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Cytokinins 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), *ahp1 ahp2 ahp3 ahp4 ahp5* (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 $\mu M.m^{-2}.s^{-1}$ 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-glc, 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-l, 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 agravitropic 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 *PIN*s 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

(Marhavý *et al.*, 2011). 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 relocalizes 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-dependent 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

M.P., J.F. and J.H. designed the research, M.P., T.P., P.G., T.N., L.S. and M.M. performed the research, M.P., T.P., P.G. and D.H. analyzed the data and M.P., D.H., P.G., J.F. and J.H. wrote the paper.

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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. Cytokinins 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.

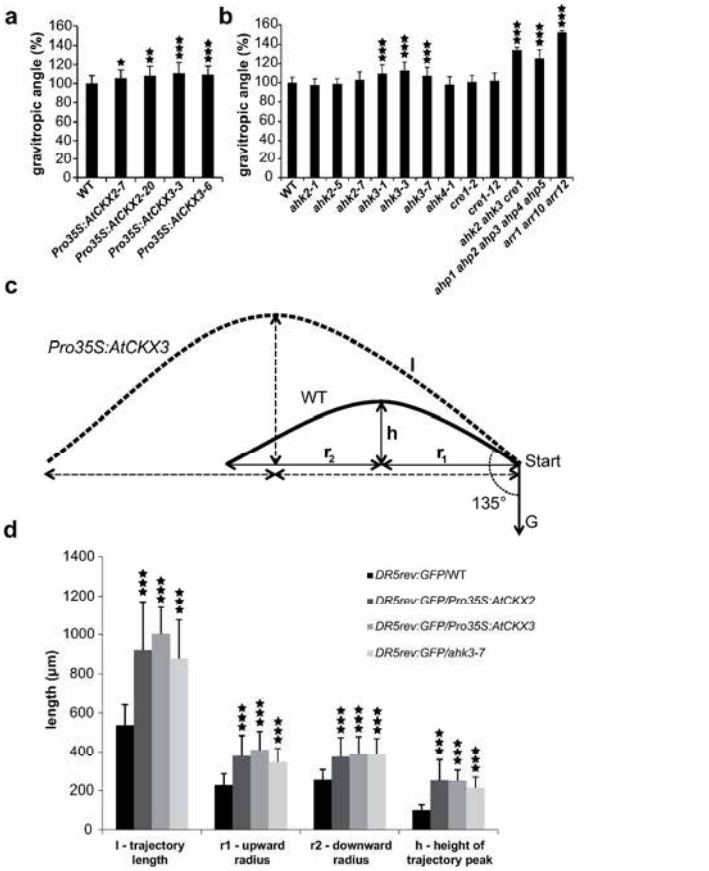

Figure 1. Endogenous cytokinins control root bending after gravistimulation.

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 ***).

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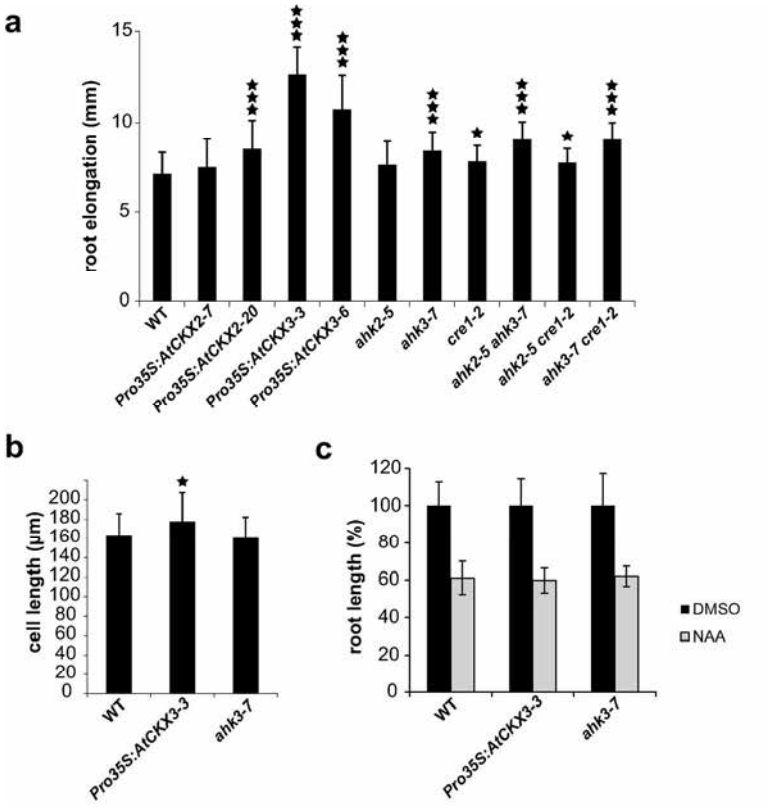

Figure 2. Endogenous cytokinins are negative regulators of root growth and cell elongation in the root differentiation zone.

(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 ***).

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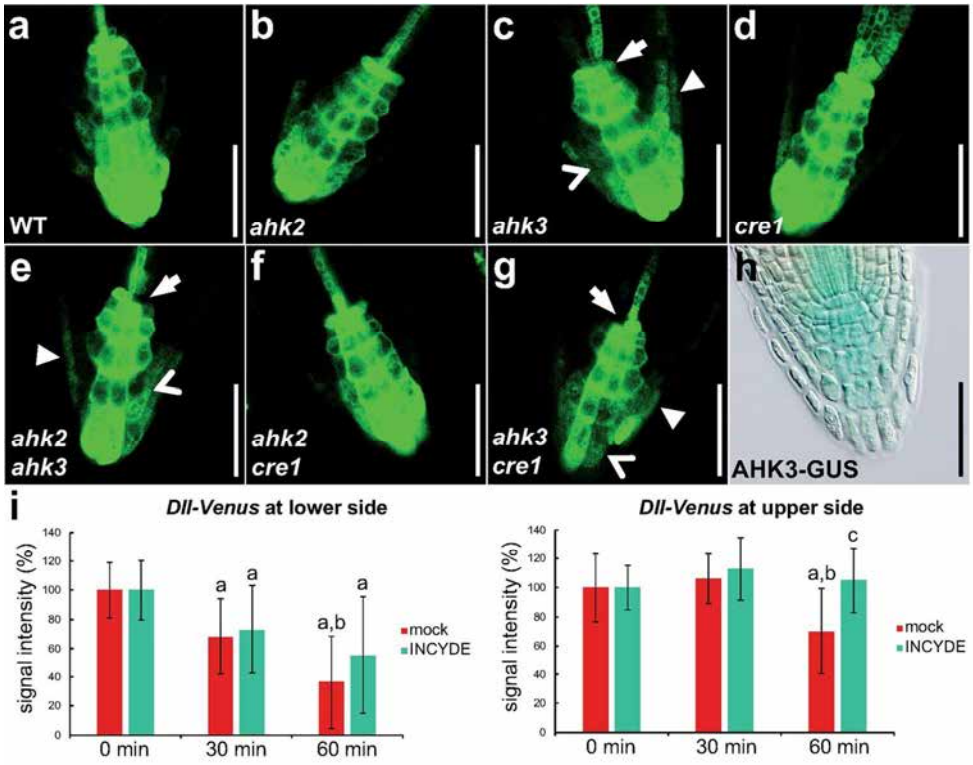

Figure 3. Cytokinins control auxin response distribution in the root tip. Expression of auxin reporter *DR5rev:GFP* in control **(a)**, single **(b-d)** and double **(e-g)** cytokinin receptor mutants. **(h)** AHK3 localization domain in root tip. Arrow - signal absence in quiescent center; triangle - 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.

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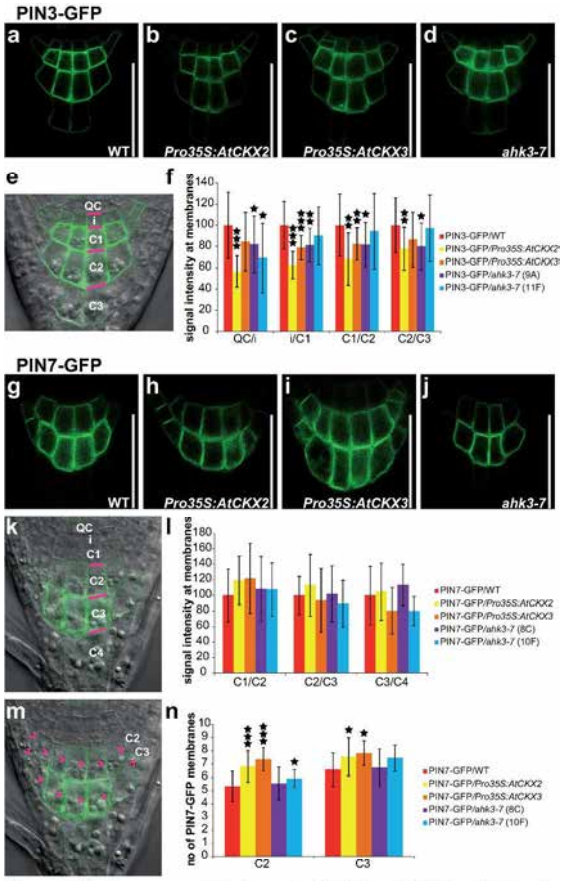

Figure 4. Endogenous cytokinins control PIN3 and PIN7 localization in the root tip.

In comparison to the control **(a)**, in *Pro35S: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 **(n,m)**. 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.

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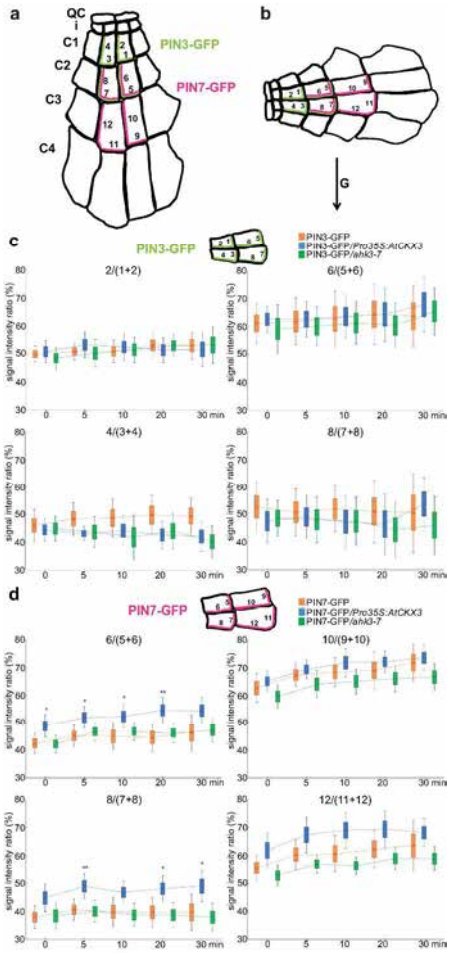

Figure 5. Endogenous cytokinins do not affect relocalization of PIN3-GFP and PIN7-GFP in columella cells during 30 minutes after gravistimulation.

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 ***).

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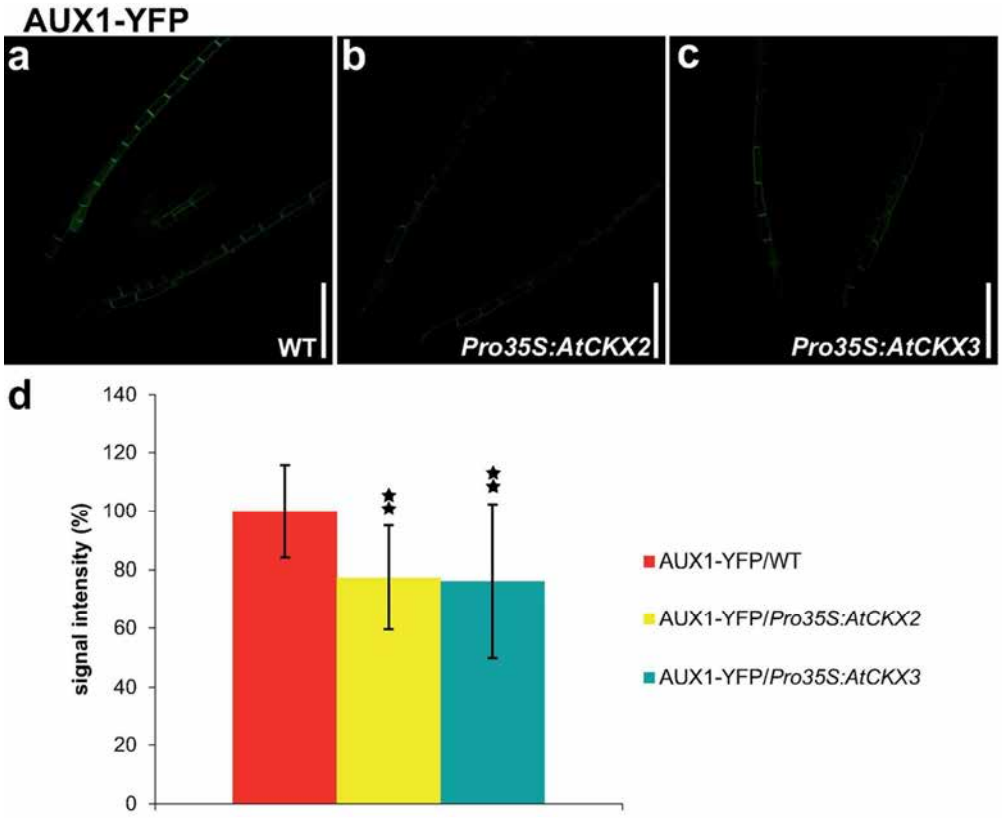

Figure 6. Cytokinins affect AUX1-YFP signal.
In comparison to the control **(a)**, in *Pro35S:AtCKX2* **(b)** and *Pro35S:AtCKX3* lines **(c)** there is a decrease of the AUX1-YFP signal measured in columella and lateral root cap below the quiescent center **(d)**. In the chart, the error bars correspond 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.

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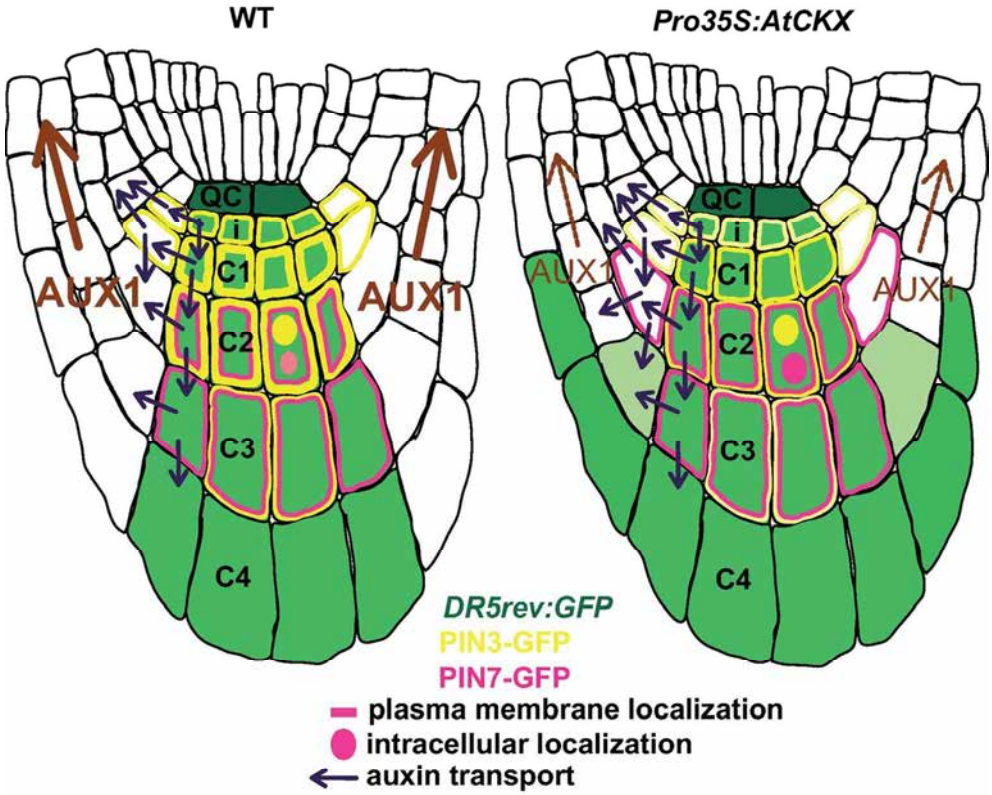

Figure 7. Model for cytokinin regulation of the auxin response distribution in the root tip.

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.

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