Cytokinins influence root gravitropism via differential regulation of auxin transporter expression and localization in *Arabidopsis*

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Cytokinin influence root gravitropism via differential regulation of auxin transporter expression and localization in *Arabidopsis*.

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SUMMARY
Redirection of intercellular auxin fluxes via relocalization of the PIN3 and PIN7 auxin efflux carriers has been suggested to be necessary for the root gravitropic response. Cytokinins have also been proposed to play a role in controlling root gravitropism, but conclusive evidence is lacking. We present a detailed study of the dynamics of root bending early after gravistimulation, which revealed a delayed gravitropic response in transgenic lines with depleted endogenous cytokinins (Pro35S:AtCKX) and cytokinin signaling mutants. Pro35S:AtCKX lines as well as a cytokinin receptor mutant ahk3 showed aberrations in the auxin response maximum in columella cells consistent with defects in the auxin transport machinery. Using in vivo real-time imaging of PIN3-GFP and PIN7-GFP in AtCKX3 overexpression and ahk3 backgrounds, we observed wild type-like relocalization of PIN proteins in columella early after gravistimulation, with gravity-induced relocalization of PIN7 faster than that of PIN3. Nonetheless, the cellular distribution of PIN3 and PIN7 and expression of PIN7 and the auxin influx carrier AUX1 was affected in AtCKX overexpression lines. Based on the retained cytokinin sensitivity in pin3 pin4 pin7 mutant, we propose the AUX1-mediated auxin transport rather than columella-located PIN proteins as a target of endogenous cytokinins in the control of root gravitropism.

Key words: Arabidopsis, auxin, cytokinins, PIN, AUX1, root gravitropism

INTRODUCTION
Plants as sessile organisms have developed a plethora of mechanisms allowing developmental adaptation to a wide range of environmental conditions. In response to changes in the gravity vector, plants redirect root growth, facilitating proper grounding and nutrient acquisition from the soil. The root gravitropic response is a consequence of several regulatory events in which the site of gravity sensing is spatially separated from the elongation zone where bending takes place (Ottenschlager et al., 2003; Swarup et al., 2005; Muday & Rahman, 2008). Perception of the gravitropic signal occurs in specialized columella cells in the root tip (Blancaflor et al., 1998; Tsugeki & Fedoroff, 1999), but the precise mechanism is still unknown (Baldwin et al., 2013). After gravistimulation, amyloplasts filled with starch grains sediment to the new bottom side of the cells, which is suggested to be the key
step in gravity sensing (Perrin et al., 2005; Leitz et al., 2009). Afterwards, alkalinization of the cytoplasm occurs (Fasano et al., 2001; Monshausen et al., 2011), followed by relocalization of auxin efflux carriers from the PIN-FORMED (PIN) family (Petrasek et al., 2006; Wisniewska et al., 2006), particularly PIN3 and PIN7 (Friml et al., 2002b; Harrison & Masson, 2008; Kleine-Vehn et al., 2010). As a result, the auxin flow is redirected preferentially to the lower side of the root (Band et al., 2012; Brunoud et al., 2012) and translocated in a PIN2-dependent manner to the elongation zone (Luschnig et al., 1998). This asymmetric auxin flow is reinforced by stabilization of PIN2 at the lower side of the root and its vacuolar-mediated degradation at the upper side (Abas et al., 2006; Baster et al., 2013). Auxin accumulating at the lower side inhibits cell elongation, whereas cells in the upper side of the root elongate normally. Differential elongation of opposite root sides results in downward bending (Ishikawa & Evans, 1993). The auxin transport and auxin-dependent root gravitropic response is regulated not only by PIN efflux carriers but also by the influx carriers from the AUX/LAX family (reviewed in (Swarup & Peret, 2012)).

Another group of phytohormones, cytokinins has been also proposed to play a role in root gravitropism (Aloni et al., 2004). Cytokinins stimulate a multistep phosphorelay signaling pathway by binding to cytokinin receptors ARABIDOPSIS HISTIDINE KINASE AHK2, AHK3 or AHK4/WOL/CRE1. The signal is subsequently transferred through ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS (AHPs) to ARABIDOPSIS RESPONSE REGULATORS (ARRs). Type-A ARRs, the cytokinin primary response genes, are promptly upregulated by cytokinins, in parallel inhibiting the cytokinin signaling pathway, and thus generating a negative feedback loop. Type-B ARRs contain a DNA binding domain and control expression of cytokinin-regulated genes, including type-A ARRs (reviewed in (Hwang et al., 2012)). Asymmetric activation of one of the type-A ARRs, ARR5, early after gravistimulation (Aloni et al., 2004) suggests that the cytokinin signaling pathway may play a role in the root gravitropic response. In support of this, lateral expansion of the auxin maxima in roots of lines with depleted endogenous cytokinins via overexpression of CYTOKININ OXIDASE DEHYDROGENASE (CKX) genes (Pro35S:AtCKX2 and Pro35S:AtCKX3) has been demonstrated (Pernisová et al., 2009).
Here, we present a possible mechanism underlying the role of endogenous cytokinin levels and cytokinin signaling in the root gravitropic response. Our detailed microscopy analysis, including in vivo real time imaging at subcellular resolution revealed a cytokinin effect on expression and/or protein localization of PIN3, PIN7 and AUX1 auxin transporters. However, using genetic manipulation we propose rather minor role of columella-located PIN proteins in controlling the root gravitropic response and suggest AUX1 in mediating the cytokinin control over root gravitropism.

**MATERIALS AND METHODS**

**Plant material**

Unless otherwise mentioned, all plant material used was *Arabidopsis thaliana*, ecotype Col. The transgenic or mutant lines have been described previously: *Pro35S:AtCKX2*, *Pro35S:AtCKX3*, *Pro35S:AtCKX2/DR5rev:GFP* and *Pro35S:AtCKX3/DR5rev:GFP* (Pernisová et al., 2009), *DR5rev:GFP* (Friml et al., 2003), *DII-Venus* (Liao et al., 2015), *ProPIN3:PIN3-GFP* (PIN3-GFP; (Zadnikova et al., 2010)), *ProPIN7:PIN7-GFP* (PIN7-GFP; (Kleine-Vehn et al., 2010)), *ProAUX1:AUX1-YFP* (AUX1-YFP; (Swarup et al., 2004)), *ProAHK3:AHK3-uidA* (AHK3-GUS; (Dello Ioio et al., 2007)), *ahk2-1* (Nishimura et al., 2004), *ahk2-5* (Riefler et al., 2006), *ahk2-7* (Rashotte et al., 2006), *ahk3-1* (Nishimura et al., 2004), *ahk3-3* (Higuchi et al., 2004), *ahk3-7* (Riefler et al., 2006), *ahk4-1* (Ws ecotype) (Ueguchi et al., 2001), *cre1-2* (Inoue et al., 2001), *cre1-12* (Higuchi et al., 2004), *ahk2-5 ahk3-7*, *ahk2-5 cre1-2*, *ahk3-7 cre1-2*, *ahk2-5 ahk3-7 cre1-2* (Riefler et al., 2006), *ahn1 ahn2 ahn3 ahn4 ahn5* (Deng et al., 2010), *arr1-3 arr10-5 arr12-1* (Mason et al., 2005), *pin3-5 pin7-1*, *pin3-5 pin4-3 pin7-1* (Blilou et al., 2005) and *aux1-7* (NASC N3074).

*Pro35S:AtCKX2* and *Pro35S:AtCKX3* were crossed with PIN3-GFP, PIN7-GFP, AUX1-YFP and double homozygous lines were analyzed. Mutant lines *ahk2-5*, *ahk3-7*, *cre1-2*, *ahk2-5 ahk3-7*, *ahk2-5 cre1-2* and *ahk3-7 cre1-2* were crossed with *DR5rev:GFP*, *ahk3-7* was crossed with PIN3-GFP and PIN7-GFP; and homozygous lines were used in the experiments.

**Growth conditions**

The growth media used comprised 0.5 MS (Duchefa) with 1% sucrose and 1% Plant agar (Duchefa), pH 5.7. 100 nM 1-naphthalene acetic acid (NAA; Sigma), 100 nM
benzyladenine (BA; Sigma) and 100 nM INCYDE (Zatloukal et al., 2008) were used for exogenous hormonal treatment. Plants were cultivated in growth chambers (CLF Plant Climatics Gmb) under long day conditions (16 h light/8 h dark) at 21°C in Petri dishes on 0.5 MS medium or in soil, with a light intensity of 150 µM.m⁻².s⁻¹ and 60% relative humidity.

**Root gravitropic angle measurement**

Five-day-old plants on Petri dishes were gravistimulated by rotation of 90°. The root gravitropic angle between the longitudinal axis of the root apical meristem and elongation zone of the primary root was measured. The gravitropic angle was approximately 180° immediately after gravistimulation and decreased with time until it reached that for downward growth (90°). Thus, the gravitropic angle reflected the velocity of root tip bending after gravistimulation.

**Histochemical staining**

AHK3-GUS seedlings were stained in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% X-gluc, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆] and 0.05 % Triton X-100 for 30 minutes at 37°C and were destained overnight in 80% (vol/vol) ethanol. Tissue clearing was conducted as previously described (Malamy & Benfey, 1997).

**Microscopy**

DIC microscopy was performed on an Olympus BX61 microscope (Olympus Optical Co., Ltd.) equipped with a DP50 camera. Confocal microscopy was carried out on several different microscopes: an inverted Zeiss Observer.Z1 equipped with a LSM780 confocal unit and x40 water immersion objective; a vertical Zeiss microscope with confocal unit LSM700 and x20 air objective; an upright LEICA DM 2500 with TCS SPE confocal unit and x40 air objective; a Nikon AZ100 with horizontally-oriented optical axis, Nikon D-ECLIPSE C1 confocal unit and x5 air objective with long working distance (Pernisova et al., unpublished). An appropriate set of filters was used for GFP imaging (excitation 488 nm, emission 507 nm).

**Image analysis**

Root or cell growth parameters were analyzed with the ImageJ software (NIH; http://rsb.info.nih.gov/ij). Signal intensity measurements were carried out with
software accompanying the confocal microscopes: ZEN (Carl Zeiss MicroImaging GmbH), LAS AF lite (Leica Microsystems CMS GmbH) and EZ-C1 (Nikon Corporation).

**Signal intensity ratio measurement**

PIN3-GFP, PIN7-GFP, AUX1-YFP and *DII-Venus* signal intensities were measured as average grey scale values, ranging from 0 to 4096. PIN3-GFP and PIN7-GFP signal intensity ratio was calculated as a percentage of the signal intensity at the lateral membrane (new bottom membrane after gravistimulation) to the sum of signal intensities at the basal (old bottom membrane before gravistimulation) and lateral membranes. According to this definition, the signal intensity ratio showed changes in the protein redistribution between basal and lateral membranes, thus reflecting the dynamics of protein relocalization during the root gravitropic response.

**Statistical analyses**

The change in the signal intensity ratio was evaluated from 0 to 5 min after gravistimulation by calculating the signal intensity ratio at time 0 subtracted from the signal intensity ratio at time 5 min divided by five. The resulting value was always positive, suggesting that the signal intensity ratio increased over the first five minutes after gravistimulation. Similarly, the change in the signal intensity ratio was calculated from 5 to 30 min as the average of changes over the particular time period weighted by the length of the time period. The afore-mentioned variables were referred to as the increase of the signal intensity ratio from 0 to 5 min and from 5 to 30 min, respectively.

All the variables, including the signal intensity at membranes and signal intensity ratio at times 0, 5, 10, 20 and 30 minutes, as well as the increase in the signal intensity ratio from 0 to 5 min, and from 5 to 30 min, respectively, were tested for a normal distribution within the WT, *Pro35S:AtCKX3* line and *ahk3-7* mutant by means of the Shapiro-Wilk test. Since almost all tests showed a normal distribution, the two sample *t*-test was used to analyze differences between the WT and *Pro35S:AtCKX3* line and between the WT and *ahk3-7* mutant. Owing to multiple usage of the *t*-test, the hypotheses were tested at an adjusted significance level of $\alpha = 0.025$ ($0.05/2$; Bonferroni’s correction). The one-sample *t*-test was used to analyze the increase in
the signal intensity ratio from 0 to 5 min and from 5 to 30 min, respectively, comparing the values against a reference value 0. In cases of a small number of samples, the nonparametric Mann-Whitney U test and one sample Wilcoxon test were used. Data analyses were performed with IBM SPSS Statistics, version 22 and Statistica software, version 12 (StatSoft, 2013).

RESULTS

Cytokinins regulate the root gravitropic response

To investigate the potential role of cytokinins in root gravitropism, we used transgenic lines with depleted endogenous cytokinin levels (Pro35S:AtCKX2 and Pro35S:AtCKX3) and single and multiple cytokinin signaling mutants (ahk, ahp, arr). The root gravitropic angle between the root meristem and elongation zone (Materials and Methods, Fig. S1a) was measured 3 hours after gravistimulation. The angle was larger in all Pro35S:AtCKX2 and Pro35S:AtCKX3 lines compared with WT, suggesting slower root bending (Fig. 1a). A comparable effect was observed in single and double mutants of cytokinin receptors carrying the ahk3 allele (Fig. 1b, Fig. S1b). An increased angle was also observed in multiple ahk2 ahk3 ahk4, ahp1 ahp2 ahp3 ahp4 ahp5 and arr1 arr10 arr12 mutants (Fig. 1b), however probably reflecting the general root growth defects observed in these plants (Nishimura et al., 2004; Hutchison et al., 2006; Ishida et al., 2008). We also tested exogenous cytokinin application in seedlings grown on MS media supplemented by benzyladenine. However, no difference between mock and cytokinin treated plants was identifiable after 3 hours of gravistimulation (Fig. S1c), suggesting dominant role of endogenous cytokinins in the root gravitropic response.

We also investigated a longer time of gravistimulation, specifically 6 and 24 hours. Differences in the gravitropic response of cytokinin receptor single and double mutants after 6 hours were similar to those seen after 3 hours. On the other hand, the gravitropic response was comparable to WT in all ahk single and double mutants after 24 hours of gravistimulation (Fig. S1d), suggesting the transient nature of the observed phenomenon.

For detailed characterization of the early events during root gravitropic bending, we developed a novel approach allowing in vivo real-time imaging using a confocal microscope Nikon with horizontal-oriented optical axis (Pernisova et al., unpublished). We measured several parameters of gravitropic root bending, i.e.,
length of trajectory, height of trajectory peak, and upward and downward radius (Fig. 1c). In comparison to the control, all parameters were increased in both Pro35S:AtCKX2 and Pro35S:AtCKX3 lines and in the ahk3-7 mutant (Fig. 1d), further supporting our previous observations.

Altogether, our findings suggest that cytokinins control the dynamics of the root gravitropic response in Arabidopsis but not the ability of the root to react to changes in the gravitropic vector per se. This effect is mediated through the cytokinin signaling pathway, primarily via the cytokinin receptor AHK3.

Cytokinins affect the root gravitropic response via gravitropic signal perception or transduction

The velocity of root growth is determined by the rate of cell division in the root apical meristem, cell differentiation and, in particular, elongation of differentiated cells leaving the root apical meristem. Cytokinins have been shown to initiate cell differentiation, leading to shortening of the meristem, and thus reduction of the root length (Dello Ioio et al., 2007). However, cytokinins do not seem to control the cell division rate in the root apical meristem (Beemster & Baskin, 2000; Werner et al., 2003; Dello Ioio et al., 2007) but instead negatively regulate the number of dividing cells (Werner et al., 2003; Dello Ioio et al., 2007).

To decipher the cytokinin impact in delayed root gravitropic bending, we first compared root elongation without any gravistimulation in WT, Pro35S:AtCKX2, and Pro35S:AtCKX3 lines and cytokinin receptor single and double mutants. Interestingly, we observed that root growth was faster in all tested lines in comparison to WT (Fig. 2a). Next, we investigated the potential effect of cytokinins on cell elongation, which is the dominant mechanism of root bending after gravitropic stimulation. We found that fully differentiated cells were significantly longer in Pro35S:AtCKX3 roots but not in ahk3-7 in comparison to WT (Fig. 2b). We also explored the potential influence of endogenous cytokinins or cytokinin signaling on auxin-mediated reduction of root elongation. However, root elongation of Pro35S:AtCKX3 and ahk3-7 lines after exogenously applied auxin NAA displayed a WT-like response (Fig. 2c).

Taken together, our results suggest that delayed gravitropic bending is due to the cytokinin impact on gravitropic signal perception in the columella cells or gravitropic signal transduction through the proximal meristem to the root elongation zone rather than on the cell elongation.
Cytokinins regulate the auxin response distribution in the root tip via AHK3 signaling

Endogenous cytokinins have previously been shown to affect the auxin distribution in the root tip (Pernisová et al., 2009). To investigate the potential role of cytokinin signaling in controlling the intercellular auxin distribution, we crossed the auxin response reporter DR5rev:GFP with single and double mutants in cytokinin receptors. Even without any gravistimulation, we observed an altered auxin response distribution in the columella cells of mutant lines (Fig. 3a-g, Table S1). Three effects on the DR5rev:GFP expression pattern were distinguishable in the ahk lines: absence of the signal in the quiescent center, signal asymmetry in columella cells and its expansion from columella into the lateral root cap. These differences were visible in single ahk3-7 mutant and were more pronounced in both double mutant combinations containing ahk3-7 allele, ahk2-5 ahk3-7 and ahk3-7 cre1-2. Expression of AHK3-GUS revealed localization of AHK3 in the quiescent center, stem cell niche, columella and stele in the root tip (Fig. 3h), consistent with the aberrations of DR5rev:GFP expression observed in the ahk3-7 mutant backgrounds.

Thus, it seems that cytokinin signaling controls the auxin response distribution in the quiescent center and columella cells of the root tip. The cytokinin receptor AHK3 appears to play a dominant role in this process, but two other cytokinin receptors, AHK2 and AHK4, have additive effects.

Endogenous cytokinins affect auxin signaling distribution in meristematic zone after gravistimulation

Asymmetric auxin distribution after gravistimulation is hypothesized to be important for unequal cell elongation at the upper and lower sides of the root, thus facilitating the root bending (Boonsirichai et al., 2002; Morita, 2010). We visualized auxin signaling distribution early after root gravistimulation by the DII-Venus reporter (Liao et al., 2015). The reporter allows sensitive visualization of auxin distribution via fast auxin-induced downregulation of the Venus signal (Band et al., 2012; Brunoud et al., 2012). In WT, we observed auxin accumulation at the lower side of the root 30 min after gravistimulation, while at that time there was no change in the auxin concentration identifiable at the upper side of the root (Fig. 3i). Interestingly, we spotted slight increase in the auxin concentration 60 min after gravistimulation at both
the upper and lower sides of the root. Upregulation of endogenous cytokinins via application of INCYDE, an inhibitor of cytokinin oxidase activity (Zatloukal et al., 2008; Antoniadi et al., 2015), delimited the increase of the auxin accumulation at the lower side of the root and completely inhibited the auxin increase at the upper side of the root gravistimulated for 60 minutes (Fig. 3i). This is in a good agreement with slower root bending on INCYDE after gravistimulation (Fig. S8).

In a conclusion, these data suggest impaired ability to redistribute auxin and delayed root bending in the gravistimulated root in a response to the increase of endogenous cytokinin content.

Endogenous cytokinins control the abundance and localization of PIN3 and PIN7 in the root tip

The auxin response maximum in the root meristem is to a large extent mediated by the activity of PIN auxin transporters (Friml et al., 2002a; Petrasek et al., 2006). PIN3 and PIN7 auxin transporters are localized in columella cells, and thus presumably involved in the auxin distribution in the root tip (Friml et al., 2002b; Blilou et al., 2005).

Both of these transporters have been suggested to have an important role in root gravitropism (Friml et al., 2002b; Harrison & Masson, 2008; Kleine-Vehn et al., 2010). Hence, we investigated the role of cytokinins in regulating the expression and localization of PIN3-GFP and PIN7-GFP in columella cells of the Pro35S:AtCKX2, Pro35S:AtCKX3 and ahk3-7 lines.

The PIN3-GFP signal at plasma membranes of columella cells was lower in both the Pro35S:AtCKX2 and Pro35S:AtCKX3 lines and in one of the tested ahk3 mutant lines (Fig. 4a-f, Fig. S2a, Fig. S3, Table S2). Nonetheless, the signal intensity of intracellular PIN3-GFP was comparable to the control in all tested lines (Fig. S2b) and the PIN3 expression niche was not changed either. In contrast to PIN3, the PIN7-GFP signal intensities at plasma membranes were comparable in the WT, Pro35S:AtCKX2 and Pro35S:AtCKX3 lines (Fig. 4g-i, Fig. S4a, Fig. S5, Table S3). However, the amount of intracellular PIN7 protein was increased in the C2 layer (Fig. S4b) and the PIN7 expression domain had expanded laterally into the root cap of the Pro35S:AtCKX2 and Pro35S:AtCKX3 lines (Fig. 4m, n). The PIN7-GFP signal in the ahk3-7 mutant was variable and no statistically significant differences were detected (Fig. 4, Fig. S4).
In line with that, increasing the endogenous cytokinin content via application of INCYDE led to upregulation of PIN3 signal while narrowing the PIN7 expression niche, i.e. the opposite effect in comparison to transgenic lines with depleted endogenous cytokinins (Pro35S:AtCKX2 and Pro35S:AtCKX3) (Fig. S6a). Furthermore, PIN3-GFP and PIN7-GFP signal in columella as well as the gravitropic bending (Fig. S6b) was reverted to WT phenotype in Pro35S:AtCKX2 and Pro35S:AtCKX3 lines after INCYDE treatment.

To summarize, our results suggest that endogenous cytokinin levels may potentially influence the intercellular auxin distribution via differential regulation of PIN3 and PIN7 abundance and/or localization. In addition, endogenous cytokinin levels delimit PIN7 expression niche. AHK3 alone seems to have no major role in these processes.

Cytokinins have no impact on PIN3 and PIN7 relocalization in response to gravistimulation

PIN3 and PIN7 proteins are localized uniformly in columella cells. After gravistimulation, they accumulate on the new bottom side of the cells via transcytosis. This relocalization is fast and independent of de novo protein synthesis (Kleine-Vehn et al., 2010). To assay changes in PIN protein localization, we applied in vivo real-time imaging using two different confocal microscopes: Nikon with horizontal-oriented optical axis and vertical-oriented Zeiss. PIN3-GFP and PIN7-GFP signals were measured at plasma membranes in columella cells of the WT, Pro35S:AtCKX3 line and ahk3-7 mutant at times 0, 5, 10, 20 and 30 minutes after gravistimulation (Fig. 5a,b, Fig. S7). To eliminate the effect of bleaching apparent during the time lapse imaging, we evaluated the data as a signal intensity ratio between lateral and basal membranes (Materials and Methods).

The PIN3-GFP signal intensity ratio was comparable in the WT and Pro35S:AtCKX3 lines and remained nearly unchanged for up to 30 minutes after gravistimulation in innermost columella cells (Fig. 5c). In outer columella cells at bottom side of the root tip, the PIN3-GFP signal intensity ratio was slightly increased after 30 minutes (Fig. S7b, Table S4). On the other hand, the PIN7-GFP signal intensity ratio increased rapidly within 5 minutes after gravistimulation in both the WT and Pro35S:AtCKX3 lines (Fig. 5d, Fig. S5, Fig. S7d, Table S3, Table S5), suggesting fast accumulation of PIN7 at the lateral membranes. After the rapid change observed during the first 5 minutes, the signal intensity ratio rose only slightly over the subsequent time points.
In the Pro35S:AtCKX3 line, the PIN7-GFP signal intensity was slightly stronger at the lateral membranes (Fig. S5, Table S3), resulting in elevation of the signal intensity ratio compared to the control (Fig. 5d). However, the relocalization dynamics of PIN7-GFP in Pro35S:AtCKX3 was similar to the control after gravistimulation (Fig. 5d, Fig. S7c, Table S5).

In the ahk3-7 line, all the tested parameters for PIN3-GFP and PIN7-GFP were similar to control (Fig. 5c, d, Fig. S3, Fig. S5, Table S2, Table S3), suggesting that AHK3 signaling plays only a minor function in PIN3 and PIN7 relocalization in gravistimulated columella cells.

Interestingly, the pin3 pin7 mutant showed WT-like root gravitropic bending and retained sensitivity to INCYDE comparable to WT as well (Fig. S8). PIN4, another member of the PIN family, was shown to laterally expand in the columella in the pin3 pin7 background, potentially masking the absence of PIN3 and PIN7 (Blilou et al., 2005). To inspect possible PIN4-mediated rescue of the pin3 pin7 gravitropic response, we tested pin3 pin4 pin7 triple mutant. Our results show partial defect of pin3 pin4 pin7 in the root bending after gravistimulation. However, the triple pin mutant still displayed cytokinin sensitivity in gravitropic bending (Fig. S8).

Altogether, we conclude that the ability of PIN3 and PIN7 to relocalize to lateral membranes in a response to a gravitropic stimulus remains unaffected by decreased endogenous cytokinin levels and/or attenuated AHK3-mediated cytokinin signaling. Notably, PIN7 relocalization after gravistimulation is more dynamic than that of PIN3, suggesting the importance of PIN7 at very early stages after gravistimulation, a notion that has not been elaborated on before. Our data also predict existence of another cytokinin target in the control of early root gravitropic response than the PIN-mediated auxin transport in columella.

**Cytokinins influence AUX1 expression in the root tip**

The expansion of the auxin response from the columella to lateral root cap in cytokinin deficient plants (Pernisová et al., 2009) together with the absence of PIN3 and PIN7 in adjacent cells and the aforementioned cytokinin sensitivity of pin3 pin4 pin7, implies involvement of another transporter in the cytokinin-mediated control over auxin distribution in the root tip. An auxin influx carrier AUX1 is localized in the columella cells, lateral root cap, epidermis and stele (Swarup et al., 2004) and was shown to be regulated by exogenous cytokinin application (Zhang et al., 2013). That,
together with strong gravitropic phenotype of aux1 (Fig. S8) (Marchant et al., 1999), made AUX1 a good candidate in our search. We compared AUX1-YFP signal in WT, Pro35S:AtCKX2 and Pro35S:AtCKX3 lines and found decrease of the AUX1-YFP in both cytokinin-deficient lines (Fig. 6).

Altogether, our results indicate that endogenous cytokinins regulate AUX1 expression, thus controlling velocity of the auxin transport in the root tip.

DISCUSSION

Cytokinins regulate root gravitropic bending via AHK3 signaling

Our work addresses the role of endogenous cytokinins and cytokinin signaling in early events of root gravitropism in Arabidopsis. We showed that endogenous cytokinins affect gravitropic bending preferentially via AHK3 signaling. Faster root elongation in Pro35S:AtCKX2, Pro35S:AtCKX3 and ahk3-7 plants may suggest faster gravitropic bending, but our results showed the opposite situation. Moreover, the sensitivity of the Pro35S:AtCKX3 and ahk3-7 lines to exogenously applied auxin was comparable to that of WT. Thus, neither cell elongation nor the sensitivity to auxin could explain the observed delay in root bending after gravistimulation, suggesting that cytokinins affect gravitropic signal perception and/or transduction in the root tip.

AHK3 signaling is required for the auxin response pattern in the root tip

Depletion of endogenous cytokinin levels has been shown to be responsible for expansion of the auxin response pattern from the columella to the lateral root cap (Pernisová et al., 2009), suggesting that endogenous cytokinins play a role in regulating the auxin response distribution. However, compared with AtCKX overexpressing lines, attenuation of AHK3-mediated cytokinin signaling causes different and specific aberrations of the DR5rev:GFP signal in the root tip, namely, absence of the signal in the quiescent center, signal asymmetry in columella cells and its expansion from the columella into the lateral root cap. More pronounced aberrations of the DR5rev:GFP in double mutants carrying ahk3-7 allele suggest partial contribution of AHK2 and AHK4, too. The role of AHK3 signaling has been shown in the root transition zone, where AHK3 induces expression of SHY2/IAA3, thus repressing auxin signaling and PINs expression, leading to auxin redistribution (Dello Ioio et al., 2008). However, it remains to be clarified whether a mechanism
similar to the AHK3/SHY2 pathway also mediates regulation of the auxin signaling
distribution in the root tip.

**Endogenous cytokinins control auxin redistribution after gravistimulation**

Our data suggest that proper levels of endogenous cytokinins are necessary for the
gravity-induced auxin redistribution. We show that upregulating endogenous
cytokinins results into slight downregulation of auxin transport to the lower part of the
root 60 minutes after gravistimulation. Interestingly, at the same time interval, we
observed increase of auxin concentration not only at the lower, but also at the upper
side of the root. That might imply existence of feedback regulatory mechanism,
slowing down the root bending at the later stages of the root gravitropic response.
This mechanism seems to be strongly affected following increase of endogenous
cytokinins via downregulation of CKX activity.

In summary, our data show sensitivity of gravity–induced auxin redirecting
mechanisms to endogenous cytokinin levels.

**Cytokinins differentially regulate the expression and localization of PIN3 and
PIN7**

Expression patterns of *PIN3* and *PIN7* have been shown to partially overlap in
columella cells and reportedly play an important role in the root tip during gravitropic
responses (Friml et al., 2002b; Harrison & Masson, 2008) with possible functional
redundancy (Kleine-Vehn et al., 2010). Furthermore, PIN3 is most closely related to
PIN7 in the *Arabidopsis* PIN family of auxin efflux carriers (Adamowski & Friml,
2015). Thus, it is interesting that cytokinins appeared to control both proteins
differently. Such effects were observed also after exogenous cytokinin application.

After treatment with the cytokinin benzyladenine, PIN3-GFP and PIN7-GFP were
found to be regulated in opposite way in the root tip (Růžička et al., 2009; Zhang et
al., 2011). Analysis of PIN transcription in root tips has also revealed a similar trend
of regulation (Růžička et al., 2009; Zhang et al., 2011).

Here, we showed the impact of endogenous cytokinins on *PIN7* expression and PIN3
and PIN7 localization. A lower amount of membrane-associated PIN3-GFP was
detected in the *Pro35S:AtCKX3* line, which could slow down auxin redistribution after
gravistimulation, thus delaying root bending. Cytokinins were shown to control
endocytic recycling and abundance of PIN1 by its redirecting to lytic vacuoles
Here we spotted slight increase in the amount of intracellular PIN7. However, we do not see any effects on the relocalization of PIN7-GFP to the lower membranes after gravistimulation. That might imply existence of separated pathways, acting in gravistimulation-independent vesicular trafficking and gravity-induced PIN7 transcytosis. That, however, remains to be identified.

Taken together, our results suggest distinct and mostly opposite regulation of PIN3 and PIN7 proteins by endogenous cytokinin levels, particularly in the root tip, similar to previous findings for exogenous cytokinin treatments (Růžička et al., 2009).

**AHK3 signaling is not essential for PIN3 and PIN7 expression in columella cells**

In contrast to the AtCKX overexpressing lines, we observed that AHK3 signaling has nearly no effect on PIN3 and PIN7 expression and protein localization in columella cells. This implies that the delay in gravitropic response we observed in ahk3 is due to as yet unknown regulation. Similarly to PIN3 and PIN7, another auxin efflux carrier, PIN4, has been shown to localize to the columella cells (Friml et al., 2002a) and can also be influenced by cytokinins (Zhang et al., 2011). Our data showing sensitivity of gravitropic response in WT and pin3 pin4 pin7 triple mutant to cytokinins, however, seem to argue rather against this possibility. Other possible mechanism of regulation of gravitropic bending in ahk3-7 mutant could be AHK3-dependent regulation of auxin transporters PIN2 or AUX1, whose corresponding mutants are agravitropic (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Marchant et al., 1999). Our data imply the latter being possible, as the AUX1 is downregulated in the cytokinin-deficient lines.

**Gravity-induced relocalization of PIN3 and PIN7 is cytokinin-independent; PIN7 relocates faster than PIN3**

Polarization of PIN3 and PIN7 proteins toward the lower cell sides after gravistimulation has been demonstrated and both proteins have been suggested to be important players in the root gravitropic response (Friml et al., 2002b; Kleine-Vehn et al., 2010). Here, we compared the in vivo dynamics of PIN3 and PIN7 relocalization at several time points during the first 30 minutes after gravistimulation. According to our findings, PIN3 localization at plasma membranes in columella cells was more stable than that of PIN7, which relocalized more rapidly. This rapid relocalization of PIN7 could facilitate the redirection of auxin flow during the
immediate stages after gravitropic stimulation. However, relocalization of both PIN3 and PIN7 seems to be independent of cytokinin signaling and endogenous cytokinin levels. In line with that, the WT-like gravitropic response observed in pin3 pin7 suggests rather minor regulatory function of PIN3 and PIN7 auxin efflux carriers and/or ability of other PIN proteins (e.g. PIN4) to complement their role during early gravitropic response in the root tip. This seems to be in contrast to the gravitropic response of hypocotyl (Rakusova et al., 2011).

Suggested model
Based on our results, we propose a model (Fig. 7) in which endogenous cytokinins differentially regulate the expression and localization of auxin efflux carriers PIN3 and PIN7 and the auxin response distribution in the root tip independently of gravistimulation. Endogenous cytokinins positively regulate PIN3 but negatively control PIN7 in columella cells. Moreover, proper endogenous cytokinin levels maintain the PIN7 expression niche. The high complexity of PIN-mediated auxin transport in the root tip (Vieten et al., 2005), including compensatory mechanism at the level of ectopic expression of other PINs in multiple PIN mutants (Blilou et al., 2005) makes difficult to assess the developmental importance of the cytokinin-mediated control over PIN-dependent auxin redistribution in columella upon gravistimulation. However, the WT-like gravitropic response of pin3 pin7 and retained cytokinin sensitivity of pin3 pin4 pin7 mutant suggests rather limited importance of efflux-regulated intercellular auxin distribution in columella for the cytokinin-independent control of the root gravitropism. In support of that, we identified another important non-redundant regulator of the root gravitropism, the auxin influx carrier AUX1, being under control of endogenous cytokinins.

Taken together, cytokinins seem to differentially regulate expression and localization of auxin transporters in the root tip, leading to the gravistimulation-independent changes in the auxin intercellular distribution. However, the AUX1-mediated auxin transport from the columella towards the transition zone seems to be the target in the cytokinin–controlled root gravitropic response.

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AUTHOR CONTRIBUTION

REFERENCES


FIGURE LEGENDS

Figure 1. Endogenous cytokinins control root bending after gravistimulation.
Gravitropic response of 5-day-old seedlings in cytokinin-deficient lines Pro35S:AtCKX2 and Pro35S:AtCKX3 (a) and in cytokinin signaling mutants (b) 3 hours after gravistimulation.
(c) Schematic description of gravitropic trajectory of WT (full line) and Pro35S:AtCKX3 background (dashed line). l – length of trajectory, h - height of trajectory peak, r1 - upward radius, r2 - downward radius.

(d) Gravitropic parameters of WT, Pro35S:AtCKX2, Pro35S:AtCKX3 and ahk3-7 background in 6-day-old seedlings gravistimulated by 135°.

In all charts, the error bars correspond to standard deviation from the mean values. Statistical significance tested by t-test at alpha levels of 0.05, 0.01 and 0.001 is shown (*, ** and ***).

Figure 2. Endogenous cytokinins are negative regulators of root growth and cell elongation in the root differentiation zone.

(a) Root elongation between the third and fifth day of cultivation in WT, Pro35S:AtCKX2, Pro35S:AtCKX3 and ahk single and double mutant lines.

(b) Length of fully differentiated root cells in 5-day-old seedlings of WT, Pro35S:AtCKX3 and ahk3-7.

(c) Sensitivity to exogenously applied auxin NAA is comparable in WT, Pro35S:AtCKX3 and ahk3-7 in 5-day-old roots.

In all charts, the error bars correspond to standard deviation from the mean values. Statistical significance tested by t-test at alpha levels of 0.05, 0.01 and 0.001 is shown (*, ** and ***).

Figure 3. Cytokinin signaling controls auxin response distribution in the root tip.

Expression of auxin reporter DR5rev:GFP in control (a), single (b-d) and double (e-g) cytokinin receptor mutants. (h) AHK3 localization domain in root tip.

Arrow - signal absence in quiescent center; triangle - lateral signal expansion; arrowhead - signal asymmetry; scale bars: 50 µm.

(i) DII-Venus relative signal intensities (normalized to time 0 minutes) at the upper and lower side of the root. Signal was measured 0, 30 and 60 minutes after gravistimulation. In the chart, the error bars correspond to standard deviation from the mean values. Letters above the bars indicate the value is significantly different: “a” – from time 0 minutes, “b” – 30 minutes from 60 minutes in an appropriate treatment, “c” – mock from INCYDE.
Figure 4. Endogenous cytokinins control PIN3 and PIN7 localization in the root tip.

In comparison to the control (a), in Pro35S:AtCKX2 (b), Pro35S:AtCKX3 (c) and ahk3-7 line (d) there is an apparent decrease of the PIN3-GFP signal at plasma membranes in columella cells (e,f). In contrast, compared to the control (g), changes in the PIN7-GFP signal intensity at membranes of both Pro35S:AtCKX2 (h), Pro35S:AtCKX3 (i) and ahk3-7 mutant (j) are statistically insignificant (l). However, lateral expansion of the PIN7 localization domain is apparent (m,n).

QC – quiescent center, i – columella initials, C1, C2, C3, C4 – columella cell layers.

In all charts, the error bars correspond to standard deviation from the mean values.

Statistical significance tested by t-test at alpha levels of 0.05, 0.01 and 0.001 is shown (*, ** and ***). Scale bars: 50 µm.

Figure 5. Endogenous cytokinins do not affect relocalization of PIN3-GFP and PIN7-GFP in columella cells during 30 minutes after gravistimulation.

Membrane numbering in columella cells and schematic description of signal measurement before (a) and after (b) gravistimulation. QC – quiescent center, i – columella initials, C1, C2, C3, C4 – columella cell layers.

(c) The PIN3-GFP signal intensity ratio is constant during 30 min after gravistimulation and remains comparable in all tested lines. (d) The PIN7-GFP signal intensity ratio increases within 5 minutes after gravistimulation; the signal intensity ratio is higher in Pro35S:AtCKX3 when compared to control.

In all charts, the middle point corresponds to the mean, the box value corresponds to the standard error and the whisker value corresponds to the confidence interval.

Statistical significance tested by t-test at alpha levels of 0.05, 0.01 and 0.001 is shown (*, ** and ***).

Figure 6. Cytokininns affect AUX1-YFP signal.

In comparison to the control (a), in Pro35S:AtCKX2 (b) and Pro35S:AtCKX3 lines (c) there is a decrease of the AUX1-YFP signal measured in columella and lateral root cap below the quiescent center (d). In the chart, the error bars correspond to standard deviation from the mean values. Statistical significance tested by t-test at alpha levels of 0.01 is shown (**). Scale bars: 50 µm.
Figure 7. Model for cytokinin regulation of the auxin response distribution in the root tip.

Yellow, magenta and brown colors represent the localization and intensity of the PIN3-GFP, PIN7-GFP and AUX1-YFP signal, respectively. Lines and circles indicate plasma membrane and intracellular localization, respectively. Dashed lines represent predicted decrease of AUX1-mediated auxin transport in Pro35S:AtCKX lines. The resulting auxin response distribution is visualized by DR5rev:GFP (green) in Pro35S:AtCKX2 and Pro35S:AtCKX3 plants (Pernisová et al., 2009) suggesting an additional regulator of the auxin transport to be under cytokinin control (violet arrow). QC – quiescent center, i – columella initials, C1, C2, C3, C4 – columella cell layers.

Supporting Information

Figure S1. Cytokinins affect root bending after gravistimulation.
Figure S2. Endogenous cytokinins affect the PIN3-GFP signal intensity at plasma membranes of columella cells.
Figure S3. PIN3-GFP signal intensity measurement during relocalization.
Figure S4. Endogenous cytokinins control the PIN7-GFP signal intensity in columella cells.
Figure S5. PIN7-GFP signal intensity measurement during relocalization.
Figure S6. INCYDE reverts Pro35S:AtCKX2 and Pro35S:AtCKX3 phenotype to WT.
Figure S7. PIN7-GFP relocalization is more dynamic than that of PIN3-GFP in columella cells during 30 minutes after gravistimulation.
Figure S8. Root bending and phenotype of pin multiple mutants and aux1.
Table S1. Quantification of DR5rev:GFP aberrations in the root tip of cytokinin receptor mutants.
Table S2. Statistical evaluation of PIN3-GFP signal intensities.
Table S3. Statistical evaluation of PIN7-GFP signal intensities.
Table S4. PIN3-GFP signal intensity measurement.
Table S5. PIN7-GFP signal intensity measurement.
Figure 1. Endogenous cytokinin control root bending after gravistimulation.
Gravitropic response of 5-day-old seedlings in cytokinin-deficient lines Pro35S::ACKX2 and Pro35S::ACKX3 (a) and in cytokinin signaling mutants (b) 3 hours after gravistimulation.
(c) Schematic description of gravitropic trajectory of WT (full line) and Pro35S::ACKX3 background (dashed line). $l$ - length of trajectory, $h$ - height of trajectory peak, $r_1$ - upward radius, $r_2$ - downward radius.
(d) Gravitropic parameters of WT, Pro35S::ACKX2, Pro35S::ACKX3 and ahk3-7 background in 6-day-old seedlings gravistimulated by 130°.
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Figure 4. Endogenous cytokinins control PIN3 and PIN7 localization in the root tip.
In comparison to the control (a), in Pro35S:AI/DOCK2 (b), Pro35S:AI/DOCK3 (c) and ahl3-7 line (d) there is an apparent decrease of the PIN3-GFP signal at plasma membranes in columnella cells (e,f). In contrast, compared to the control (g), changes in the PIN7-GFP signal intensity at membranes of both Pro35S:AI/DOCK2 (h), Pro35S:AI/DOCK3 (i) and ahl3-7 mutant (j) are statistically insignificant (f). However, lateral expansion of the PIN7 modification domain is apparent (n,m). QC – quiescent center, i – columnella initials, C1, C2, C3, C4 – columnella cell layers.
In all charts, the error bars correspond to standard deviation from the mean values. Statistical significance tested by t-test at alpha levels of 0.05, 0.01 and 0.001 is shown (*, ** and ***). Scale bars: 50 μm.
Figure 3. Endogenous synctiathria and the effect of colchicine of PIN3-GFP and PIN7-GFP in columnella cells during 30 minutes after gravistimulation.  
Membrane staining of column cells and schematic description of signal measurement before (a) and after (b) gravistimulation. QC – quiescent center, i – columnella interior, C1, C2, C3, C4 – columnella cell layers.  
(c) The PIN3-GFP signal intensity ratio is constant during 30 min after gravistimulation and remains comparable in all tested lines. (d) The PIN7-GFP signal intensity ratio increases within 5 minutes after gravistimulation, the signal intensity ratio is higher in Pro35S:YFP::PIN7 when compared to control.  
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