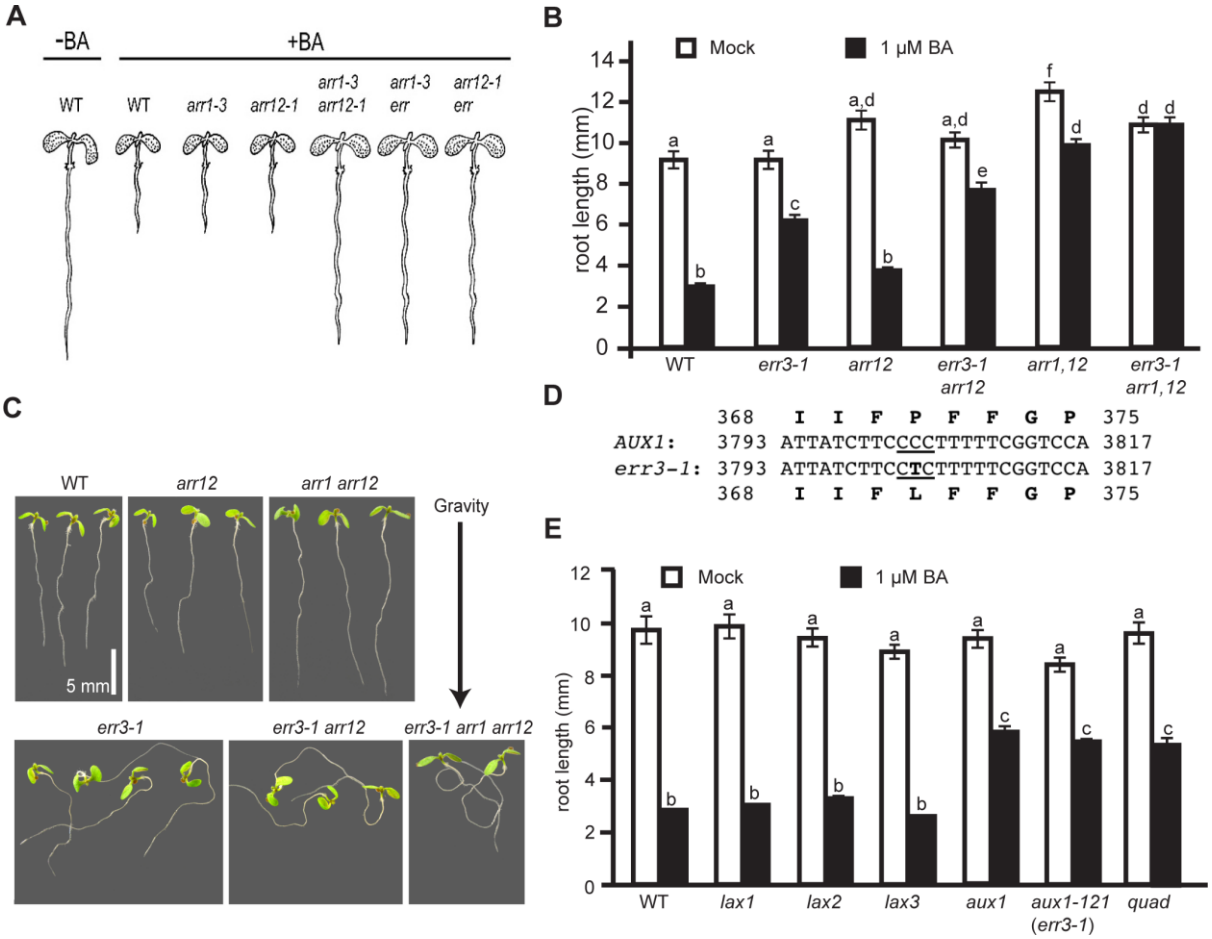


Fig. 1. A genetic screen identifies *AUX1* as a positive regulator of cytokinin signaling in the primary root. (A) Design of the mutant screen to identify *enhancer of response regulator* (*err*) mutants; these enhance the cytokinin insensitivity of type-B *ARR* single mutants**. (B)** Root growth response to cytokinin of wild type (WT), single mutants *err3-1* and *arr12,* double mutants *err3-1 arr12* and *arr1 arr12,* and triple mutant *err3-1 arr1 arr12*. Seedlings were grown on vertical plates in the absence or presence of 1 µM BA ($n \geq 20$) 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 wild type (WT) compared to the *AUX1/LAX* auxin importer family single mutants *lax1, lax2, lax3, aux1-21, aux1-121* (*err3-1*), and the quadruple mutant *lax1 lax2 lax3 aux1* ($n \geq 20$). Same letters in (B) and (E) indicate no statistical significance ($p < 0.05$). Error bars represent SE.

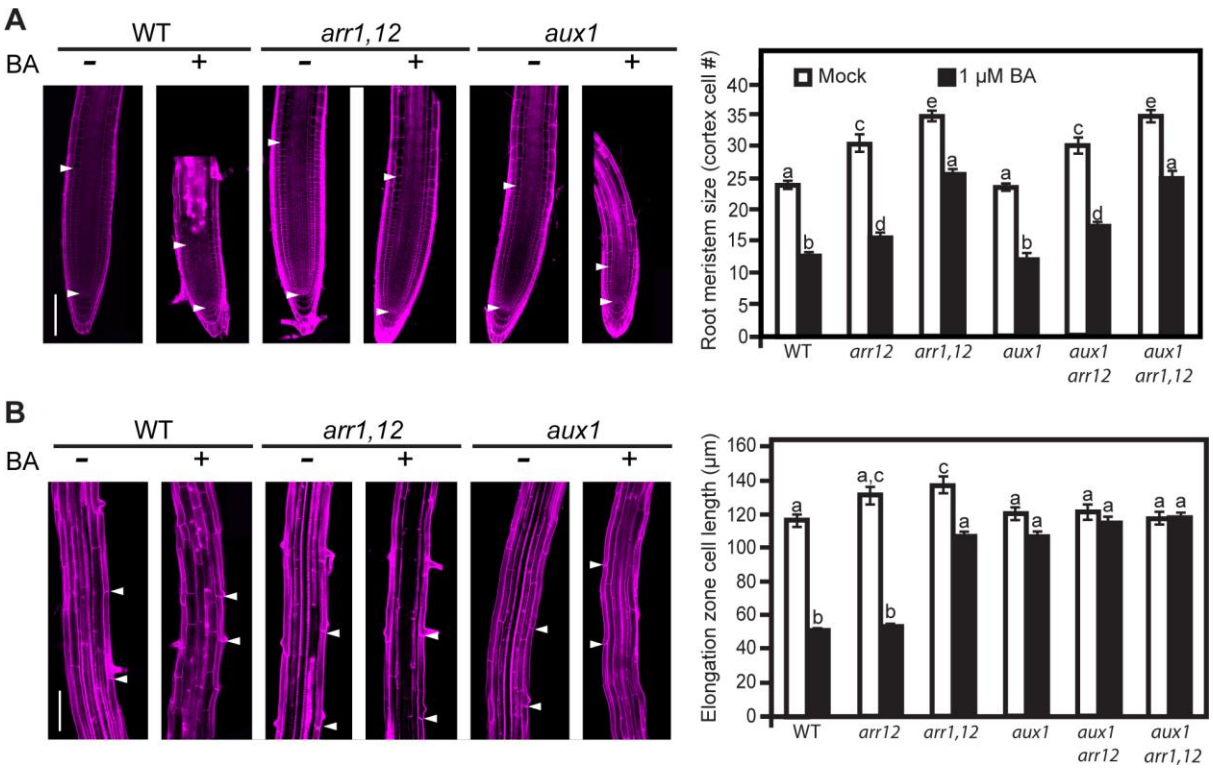


Fig. 2. Cytokinin requires *AUX1* to inhibit cell elongation but not cell proliferation at the root tip. Seedlings of wild type (WT), the single mutants *arr12* and *aux1-121,* the double mutants *arr1 arr12* and *aux1-121 arr12*, and the triple mutant *aux1-121 arr1 arr12* were grown in the absence or presence of 1 µM BA for 5 days. Representative images stained with propidium iodide (scale bar = 100 µm) and quantification are given. (**A**) Effect on root meristem size. Meristem size was quantified by cortical layer cell counts (n≥10). Results of one representative experiment are shown. (**B**) Effect on root epidermal cell size. Cell lengths were quantified on the shootward side of the elongation zone at the region of root hair initiation (n≥20). Same letters indicate no significant difference ($p < 0.05$). Error bars represent SE.

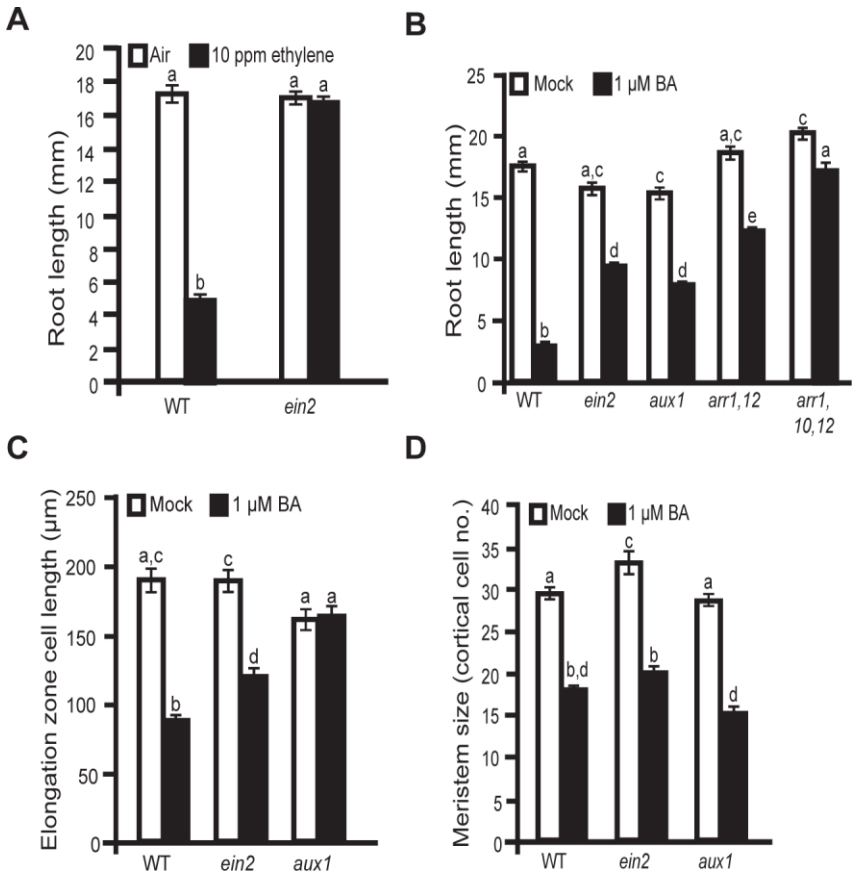


Fig. 3. The ethylene insensitive *ein2* mutant confers partial insensitivity to cytokinin. (A) Root lengths of wild-type and *ein2-5* seedlings grown in the absence or presence of 10 µL L^{-1} ethylene (n≥20) for 7 days. (**B**) Root lengths of wild type as well as *aux1-7, ein2-5, arr1 arr12*, and *arr1 arr10-2 arr12* mutants grown in the absence or presence of 1 µM BA (n ≥ 20) for 7 days. (**C**) Elongation zone cell lengths of roots from wild-type, *ein2-5*, and *aux1-7* seedlings grown in the absence or presence of 1 µM BA (n ≥ 20) for 7 days. (**D**) RAM cortex layer cell counts of *ein2-5* and *aux1-7* seedlings grown in the absence or presence of 1 µM BA (n ≥ 8) for 7 days. Same letters indicate no significant difference ($p < 0.05$). Error bars represent SE.

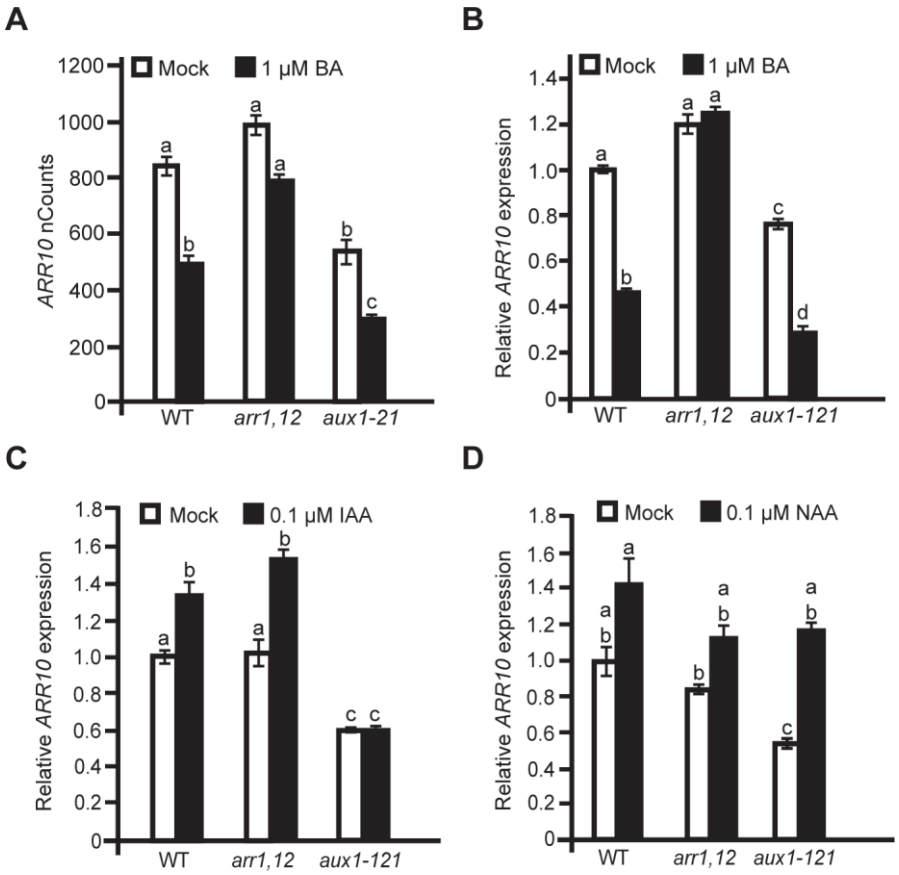


Fig. 4. Effects of auxin and cytokinin on expression of *ARR10*. (A) Cytokinin inhibits expression of *ARR10*. NanoString analysis of *ARR10* levels in 7-day-old 1-mm primary root tips of wild type, *arr1 arr12*, and *aux1-21* treated in the absence or presence of 1 μM cytokinin for 24 hours. Same letters indicate no significant difference ($p < 0.05$). **(B)** qRT-PCR validation of (**A**) in wild-type, *arr1 arr12*, and *aux1-121*. **(C)** Auxin stimulates expression of *ARR10* in an *aux1*-dependent manner. Expression of *ARR10* in root tips of wild-type, *arr1 arr12,* and *aux1-121* of 7-day-old seedlings in the presence or absence of 100 nM IAA. **(D)** Expression of *ARR10* in root tips of wild-type, *arr1 arr12,* and *aux1-121* of 7-day-old seedlings in the presence or absence of membrane-permeant 100 nM NAA. Relative levels of *ARR10* in (**B-D**) are normalized to the untreated wild-type control. Error bars in (**A-D**) represent SE based on three biological replicates.

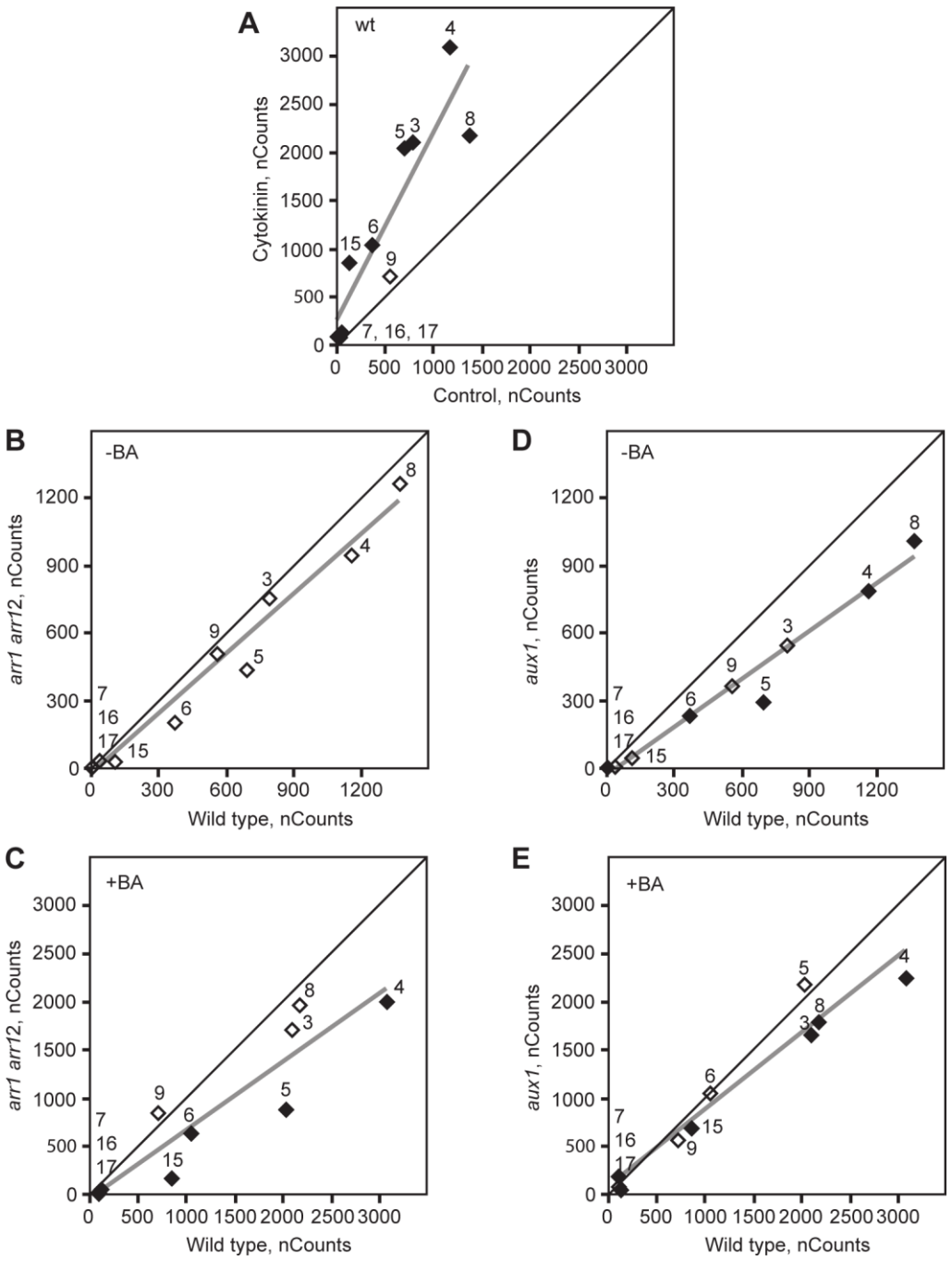


Fig. 5. Type-A *ARR* expression is attenuated in *aux1-21* and *arr1 arr12* mutant backgrounds. Seven-day-old seedlings were treated in the absence or presence of 1µM BA for 24 hours, and NanoString expression analysis of type-A *ARR*s performed on 1-mm primary root tips. **(A)** Type-A *ARR* induction by cytokinin in wild type. **(B)** Type-A *ARR* expression in *arr1 arr12* compared to wild type in the absence of cytokinin (-BA). **(C)** Type-A *ARR* expression in *arr1 arr12* compared to wild type in the presence of cytokinin (+BA). **(D)** Type-A *ARR* expression in *aux1-21* compared to wild type in the absence of cytokinin (-

BA). **(E)** Type-A *ARR* expression in *aux1-21* compared to wild type in the presence of cytokinin (+BA). Points represent the mean of three biological replicates. Black lines at 45˚ angles in plots indicate position of equivalent expression level. Light gray lines are best-fit lines to indicate overall skewing of type-A *ARR* expression. Numbers next to points label individual type-A *ARR* genes. Filled symbols indicate significant differences for type-A ARR expression between the conditions examined ($p < 0.05$).

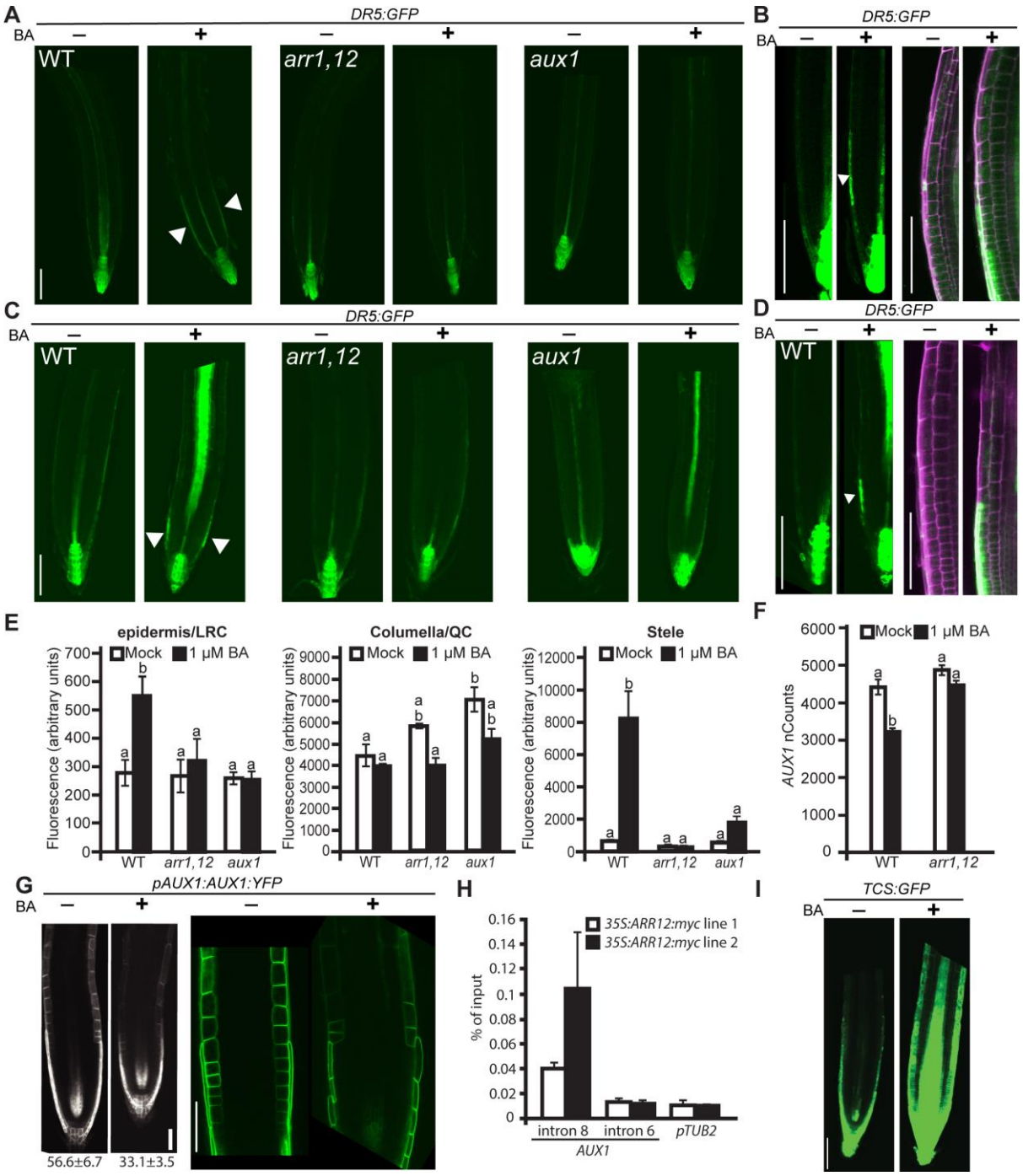


Fig. 6. Cytokinin induction of auxin activity in the root is dependent on type-B *ARRs* and *AUX1*. (A, B) Representative images of *DR5:GFP* reporter activity in wild-type, *arr1 arr12,* and *aux1-7* backgrounds after 24 hours in the absence or presence of 1 µM BA. Scale bar = 100 µm. Arrowheads indicate induction of DR5 reporter in the outer cell layers. **(C, D)** Representative images of *DR5:GFP* reporter activity in wild-type, *arr1 arr12,* and *aux1-7* backgrounds grown in the presence or absence of constant 1µM BA. Scale bar = 100 µm. **(E)**

Quantification of tissue specific *DR5:GFP* signal from experiment in (**C**). From left to right: the epidermis/LRC, QC/columella, and stele tissues (n=5). Same letters indicate no significant difference by ANOVA applying Bonferroni correction post-test comparisons ($p< 0.05$). **(F)** NanoString expression analysis of *AUX1* transcript from 1-mm root tips of 7-day-old seedlings after 24 hours in the absence or presence of 1 µM BA. **(G)** Representative images of *pAUX1:AUX1:YFP* expression after growth in the absence or presence of 1 µM BA. Numbers are relative quantification of *pAUX1:AUX1:YFP* fluorescence (±SE; n=5; $p<0.05$). Scale bar = 50 µm. **(H)** ARR12 binds to intron 8 of *AUX1* based on ChIP-qPCR analysis. ChIP analysis was performed on 3-week-old seedlings with two independent *35S:ARR12:myc* lines treated with 5 µM BA for 30 min. Three biological replicates of each line were analyzed (error bars = SD). *TUB2* and intron 6 of *AUX1* are negative controls. **(I)** Representative images of *TCS:GFP* reporter showing induction of cytokinin activity in 7-day-old root tips treated for 24 h with 1 µM BA or a vehicle control.

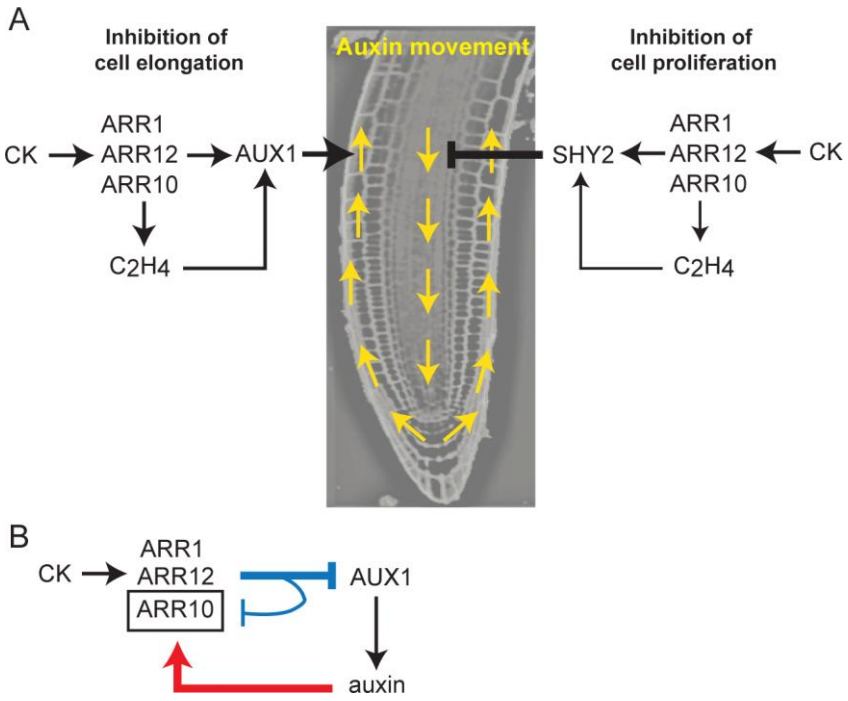
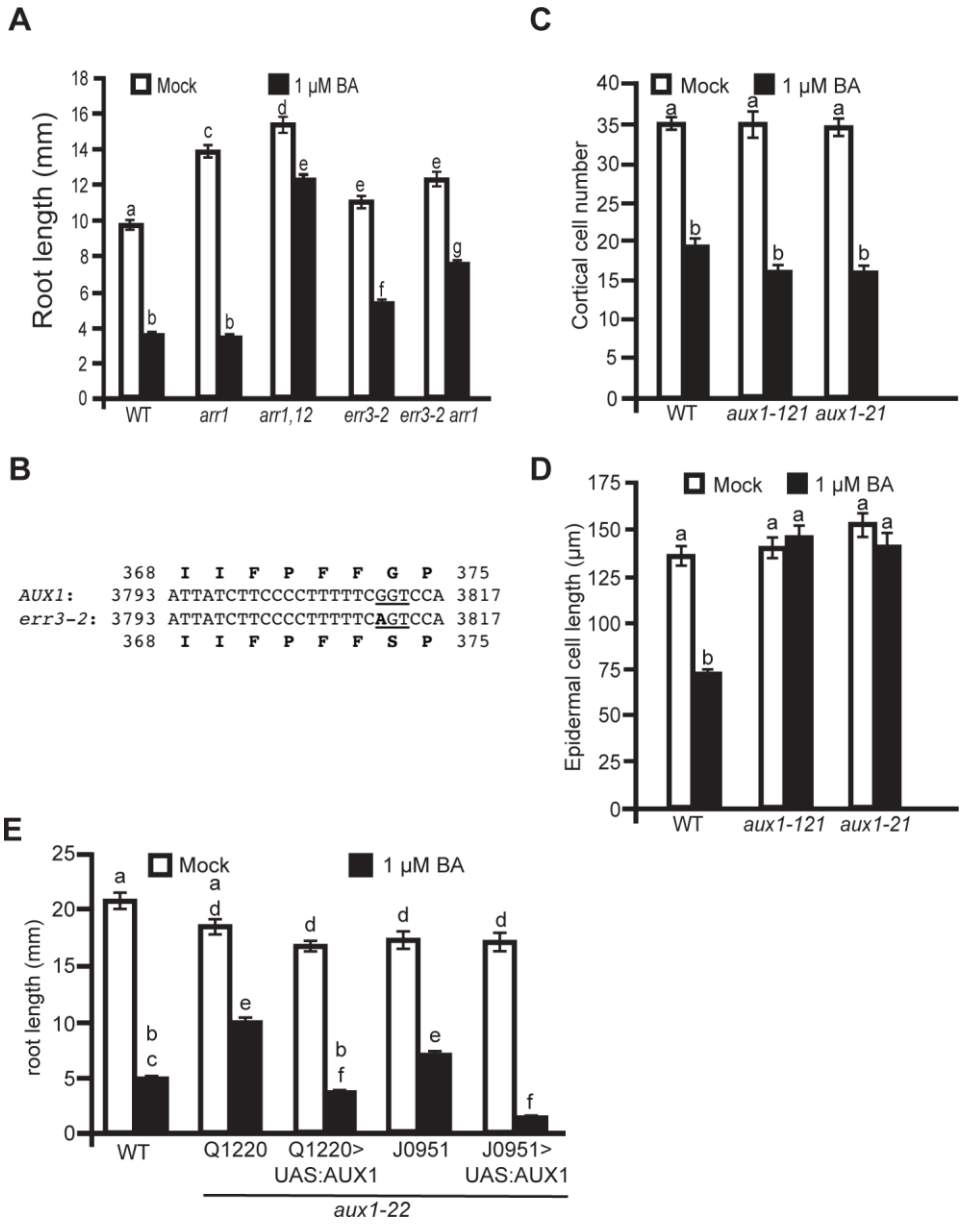


Fig. 7. Model for cytokinin inhibition of root cell elongation and proliferation. (A) Genetic model for control of root cell elongation and proliferation by cytokinin (CK). The cytokinin signal is transmitted through a two-component signaling pathway (AHKs, AHPs, type-B ARRs). The auxin influx carrier 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, Ruzika 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*.

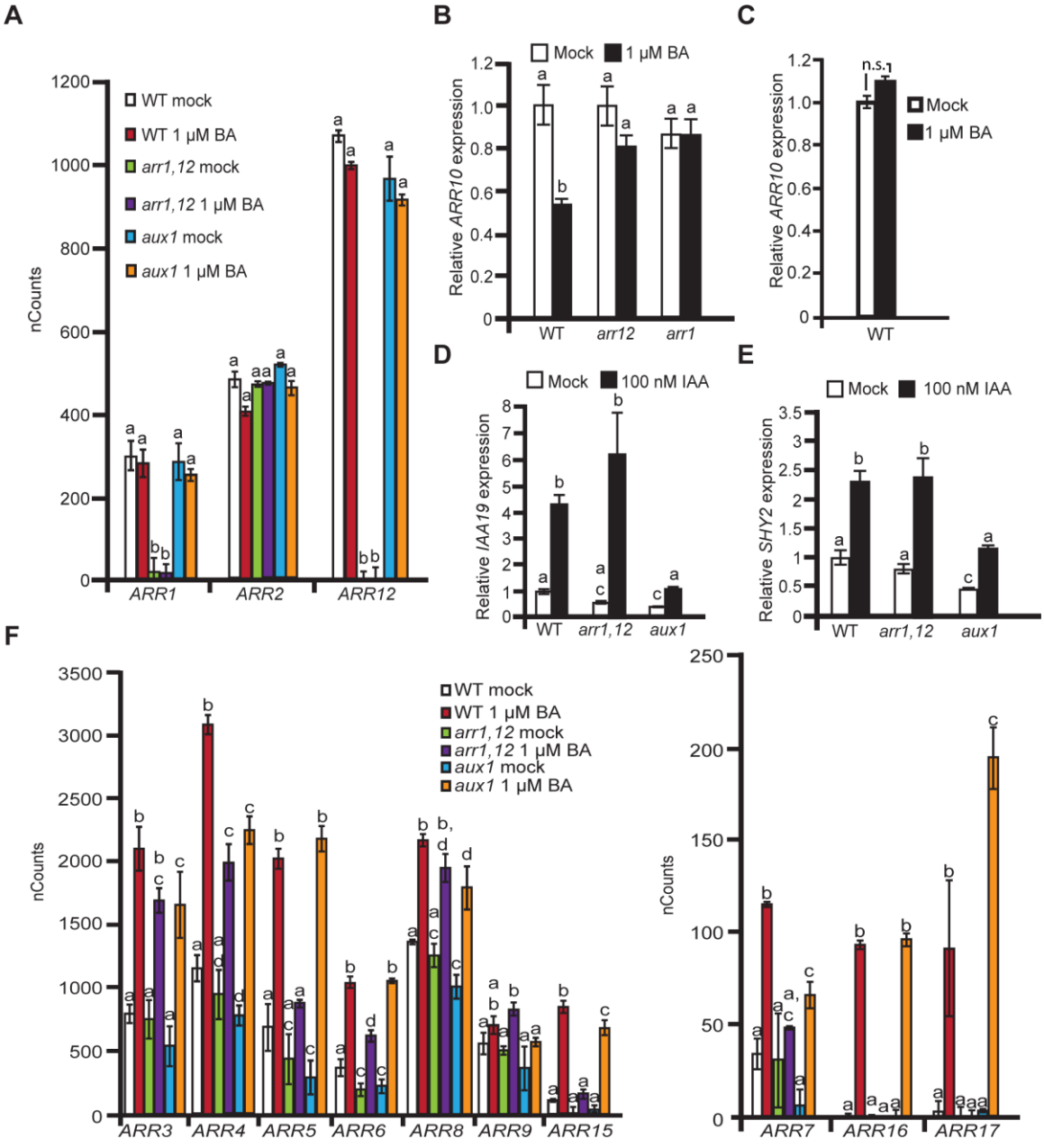
SUPPLEMENTAL DATA

Supplemental Figures



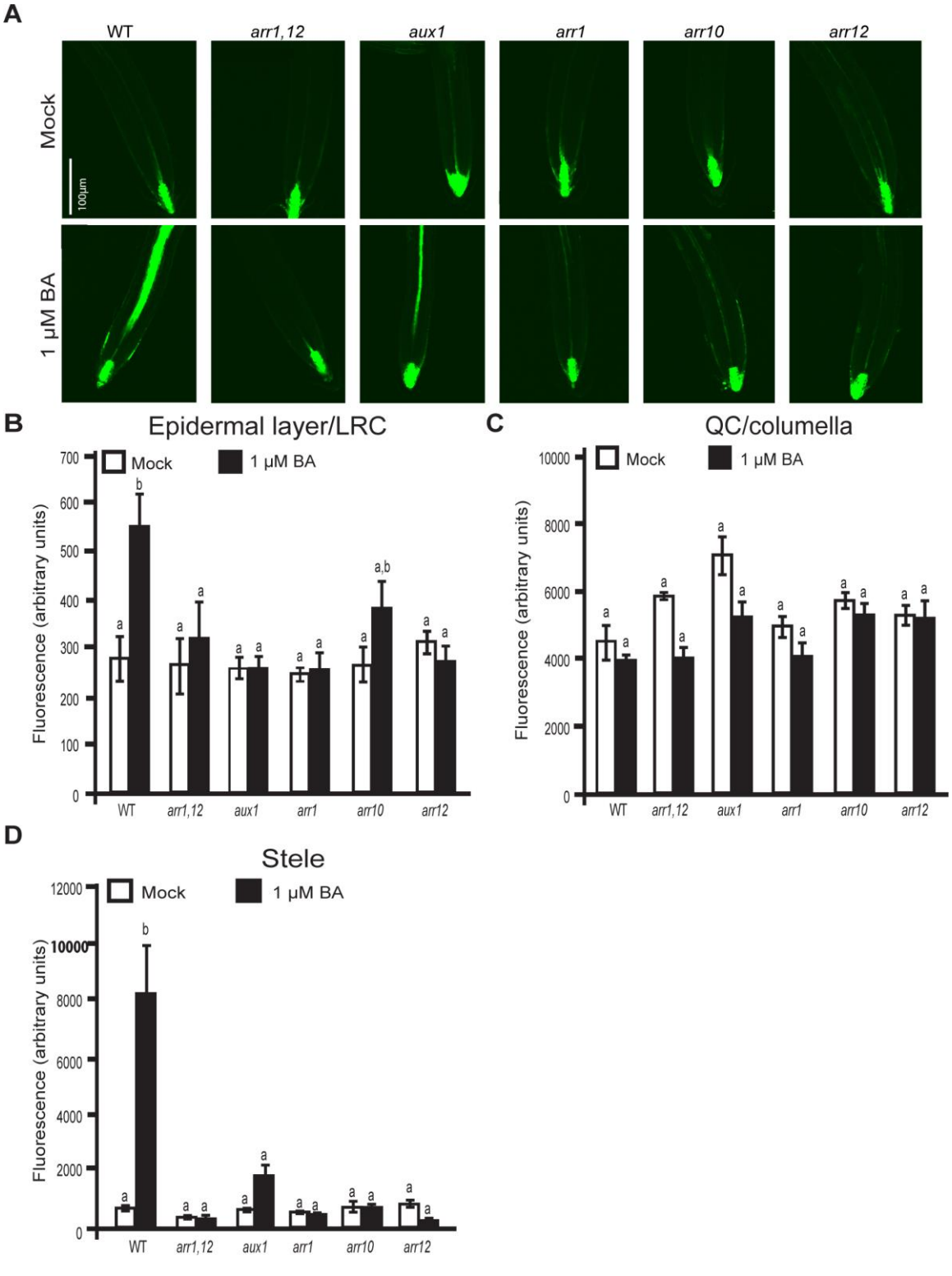
Supplemental Fig. S1. Cytokinin requires *AUX1* to inhibit primary root growth. (A) The *err3-2* mutant allele of *AUX1* results in reduced sensitivity to cytokinin in a root growth assay. Root lengths of wild type (WT), *arr1*, *arr-1-3 arr12*, *aux1-122 (err3-2)*, *aux1-122 (err3-2) arr1* (n ≥20). Plants were grown on vertical plates in the absence or

presence of 1 μM BA for seven days. Same letters indicate no statistical significance by an analysis of variance applying Bonferroni correction post-test comparisons ($p < 0.05$). Error bars represent SE. (B) The G to A missense mutation in *err3-2* induces a Gly374Ser amino acid change in AUX1. (C) The *aux1-121* and *aux1-21* mutants do not affect the ability of cytokinin to inhibit cell proliferation at the root apical meristem. (D) Both *aux1-121* and *aux1-21* mutants affect the ability of cytokinin to inhibit root cell elongation. (E) The sensitivity of *aux1-22* root growth toward cytokinin is restored by targeted expression of *AUX1* in the LRC and elongating epidermal cells in the *aux1-22 Q1220>>UAS:AUX1* and *aux1-22 J0951>>UAS:AUX1* lines using a GAL4-based transactivation expression approach (Swarup et al., 2005).

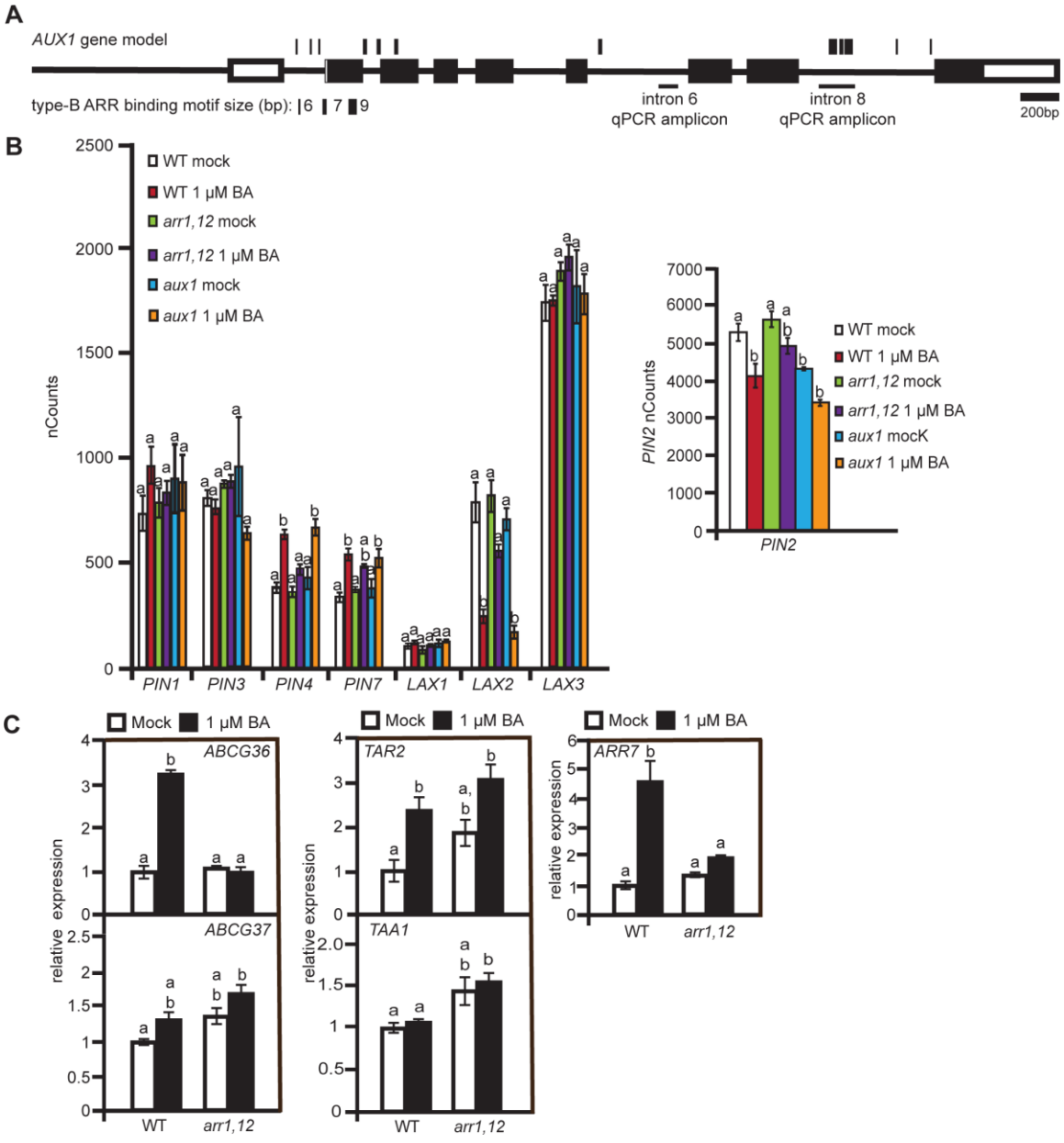


Supplemental Fig. S2. Cytokinin and auxin regulation of genes in the primary root tip. (A) Cytokinin does not affect expression of *ARR1*, *ARR2*, and *ARR12* based on NanoString analysis. nCounts of type-B *ARR1*, *ARR2*, and *ARR12* expression levels in 7-day-old 1-mm primary root tips of wild-type, *arr1,12* and *aux1-21* treated in the absence or presence of 1 µM cytokinin for 24 hours. Same letters indicate no significant difference by an analysis of variance applying Bonferroni correction post-test

comparisons ($p < 0.05$). (B) The cytokinin-mediated reduction in *ARR10* expression is dependent on both *ARR1* and *ARR12*. Expression was determined by qRT-PCR in 7-day-old 1-mm primary root tips of wild-type, *arr1*, and *arr12* treated in the absence or presence of 1 µM cytokinin for 24 hours. (C) Cytokinin does not inhibit *ARR10* expression in roots lacking the root tip. Expression was determined by qRT-PCR in 7-day-old primary roots from which the 1-mm root tip was excised, the seedlings having been treated in the absence or presence of 1 µM cytokinin for 24 hours. n.s. = not significant by student's t-test ($p < 0.05$). (D, E) Known auxin-inducible genes *IAA19* and *SHY2* are still auxin inducible in an *aux1* background; expression determined in 7-day-old 1-mm primary root tips of wild-type, *arr1 arr12* and *aux1-21* treated in the absence or presence of 100 nM IAA for 24 hours. (F) Histograms of NanoString data presented in Figure 5 with higher (left) and lower (right) abundant type-A *ARRs* in wild-type, *arr1 arr12*, and *aux1-21* after 24-hours treatment in the presence or absence of 1µM BA. Same letters indicate no significant difference by an analysis of variance applying Bonferroni correction post-test comparisons ($p < 0.05$). Error bars represent SE.



Supplemental Fig. S3. Single type-B *ARR* mutants have attenuated epidermal and stele expression of the *DR5:GFP* auxin reporter after cytokinin treatment. (A) Representative images of 7-day-old *DR5:GFP* lines in wild-type, *arr1*, *arr10*, *arr12*, *arr1,12*, and *aux1-7* mutant backgrounds grown in the presence or absence of 1 μM BA. **(B-D)** Quantification of *DR5:GFP* signal in the root tip (n=5). Same letters in **(B-D)** indicate no significant difference by an analysis of variance applying Bonferroni correction post-test comparisons ($p < 0.05$). Error bars represent SE.



Supplemental Fig. S4. Cytokinin regulates expression of auxin transport and biosynthesis genes in the root tip. (A) Intron 8 of the *AUX1* gene is enriched for extended type-B ARR binding motifs. The positions of 6-, 7-, and 9-bp extended binding motifs for type-B ARRs are shown based on motifs determined by protein-binding microarray analysis (Franco-Zorrilla et al., 2014)**.** Intron 8 contains one 7-bp (CGGATCC) and two 9-bp (AAGATACGG and TAGATACGG) binding motifs in close proximity. No 8-bp binding motifs were found. **(B)** NanoString expression analysis of

auxin transporter transcripts from 1-mm root tips of 7-day-old seedlings after 24 hours in the presence or absence of 1 µM BA in wild-type, *arr1 arr12* and *aux1-21*. Same letters indicate no significant difference by an analysis of variance applying Bonferroni correction post-test comparisons ($p < 0.05$). **(C)** Cytokinin induces expression of *ABCG36* and *TAR2* in root tips based on qRT-PCR analysis, these encoding an IBA efflux carrier and an auxin biosynthetic enzyme, respectively. Expression of the related *ABCG37* and *TAA1* were not affected by cytokinin. *ARR7* served as a positive control for cytokinin induced gene expression. Cytokinin treatment was for 24-hr with 1 µM BA.

Supplemental Table S1. Primers used in this study.

Genotyping primers:	
pROK2 left border T-DNA primer	5'- TGGTTCACGTAGTGGGCCATCG
ARR1 genotyping	5'- CAACAGCAACCACAGATGAACTTAC 5'- CATAAACCTGATGGTGCTGTACCATTC
ARR12 genotyping	5'- ATCTCTCCTTCGTCACTGCCAATAC 5'- TCATATGCATGTTCTGAGTGAACTAAAC
aux1-121/122 CAPS genotyping (mutations cause loss of *BslI* restriction site)	5'- TGCTACCAAAGCACTACTACTAC 5'- GAAATGGCTGAAACCAACTCAA
qRT-PCR primers:	
TUB4	5'- TGGTGGAGCCTTACAACGCTACTT 5'- TTCACAGCAAGCTTACGGAGGTCA
ARR10	5'-ATATCACGTTACAACAACGAATCAGGCAC 5'- CATACTTTGGATCGCTATGCGCAG
AUX1	5'- GTCACGCGGTTACTGTTGAGATTATG 5'- GCGTTCTTGGGCATAAGAGAGAA
SHY2	5'- AAACAGAGCTGAGGCTGGGATTAC 5'- AACTGGTGGCCATCCAACAATCT
IAA19	5'- TTCATCTGGTGGTGACGCTGAGAA 5'- TAAGGCACACCATCCATGCTCACT
TAR2	5'- TGCTATGTATGAGAGGTGGAAAC 5'- CTCAAAGACCCTGCCAAAGA
TAA1	5'- AGATGATGAAGAATCGGTGGG 5'- ACGCAAACGCAGGGTAAGA
ABCG36	5'- GCCAACCCTTCCATCATCTT 5'- CTTACCGCCCTCATCACAATAG
ABCG37	5'- CGGTTTCTATCCTTCAGCCTAC 5'- GAGGAACATCGCAAGGTAGTT

ARR7	5’- AGAGTGGAACTAGGGCTTTGCAGT 5’-CTCCTTCTTTGAGACATTCTTGTATACGAGG
ChIP primers:	
TUB2 promoter	5’- CACAAAGGAAGTTGACGAGCAG 5’- CGCCTGAACATCTCTTGGATTG
AUX1 intron 7	5’- GAGTCGGTGGAGGAATA 5’- GAAATGGCTGAAACCAACTCAA
AUX1 intron 6	5’- TGCTACCAAAGCACTACTACTAC 5’- GATTGGATTCACAACAATCATTGAC

Supplemental Table S2. Mutant alleles used in this study.

Line	Nature of allele	Reference
err3-1/aux1-120	Point/null	This study
err3-2/aux1-121	Point/null	This study
aux1-7	Point/null	Bennett et. al. 1996
aux1-21	Point/null	Bennett et. al. 1996
aux1-22 J0951	Null *aux1* plus driver	Swarup et al., 2005
aux1-22 J0951>>UAS:AUX1	Null *aux1* plus LRC/epidermal *AUX1*	Swarup et al., 2005
aux1-22 Q1220	Null *aux1* plus driver	Swarup et al. 2007
aux1-22 Q1220 Q1220>>UAS:AUX1	Null *aux1* plus LRC/epidermal *AUX1*	Swarup et al. 2007
arr12-1	T-DNA	Mason et. al. 2005
arr1-3	T-DNA	Mason et. al. 2005
arr10-5	T-DNA	Argyros et. al. 2008
arr10-2	T-DNA/partial lof	Mason et. al. 2005
lax1	Transposon	Ugartechea-Chirino et. al. 2010
lax2	Transposon	Ugartechea-Chirino et. al. 2010
lax3	Transposon	Ugartechea-Chirino et. al. 2010
ein2-5	Frame shift/null	Alonso et. al. 1999

Supplemental Table S3. Nanostring codeset.

Gene name	
Endogenous Controls	
PP2A-A3	AT1G13320
UBQ10	AT4G05320
TUB4	AT5G44340
Type-A *ARRs*	
ARR3	At1g59940
ARR4	At1g10470
ARR5	At3g48100
ARR6	At5g62920
ARR7	At1g19050
ARR8	At2g41310
ARR9	At3g57040
ARR15	At1g74890
ARR16	At2g40670
ARR17	At3g56380
Type-B *ARRs*	
ARR1	At3g16857
ARR2	At4g16110
ARR10	At4g31920
ARR11	At1g67710
ARR12	At2g25180
ARR14	At2g01760
ARR18	At5g58080
Auxin transport	
AUX1	At2g38120
LAX1	At5g01240
LAX2	At2g21050
LAX3	At1g77690
PIN1	At1g73590
PIN2	At5g57090
PIN3	At1g70940
PIN4	At2g01420
PIN7	At1g23080