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Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation [W]

Laurent Laplaze,[a,1] Eva Benkova,[b,c] Ilda Casimiro,[d] Lies Maes,[b,c] Steffen Vanneste,[b,c] Ranjan Swarup,[e] Dolf Weijers,[f,2] Vanessa Calvo,[d] Boris Parizot,[b,c,g] Maria Begoña Herrera-Rodriguez,[a] Remko Offringa,[f] Neil Graham,[h] Patrick Doumas,[a] Jiri Friml,[b,c] Didier Bogusz,[a] Tom Beeckman,[b,c] and Malcolm Bennett[e]

[a] Institut de Recherche pour le Développement, Unité Mixte de Recherche Diversité et Adaptation des Plantes Cultivées (Agro.M/Institut National de la Recherche Agronomique/Institut de Recherche pour le Développement/Université Montpellier 2), Equipe Rhizogenèse, 34394 Montpellier cedex 5, France

[b] Department of Plant Systems Biology, Root Development Group, Vlaams Instituut voor Biotechnologie, B-9052 Gent, Belgium

[c] Department of Molecular Genetics, Ghent University, B-9052 Gent, Belgium

[d] Departmento de Ciencias Morfologicas y Biologia Celular y Animal, University of Extramadura, E-06071 Badajoz, Spain

[e] Centre for Plant Integrative Biology, School of Biosciences, University of Nottingham, Loughborough, LE12 5RD, United Kingdom

[f] Institute of Biology, Leiden University, Wassenaarseweg 64 2333 AL Leiden, The Netherlands

[g] Laboratoire de Biologie du Développement des Plantes (Centre National de la Recherche Scientifique/Commissariat à l'Energie Atomique/Aix-Marseille II), Commissariat à l'Energie Atomique Cadarache, 13108 St. Paul les Durance, France

[h] Nottingham Arabidopsis Stock Centre, School of Biosciences, University of Nottingham, Loughborough, LE12 5RD, United Kingdom

In *Arabidopsis thaliana*, lateral roots are formed from root pericycle cells adjacent to the xylem poles. Lateral root development is regulated antagonistically by the plant hormones auxin and cytokinin. While a great deal is known about how auxin promotes lateral root development, the mechanism of cytokinin repression is still unclear. Elevating cytokinin levels was observed to disrupt lateral root initiation and the regular pattern of divisions that characterizes lateral root development in *Arabidopsis*. To identify the stage of lateral root development that is sensitive to cytokinins, we targeted the expression of the *Agrobacterium tumefaciens* cytokinin biosynthesis enzyme isopentenyltransferase to either xylem-pole pericycle cells or young lateral root primordia using GAL4-GFP enhancer trap lines. Transactivation experiments revealed that xylem-pole pericycle cells are sensitive to cytokinins, whereas young lateral root primordia are not. This effect is physiologically significant because transactivation of the *Arabidopsis* cytokinin degrading enzyme cytokinin oxidase 1 in lateral root founder cells results in increased lateral root formation. We observed that cytokinins perturb the expression of *PIN* genes in lateral root founder cells and prevent the formation of an auxin gradient that is required to pattern lateral root primordia.

INTRODUCTION

The plant root system is made of a primary root that originates during embryogenesis and lateral roots that form throughout the life of the plant. Root architecture is influenced by numerous environmental parameters. For example, the availability of nutrients such as nitrate (Leyser and Fitter, 1998; Zhang and Forde, 2000), phosphate (Lopez-Bucio et al., 2002), and sulfate (Kutz et al., 2002) has a strong effect on *Arabidopsis thaliana* lateral root development (for review, see Lopez-Bucio et al., 2003; Malamy, 2005). This plasticity of the root system is essential to optimize nutrient acquisition in a heterogeneous and changing environment and therefore represents an important agronomical trait (Hodge, 2004).

Lateral roots originate from a small number of differentiated cells situated at the periphery of the vascular tissues. In *Arabidopsis*, lateral roots are derived from pericycle cells adjacent to the xylem poles, called pericycle founder cells (Casimiro et al., 2001; Dubrovsky et al., 2001). These cells undergo a defined program of oriented cell divisions and expansion to form a lateral root primordium (LRP; Malamy and Benfey, 1997; Dubrovsky et al., 2001; Casimiro et al., 2003). The first step in lateral root development (lateral root initiation) occurs in three adjacent pericycle cell files. A polarized asymmetrical anticlinal division takes place in two founder cells per cell file leading to the formation of two short daughter cells surrounded by two larger cells (stage I). Anticlinal divisions, cell expansion, and periclinal divisions give rise to a simple four-layered LRP (stage IV). More divisions and expansion result in the formation of a complex stage VI LRP whose organization is similar to the primary root

[1] Address correspondence to laplaze@mpl.ird.fr.

[2] Current address: Laboratory of Biochemistry, Wageningen University and Research Centre, Dreijenlaan 3, 6703 HA, Wageningen, The Netherlands.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Laurent Laplaze (laplaze@mpl.ird.fr).

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meristem. Cell expansion then drives the emergence of the LRP from the parent root (stage VIII) before cell divisions resume in the tip (lateral root meristem activation).

Auxin plays a central role during lateral root development (reviewed in Casimiro et al., 2003). External auxin is required by LRPs for initiation and development until they become self-sufficient between stages III and V (Laskowski et al., 1995; Himanen et al., 2002; Marchant et al., 2002; Casimiro et al., 2003). It has been shown that for proper LRP development, establishment of an auxin gradient with its maximum at the tip is important (Benková et al., 2003). This gradient is dependent on auxin transport mediated by PIN auxin efflux facilitators. Chemical (inhibitors) or genetic (mutants) interference with auxin transport leads to defects in both auxin gradient establishment and LRP development (Benková et al., 2003, Geldner et al., 2004).

In contrast with auxin, relatively little is known about the role of cytokinins on lateral root development. Many reports describe the inhibitory effect of cytokinins on lateral root formation (Böttgor, 1974; Goodwin and Morris, 1979; Wightman et al., 1980), but the mechanism(s) of cytokinin regulation is not known. *Arabidopsis* mutants in cytokinin receptors (Riefler et al., 2006) or *Arabidopsis* response regulator genes (To et al., 2004; Mason et al., 2005) involved in cytokinin signaling have root branching phenotypes. Moreover, transgenic plants with reduced levels of cytokinins due to the overexpression of genes encoding the cytokinin-degrading enzyme cytokinin oxidase (CKX) exhibit enhanced root growth and branching (Werner et al., 2001, 2003). We can conclude from these observations that the effect of cytokinins is physiologically relevant and that endogenous cytokinins act in vivo to inhibit lateral root development.

It is currently unclear which stage of lateral root development is inhibited by endogenous cytokinins. Mahonen et al. (2006) have recently shown that cytokinin signaling is repressed in xylem-pole pericycle cells, suggesting that lateral root founder cells could be deliberately shielded from cytokinin action. *Arabidopsis CKX* genes are expressed in LRPs (Werner et al., 2003), suggesting that removal of the cytokinin signal is also important for later stages of lateral root development (Schmülling, 2002). Recently, exogenous cytokinin applications were shown to repress lateral root initiation in *Arabidopsis* (Li et al., 2006).

In this study, we report that lateral root founder cells are sensitive to cytokinins, whereas young LRPs are not. We show that cytokinins perturb the expression of *PIN* genes in lateral root founder cells, preventing the formation of an auxin gradient that is required to pattern LRP.

RESULTS

Cytokinins Inhibit Lateral Root Development

Cytokinins are known to be involved in multiple developmental processes, including rhizogenesis (Haberer and Kieber, 2002). To investigate the impact of elevating cytokinin levels during lateral root development, we initially observed the consequences of growing *Arabidopsis* seedlings in the presence of various concentrations of the cytokinins kinetin or 6-benzylaminopurine (BAP). Root growth and lateral root density (number of emerged lateral roots/cm primary root) were analyzed 10 d after germination. Kinetin concentrations of 0.1 and 0.5 μM had little effect on primary root length, reducing growth 8.2 and 13.3%, respectively (Figure 1A). A strong reduction of root growth was only observed for kinetin concentrations ≥1 μM. By contrast, cytokinins strongly reduced lateral root density at low concentration with an average fourfold reduction for 0.1 μM kinetin compared with nontreated plants (Figure 1B). BAP was found to be more active, but the trend was the same (see Supplemental Figure 1 online). Hence, lateral root development is more sensitive to cytokinin treatment than primary root growth.

Cytokinins stimulate ethylene production in certain conditions (Wang et al., 2002). To test whether the effect of elevated cytokinin on lateral root development is mediated by ethylene, we initially analyzed the effect of cytokinins in the presence of aminoethoxyvinylglycine (AVG) (an inhibitor of ethylene biosynthesis). AVG treatment prevented cytokinin inhibition of primary root elongation consistent with previous reports (Cary et al., 1995). However, the cytokinin-dependent inhibition of LRP initiation and development was not rescued by AVG (see Supplemental Figure 2 online), suggesting that the cytokinin effect on LRP formation is independent of ethylene biosynthesis. We further addressed the relation between ethylene and cytokinin in LRP formation by analyzing the effect of cytokinin on the root of the ethylene-insensitive mutant *etr1*, which is defective in ethylene perception (Chang et al., 1993). Cytokinins had no significant effect on *etr1* primary root growth (Figure 1C). By contrast, the ethylene-insensitive mutant, like control plants, showed a significant reduction in lateral root density in the presence of cytokinins (Figure 1D). Hence, results obtained using an ethylene-insensitive mutant and an inhibitor of ethylene biosynthesis indicate that cytokinins exert their effects on lateral root development independently of ethylene. However, the inhibition of primary root growth by cytokinins appears to be ethylene dependent.

Exogenously Applied Cytokinins Perturb Both Initiation and Organization of LRPs

To determine at which stage of lateral root development cytokinins are disrupted, seedling roots were cleared and the number and developmental stages of LRPs were analyzed (Figure 2). Ten-day-old seedlings treated with cytokinins showed a reduced number of LRPs compared with plants grown without cytokinins (Figure 2A), therefore indicating that cytokinins disrupt lateral root initiation. This was confirmed using an earlier described lateral root inducible system (Himanen et al., 2002). Seedlings are germinated in the presence of the polar auxin transport inhibitor naphthylphthalamic acid (NPA), which blocks lateral root initiation (Casimiro et al., 2001). Roots are then transferred to auxin-containing media, and in a period of 12 h, xylem pole cells throughout the entire root pericycle initiate their first anticlinal divisions (Himanen et al., 2002). The use of transgenic plants containing a promoter:β-glucuronidase (GUS) fusion for the *CYCB1;1* gene in this lateral root inducible system makes it possible to time precisely the very first divisions in the pericycle. Seeds of the *ProCYCB1;1:GUS* transgenic line were grown for 72 h in the presence 10 μM NPA. Seedlings were then transferred to growth medium containing 10 μM 1-naphtalene acetic acid

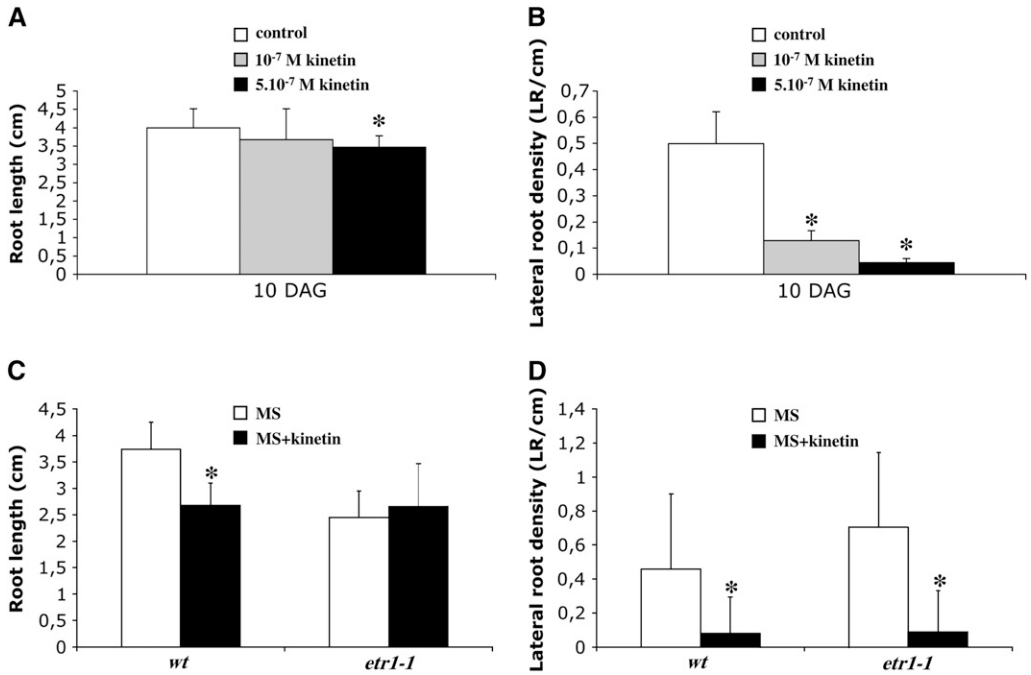

Figure 1. Cytokinins Block Lateral Root Development Independently of Ethylene.

(A) Cytokinins reduce primary root growth.

(B) Cytokinins reduce lateral root density.

(C) The *etr1* mutant is insensitive to cytokinin effect on primary root growth.

(D) The *etr1* mutant is sensitive to cytokinin-mediated inhibition of lateral root development.

(A) and **(B)** Plants were grown on vertical agar plates (half-strength Murashige and Skoog [MS] and 1.2% phytagel) supplemented with 0 (control, $n = 50$), 0.1 ($n = 45$), and 0.5 μM ($n = 45$) kinetin. Root length and the number of emerged lateral roots were measured 10 d after germination (DAG).

(C) and **(D)** Wild-type (Col-0) and *etr1-1* plants were grown for 10 d on vertical agar plates containing no (MS) or 0.5 μM kinetin (MS+kinetin). Root length and the number of emerged lateral roots were recorded using a stereomicroscope; $n = 37$ (Col-0, MS), 39 (Col-0, MS+kinetin), 27 (*etr1-1*, MS), and 34 (*etr1-1*, MS+kinetin).

The values shown are means ± SD. Significance was analyzed by analysis of variance (ANOVA) test. * $P < 0.05$ compared with untreated (**[A]** and **[B]**) or wild-type (**[C]** and **[D]**) plants. LR, lateral roots.

(NAA) or 10 μM NAA supplemented with 0.1, 1, or 10 μM BAP, respectively. Samples were tested for GUS activity by histochemical staining at 2-h intervals after transfer. NAA treatment was found to induce pericycle cell divisions from 6 h onwards starting near the root apical meristem and gradually moving basipetally as described earlier (Himanen et al., 2002). At 12 h, the complete pericycle at the protoxylem poles was activated (Figure 2C). In the presence of BAP, this activation was delayed and the first cell divisions could be detected only after 8 h of incubation on both hormones. At 12 h, only the most apical part of the pericycle appeared to be induced (Figure 2D). Therefore, cytokinin treatments clearly delayed cell divisions in the pericycle. This effect was found to be dose dependent. When plants were grown for 48 h in the presence of 10 μM NPA and then transferred to medium containing both 10 μM NPA and 10 μM BAP for another 24 h before activation in the presence of cytokinins (10 μM NAA + 10 μM BAP), a more pronounced delay in lateral root initiation was observed. The first cell cycle activity could be detected in the pericycle only at 12 h after treatment (Figure 2E). After 24 h, the pericycle was activated >50% of the primary root length (Figure 2F), and it took another 24 h to induce the entire pericycle. Hence, cytokinins perturb the first anticlinal division leading to lateral root development. This result is in agreement with previous studies (Li et al., 2006).

The effect of cytokinins on lateral root initiation does not entirely explain the reduced lateral root number in the cytokinin-treated plants (Figures 1B and 2A). We also observed that the distribution of developmental stages of primordia was altered by cytokinins, with an increased proportion of stages IV and V and a decreased proportion of later stages compared with plants grown without cytokinins (Figure 2B). This suggests that the development of some LRPs was either delayed or stopped around stages IV and V and would, together with reduced lateral root initiation, explain the reduction in the number of emerged lateral roots in cytokinin-treated plants. Detailed morphological studies revealed that cytokinin-treated plants showed abnormal patterns of cell divisions in a significant proportion of LRPs (53.7% for 0.1 μM kinetin, $n = 298$ LRP from 12 roots) that were rarely observed in control plants (2.04%, $n = 442$ LRP from 12 roots). In some stage I primordia, cytokinin treatment was observed to cause tangential and oblique divisions (Figure 2H) in place of the normal pattern of anticlinal divisions (Figure 2G). In stage II primordia,

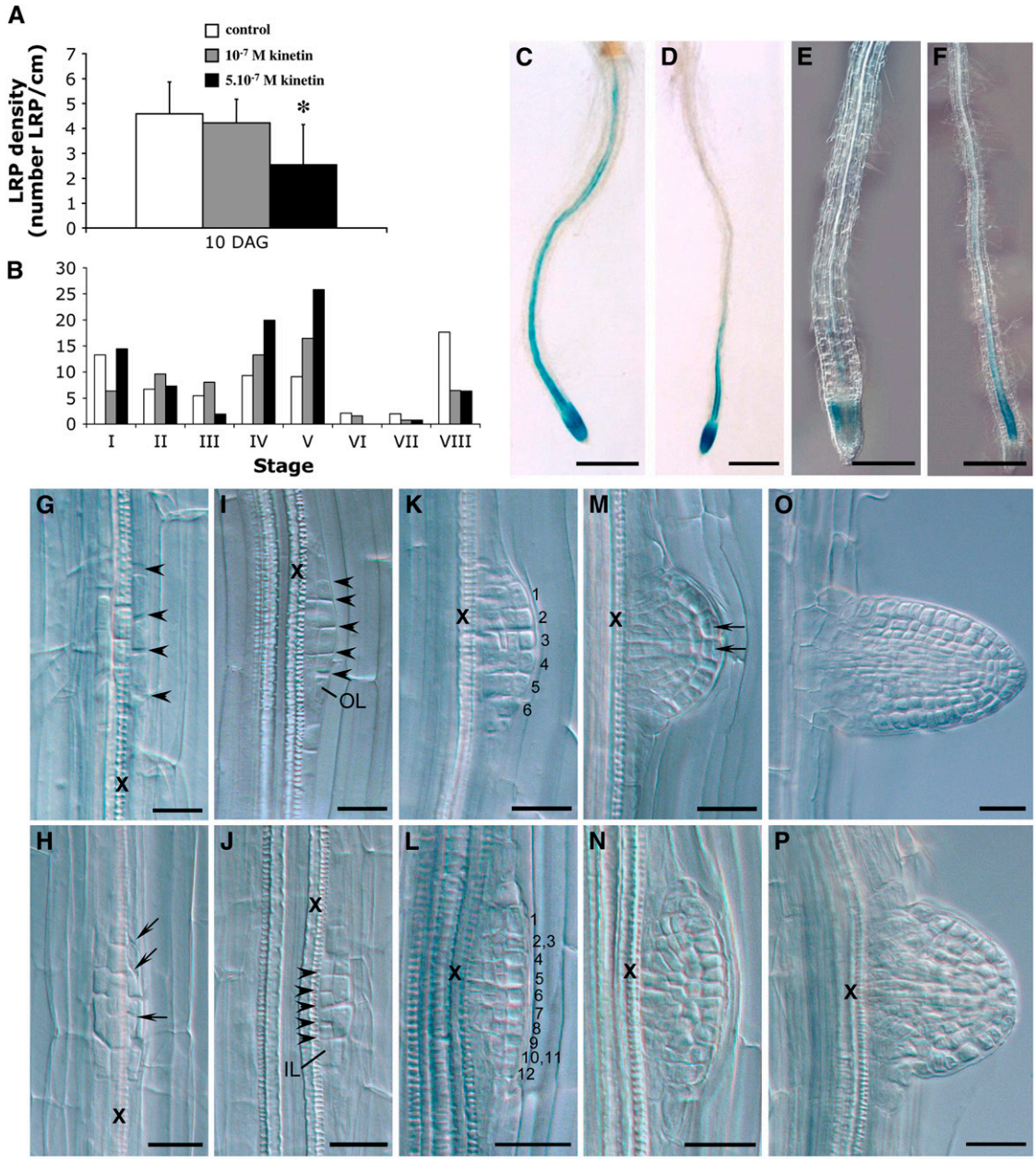

Figure 2. Cytokinin Treatment Perturbs Lateral Root Initiation and Patterning.

(A) Cytokinins affect lateral root initiation.
(B) Cytokinins alter the stage distribution of LRPs.
(C) Cell divisions occur along the entire xylem-pole pericycle 12 h after induction.
(D) Cytokinin treatment perturbs the induction of cell divisions in the pericycle 12 h after induction.
(E) Pretreatment with 10^{-5} M BAP leads to a strong inhibition of cell divisions in the root pericycle 12 h after induction.
(F) Pretreatment with 10^{-5} M BAP leads to a strong inhibition of cell divisions in the root pericycle 24 h after induction.
(G) Stage I LRPs exhibit a regular pattern of anticlinal divisions (arrowheads). X, xylem.
(H) Cytokinin treatment causes abnormal tangential and oblique divisions (arrows).
(I) In stage II primordia, anticlinal divisions only occur in the outer layer (OL).
(J) Cytokinin treatment causes ectopic anticlinal divisions within the inner layer (IL).
(K) Stage III/IV LRPs form a dome shape due to rounds of periclinal divisions in the central cells of the outer layer.
(L) Cytokinin treatment disrupts the regular pattern of periclinal divisions in central cells of the outer layer, causing the developing LRP to appear flattened. Instead, each cell in the outer layer underwent an additional round of anticlinal division giving rise to double the normal number of cells (see numbered cells).

anticlinal divisions normally only occur in the outer layer (Figure 2I), but cytokinin treatment caused ectopic anticlinal divisions in cells within the inner layer (Figure 2J). Stage III/IV LRPs normally form a dome shape due to rounds of periclinal divisions in the central cells of the outer layer (Figure 2K). However, following cytokinin treatment, central cells in the outer layer failed to undergo periclinal divisions, causing the developing LRP to appear flattened (Figure 2L). Instead, each cell in the outer layer underwent an additional round of anticlinal division giving rise to double the normal number of cells (Figure 2L). Central cells of stage VI LRP normally undergo another round of periclinal division (Figure 2M) to give rise to columella tissues (Malamy and Benfey, 1997). However, high levels of cytokinin disrupt this round of periclinal divisions (Figure 2N). As a result, the morphology of some of the cytokinin-treated LRP that emerge (stage VIII) appears disorganized particularly at the apex (Figure 2P). We conclude from our observations that cytokinin treatment results in a disorganized pattern of divisions in developing LRP leading to perturbed LRP organization.

Taken together, these results show that increasing exogenous cytokinin concentration leads to reduced lateral root initiation and disorganization of some LRPs.

Direct Effect of Cytokinins on Lateral Root Founder Cells

To precisely delimit which stage of LR development was sensitive to the cytokinin signal, we ectopically expressed the *Agrobacterium tumefaciens* cytokinin biosynthesis enzyme isopentenyltransferase (IPT; Akiyoshi et al., 1984) in either xylem-pole pericycle cells or young LRPs. This was achieved by targeting the expression of an upstream activation sequence (UAS)–linked *IPT* transgene using the xylem-pole pericycle cell or LRP-specific GAL4-GFP enhancer trap lines J0121 and J0192, respectively (Laplaze et al., 2005). Since different reports have suggested that cytokinins act where they are produced (Hewelt et al., 1994; Faiss et al., 1997; Nordström et al., 2004), we assume that the phenotypic effects of *IPT* misexpression are due to a local effect of cytokinins. However, we cannot rule out translocation of cytokinins to other sites of action. Transgenic plants harboring *UAS-IPT* did not show any phenotype in the absence of GAL4. However, when the UAS-IPT line was crossed with another transgenic line expressing *GAL4* fused to the L1-specific LTP1 promoter (Weijers et al., 2003), LTP1≫IPT F1 plants showed reduced root and hypocotyl growth, pale cotyledons, and serrated leaves (see Supplemental Figure 3 online). This phenotype is very similar cytokinin-overproducing plants (Rupp et al., 1999) or wild-type plants sprayed with cytokinins, thus confirming the functionality of the *UAS-IPT* transgene.

Homozygous J0121 or J0192 plants were crossed with homozygous UAS-IPT plants. F1 J0121≫IPT and J0192≫IPT plants were grown on vertical plates together with control plants (J0121 × Col-0 and J0192 × Col-0), and root length and lateral root number were measured 10 DAG. J0121≫IPT seedlings had shorter roots than control plants (Figure 3A). No emerged lateral roots were observed on 10-d-old J0121≫IPT plants ($n = 30$) compared with an average of 4.38 ± 2.78 lateral roots for control (J0121 × Col-0) plants ($n = 33$). Roots were cleared and the number and stages of LRPs were recorded. J0121≫IPT plants were able to develop LRPs but had a LRP density reduced by 42% (Figure 3B). This indicates that targeted cytokinin biosynthesis in the xylem-pole pericycle cells disrupts lateral root initiation. This was not due to a change in pericycle cell specification since GFP expression was not changed in J0121≫IPT compared with control J0121 × Col-0 plants (see Supplemental Figure 4 online). By contrast, J0192≫IPT seedlings did not show any significant change in root length or lateral root density compared with control (J0192 × Col-0) plants (see Supplemental Figure 5 online). Therefore, localized cytokinin biosynthesis in newly initiated primordia has no effect on lateral root development. Our study has revealed stage- and cell-specific effects of cytokinins application. Xylem-pole pericycle cells are sensitive to ectopic cytokinins biosynthesis, whereas young LRPs (stages I to IV) are not.

Surprisingly, in 10-d-old J0121≫IPT seedlings, LRPs that did form had not developed beyond stage V (Figure 3C). Closer inspection of LRP morphology revealed that the cellular organization of LRP in J0121≫IPT plants appeared disorganized compared with the wild type (see Supplemental Figure 6 online). We conclude that exposing xylem-pole pericycle cells to cytokinin disrupts an important patterning process, which later impacts the cellular organization of the developing LRP.

We tested whether the effect of cytokinins on lateral root founder cells was physiologically significant by lowering the endogenous level of cytokinins specifically in those cells. We targeted the expression of UAS-linked *Arabidopsis* cytokinin degrading enzyme cytokinine oxidase 1 (*UAS-CKX1*; Ioio et al., 2007) in xylem-pole pericycle cells using J0121. F1 J0121≫CKX1 plants were grown on vertical plates together with control (J0121 × Col-0) plants, and root length and lateral root number were recorded 11 DAG. *CKX1* expression in xylem-pole

Figure 2. (continued).

(M) Central cells in stage VI LRPs undergo another round of periclinal division (arrows).

(N) Cytokinins disrupt this round of periclinal divisions.

(O) Emerged lateral root with a sharp apex due to the formation of the central columella.

(P) Some cytokinin-treated LRPs that emerge appear disorganized particularly at the apex

(A) and **(B)** Ten-day-old plants grown on vertical agar plates containing 0 (control, $n = 12$), 0.1 ($n = 14$), and 0.5 μM ($n = 10$) kinetin were cleared, and the number and stages (Malamy and Benfey, 1997) of LRPs were recorded.

(C) to **(F)** *ProCYCB1;1:GUS* seeds were germinated and grown as described (Himanen et al., 2002). Bars = 500 μm.

(G) to **(P)** Ten-day-old plants grown on vertical agar plates containing 0, 0.1, and 0.5 μM kinetin ($n = 10$/condition) were cleared and mounted according to Malamy and Benfey (1997). Bars = 25 μm.

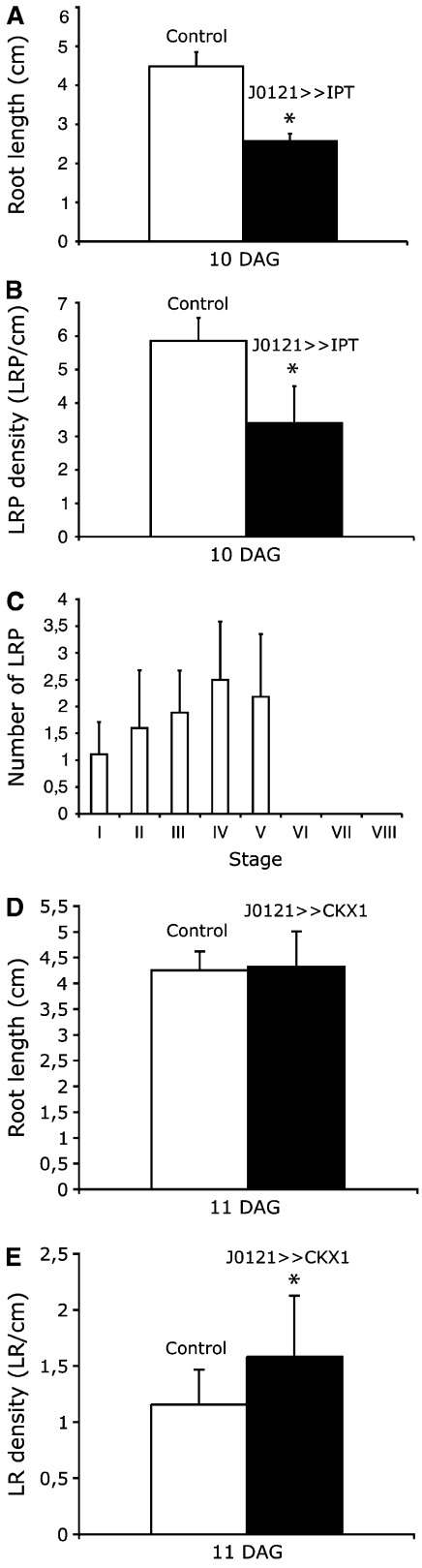

Figure 3. Targeting Cytokinin Biosynthesis or Degradation in Lateral Root Founder Cells Disrupts Lateral Root Formation.

pericycle cells had no significant effect on root growth (Figure 3D). Lateral root density was significantly higher in J0121≫CKX1 plants compared with control plants (Figure 3E). Hence, lowering the endogenous cytokinin concentration in lateral root founder cells results in increased lateral root formation. We therefore conclude that cytokinins are important endogenous regulators of lateral root initiation and that the effects we observed in our *IPT* transactivation experiments (gain of function) are physiologically significant.

Auxin-Mediated Activation of Cytokinin-Accumulating Pericycle Cells Does Not Rescue Lateral Root Formation

Given the importance of auxin to lateral root organization (Benková et al., 2003), we tested whether exogenous auxin could rescue the lateral root defect of plants producing cytokinin in the xylem-pole pericycle cells. J0121≫IPT and control (J0121 × Col-0) plants were grown for 5 d on vertical plates and then transferred to vertical plates containing different concentrations (0, 0.1, 1, and 10 μM) of the naturally occurring indole-3-acetic acid (IAA). The effects of different auxins (IAA, synthetic NAA, and 2,4-D) were also tested at 1 μM.

In contrast with control plants, none of the auxin treatments were able to induce the formation of fully developed lateral roots in J0121≫IPT plants (Figures 4A to 4C). By contrast, control plants treated with either IAA or NAA showed more lateral roots and LRPs (Figures 4A and 4B). The most dramatic phenotype was obtained with 2,4-D (Figure 4C) and was analyzed more carefully. Treatment with 1 μM 2,4-D activates cell divisions in all xylem-pole pericycle cells, leading to lateral root formation along the entire length of the primary root (Himanen et al., 2002).

Treatment with 1 μM 2,4-D completely blocked primary root growth of control and J0121≫IPT plants (Figure 4D) and also induced pericycle cells division in both control and J0121≫IPT plants, demonstrating that J0121≫IPT plants are still sensitive to auxin. Five days after transfer to 2,4-D, LRPs were present along

(A) J0121≫IPT ($n = 30$) plants have a reduced root length compared with control (J0121 × Col-0) plants ($n = 33$). The values shown are means ± SD. Significance was analyzed by ANOVA test. * $P < 0.05$ compared with control plants.

(B) J0121≫IPT plants ($n = 12$) show a reduced LRP density compared with control (J0121 × Col-0) plants ($n = 10$). The values shown are means ± SD. Significance was analyzed by ANOVA test. * $P < 0.05$ compared with control plants.

(C) Ten-day-old J0121≫IPT plant ($n = 12$) LRP distribution. No LRPs beyond stage V were observed.

(D) J0121≫CKX1 ($n = 22$) plants show no significant change in root length compared with control (J0121 × Col-0) plants ($n = 25$). The values shown are means ± SD. Significance was analyzed by ANOVA test.

(E) J0121≫CKX1 plants ($n = 22$) show a increase lateral root density compared with control (J0121 × Col-0) plants ($n = 25$). The values shown are means ± SD. Significance was analyzed by ANOVA test. * $P < 0.05$ compared with control plants.

Plants were grown on vertical agar plates (half-strength MS and 1.2% phytagel). Ten-day-old J121≫IPT and control plants (**[B]** and **[C]**) and 11-d-old J121≫CKX1 and control plants (**[D]** and **[E]**) grown on vertical agar plates were cleared, and the number and stages (Malamy and Benfey, 1997) of LRPs were recorded.

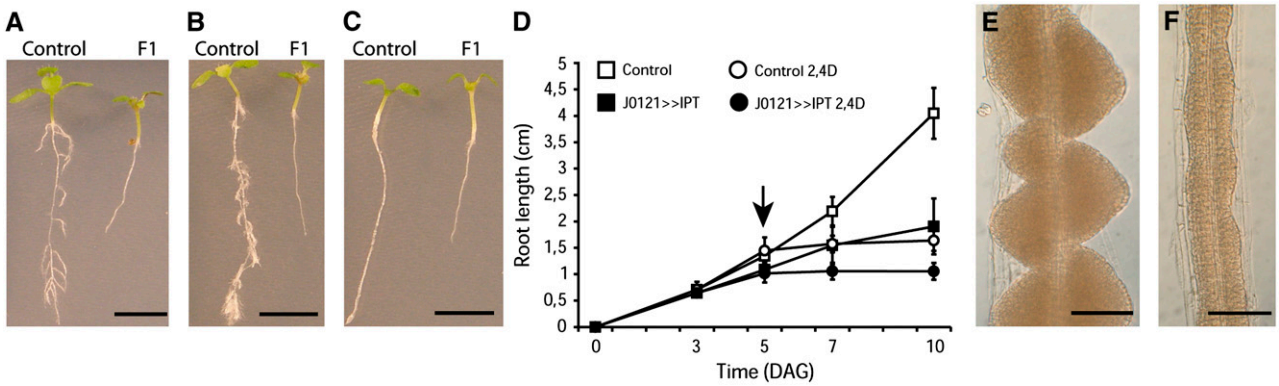

Figure 4. Auxins Do Not Rescue the J0121≫IPT Lateral Root Phenotype.

Plants were grown for 5 d on vertical plates and then transferred on new vertical plates containing 0 or 1 μM auxin (IAA, NAA, or 2,4-D). Root length and lateral root density were analyzed 0, 2, and 5 d after transfer. Bars = 5 mm in **(A)** to **(C)** and 100 μm in **(E)** and **(F)**.
(A) Control (J0121 × Col-0, left) and J0121≫IPT (right) plants 5 d after transfer on vertical plates containing IAA.
(B) Control (J0121 × Col-0, left) and J0121≫IPT (right) plants 5 d after transfer on vertical plates containing NAA.
(C) Control (J0121 × Col-0, left) and J0121≫IPT (right) plants 5 d after transfer on vertical plates containing 2,4-D.
(D) J0121≫IPT plants are still sensitive to auxin-mediated inhibition of root growth. Transfer of control and J0121≫IPT plants on auxin-containing medium 5 DAG (arrow) leads to an arrest of root growth; $n = 19$ (control), 17 (control; 2,4-D), 20 (J0121≫IPT), and 20 (J0121≫IPT; 2,4-D).
(E) View of LRPs on a control (J0121 × Col-0) plant 5 d after transfer on 2,4-D–containing medium.
(F) View of the thickening of the pericycle in J0121≫IPT plants 5 d after transfer on 2,4-D–containing plates.

the entire length of the control (J0121 × Col-0) root in front of the xylem pole (Figure 4E). By contrast, J0121≫IPT plants showed a continuous layer of cells three to four cells wide in front of the xylem poles but no discrete primordia (Figure 4F). In this system, J0121≫IPT plants were not able to develop LRP even after being exposed to 2,4-D for 1 week. Thus, auxins do not rescue the lateral root initiation defect of J0121≫IPT plants. This is consistent with previous results indicating that auxin cannot rescue the cytokinin-mediated inhibition of lateral root initiation (Li et al., 2006). Our results further suggest that cytokinin accumulation in pericycle cells does not prevent the auxin-mediated activation of cell divisions but blocks the developmental program of lateral root initiation.

Cytokinins Disrupt PIN-Dependent Formation of an Auxin Maximum during Lateral Root Development

We first tested whether cytokinins inhibit lateral root initiation by changing the sensitivity of lateral root founder cells to auxin by monitoring the expression of the auxin-sensitive *ProDR5:GUS* marker in the lateral root inducible system. Seeds of the *ProDR5:GUS* transgenic line were grown for 72 h in the presence 10 μM NPA. Seedlings were then transferred to growth medium containing 10^{-5} M NAA or 10 μM NAA supplemented either with 0.1, 1, or 10 μM BAP, respectively. Samples were tested for GUS activity by histochemical staining after transfer. We observed that the auxin-responsive promoter *ProDR5:GUS* was activated at the same time in the lateral root induction system in the presence or absence of cytokinins (see Supplemental Figure 7 online), therefore indicating that cytokinins did not perturb auxin perception in xylem-pole pericycle cells.

Benková et al. (2003) have shown that a localized auxin maximum in newly developed LRP influences the patterning of the emergent organ. To address whether cytokinins affect the patterning of LRP by modulating auxin distribution, we tested its effect on the spatial expression of the auxin-responsive reporter *ProDR5:GUS*. In control plants, *ProDR5:GUS* expression was detected in the pericycle in presumptive LRP founder cells and after the formation of short initials by anticlinal division in these cells (Figure 5A). During progression to the later stages, a gradient of GUS activity with a maximum at the tip was gradually established (Benková et al., 2003; Figure 5C). However, expression of the *ProDR5:GUS* reporter in seedlings grown on cytokinins showed a strikingly different pattern. *ProDR5:GUS* signal was occasionally detected along the root vasculature or sporadically in pericycle cells (Figure 5B). In contrast with control seedlings, this auxin response was only rarely accompanied by the anticlinal division leading to LRP initiation. *ProDR5:GUS* signal in LRP of the seedlings germinated on cytokinin was weaker and more diffuse, and the maximum at the primordia tip was often missing (68%) compared with the untreated control (23%; Figure 5D). Also, a larger portion of cytokinin-grown LRP did not show any staining (cytokinin 60%, control 40%; $n = 48$ and 44), indicating lower auxin status. Hence, cytokinins appear to perturb the formation of an auxin maximum in LRP from the first division on.

Formation of an auxin maximum in LRP has been shown to depend on the activity of PIN auxin efflux carriers (Benková et al., 2003). We therefore examined the effect of cytokinins on *PIN* gene expression during lateral root development. To monitor the effect of cytokinins on *PIN* gene expression during the initial phase of lateral root development, we made use of the lateral root inducible system (Himanen et al., 2002). The mRNA abundance of *PIN1*, *2*, *3*, *4*, *6*, and *7* was analyzed 6 h after transfer to the induction medium with or without addition of cytokinins. *PIN1*, *2*, *3*, and *7* were strongly induced 6 h after transfer on the induction medium. This PIN gene induction was reduced in the presence of

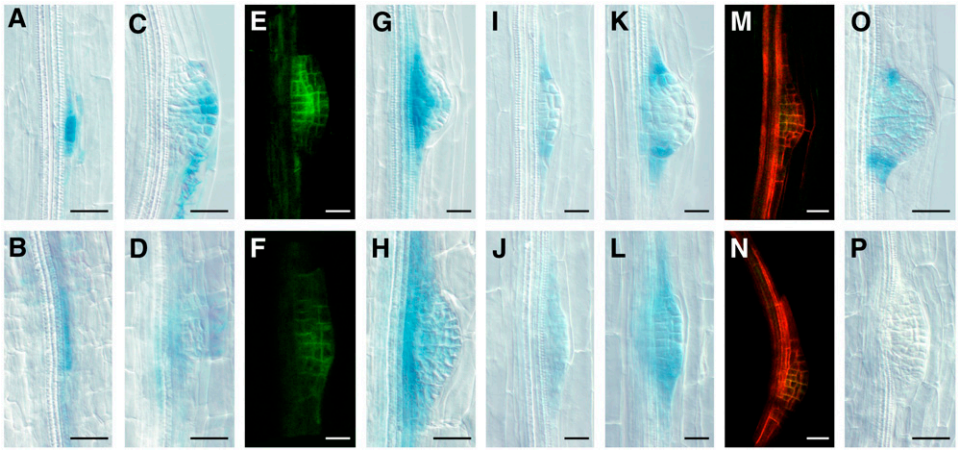

Figure 5. Cytokinin Treatment Affects *ProDR5:GUS*, *PIN*, and *CUC3* Expression.

(A) *ProDR5:GUS* is expressed in dividing pericycle cells of control roots.
(B) *ProDR5:GUS* is occasionally expressed in pericycle cells of cytokinin-grown roots but is not accompanied by initiation of LRP development.
(C) Establishment of the *ProDR5:GUS* gradient in LRPs of control roots.
(D) The *ProDR5:GUS* gradient is not established in LRPs in cytokinin-treated roots.
(E) *ProPIN1:PIN1-GFP* is associated with the central zone of LRP in control roots.
(F) *ProPIN1:PIN1-GFP* expression is more diffuse in LRPs of cytokinin-grown roots.
(G) *ProPIN1:GUS* is expressed at the base and center of LRP in control roots.
(H) *ProPIN1:GUS* is expressed at the base of LRP of cytokinin-grown roots.
(I) *ProPIN6:GUS* is expressed at the margins of stage IV LRPs in control roots.
(J) *ProPIN6:GUS* expression is not restricted to the margins of LRPs grown on cytokinins.
(K) *ProPIN6:GUS* is expressed at the base and margins of stage VI LRPs in control roots.
(L) *ProPIN6:GUS* is broadly expressed in LRPs grown on cytokinins.
(M) *ProAUX1:AUX1-YFP* is expressed throughout stage V LRPs of control roots.
(N) *ProAUX1:AUX1-YFP* expression in unaffected by cytokinin treatment.
(O) *ProCUC3:GUS* is expressed at the margins of stage VII LRPs.
(P) *ProCUC3:GUS* is not expressed in stage V LRPs grown on cytokinins.
Marker lines were grown for 10 d on MS (top row) or on MS supplemented with 0.5 μM kinetin (bottom row). Bars = 25 μm.

cytokinins (Figure 6). These results show that cytokinin treatment impacts *PIN* genes expression early during lateral root initiation. Cytokinin-induced changes in *PIN* gene expression were also examined during later stages of lateral root development using transcriptional and translational reporter fusions. Marker lines were grown on medium with or without cytokinins, and their expression pattern was recorded. *PIN1* expression in LRPs was broader, and the boundary between inner and outer layers was less clear compared with controls, where *PIN1* expression is restricted to derivatives of inner layer (Figures 5F and 5H versus 5E and 5G; Benková et al., 2003; cytokinin, 53% LRP; control, 20% LRP; $n = 28$ and 35). Cytokinin also affected the spatial expression of other *PIN* genes. For example, analysis of *ProPIN6:GUS* lines revealed more diffuse expression in LRPs (Figures 5J and 5L versus 5I and 5K; cytokinin, 68%; control, 27%; $n = 25$ and 40). Cytokinins therefore appear to perturb the pattern of *PIN* genes expression in LRP. Expression of *PIN1*, *2*, *3*, *4*, and *7* was also strongly downregulated in the shoot of cytokinin-treated plants (see Supplemental Figure 8 online). As LRP initiation is supposed to be dependent on auxin transported from the shoot (Reed et al., 1998), this general downregulation of PIN expression might be one of the causes for reduction in LRP initiation in cytokinin-grown seedlings. By contrast, cytokinin treatment had no effect on the expression of the auxin influx carrier *AUX1* (Figure 5N versus 5M) as marked by a *ProAUX1:AUX1-YFP* translational reporter fusion (Swarup et al., 2004). In conclusion, our results suggest that cytokinins inhibit lateral root initiation by interfering either directly or indirectly with PIN-dependent auxin distribution.

Cytokinins disrupt the formation of the auxin maximum, which patterns LRP. However, the absence of discrete LRP in 2,4-D–treated J121≫IPT plants suggests that cytokinins also cause defects in establishment of primordia margins. This possibility is consistent with the observed changes in *PIN6* expression in LRP (Figures 5J to 5L). Moreover, another margin marker, *CUC3* (Vroemen et al., 2003), was not expressed in LRP developing on cytokinins in contrast with controls (Figure 5P versus 5O). This altered pattern of *PIN6* and *CUC3* expression in LRP is likely to reflect defects in establishment of primordia margins during lateral root initiation.

DISCUSSION

Cytokinins Regulate Lateral Root Development in a Stage-Specific Manner

Auxin is considered the major regulator of lateral root development based on a large body of physiological, genetic, and

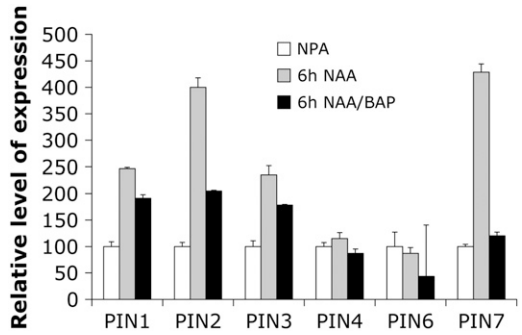

Figure 6. *PIN* mRNA Abundance Is Reduced by Cytokinins during Lateral Root Initiation.

Plants were grown in the lateral root–inducible system. RNAs were extracted from differentiated parts of roots 0 (control) and 6 h after induction in the presence of 0 or 10 μM BAP. Expression levels were normalized to *ACTIN2* and are indicated as percentages of the expression in control plants. Presented values are means ± SD.

molecular cell biological evidence (reviewed in Casimiro et al., 2003). Here, we demonstrate that cytokinins also influence lateral root development. This is in agreement with previous results showing that transgenic plants with reduced cytokinin content due to the constitutive expression of a gene encoding a cytokinin-degrading enzyme, CKX, display increased root branching (Werner et al., 2001, 2003). Similarly, *Arabidopsis* cytokinin receptor mutants show increased lateral root formation (Riefler et al., 2006). Taken together, these results indicate that cytokinins act in vivo to regulate root system architecture.

Our study has demonstrated that cytokinins influence lateral root formation independently of ethylene at a very early developmental stage. In *Arabidopsis*, lateral root founder cells are derived from three axial files of root pericycle cells that are adjacent to the two xylem poles (Dubrovsky et al., 2001). A previous study showed that exogenous applications of cytokinins inhibit lateral root initiation in those cells (Li et al., 2006). However, the main disadvantage of this experimental approach (exogenous application of plant hormones) is that it does not differentiate direct and indirect effects. We used a GAL4-based transactivation strategy to overcome this problem. This enabled us to show that lateral root founder cells are sensitive to cytokinin application, while LRPs are not. Moreover, we found that the endogenous level of cytokinins in lateral root founder cells limits lateral root formation. Our data indicate that cytokinins disrupt lateral root initiation directly in xylem pole pericycle cells in planta.

Cytokinins have recently been demonstrated to influence cell differentiation in *Arabidopsis* root apical meristem (Mahonen et al., 2006; Ioio et al., 2007). While cytokinins have been shown to repress protoxylem cell specification (Mahonen et al., 2006), these phytohormones do not appear to block xylem pole pericycle cell fate, only slightly delaying the onset of expression of the J0121 marker close to the root apical meristem. Hence, targeted expression of IPT in xylem-pole pericycle cells does not disrupt primordium formation by altering lateral root founder cell fate. Instead, our data suggest that cytokinins disrupt a later developmental event involving the polarized asymmetric cell division in two adjacent founder cells that normally leads to the formation of two short daughter cells surrounded by two larger daughter cells. Detailed morphological analyses of cytokinin-treated roots revealed an abnormal pattern of cell divisions from the earliest developmental stage onwards. For example, in stage I primordia, tangential and oblique divisions were observed in place of the normal pattern of anticlinal divisions. Similarly, in stage II primordia, anticlinal divisions normally only occur in the outer layer but cytokinin treatment caused ectopic anticlinal divisions in cells within the inner layer. We conclude that cytokinins disrupt the organization and development of LRPs.

Cytokinins Disrupt PIN-Dependent Lateral Root Initiation

How could exposing lateral root founder cells to cytokinin disrupt the subsequent organization and development of LRPs? Auxin is known to trigger the initial asymmetric cell division (Vanneste

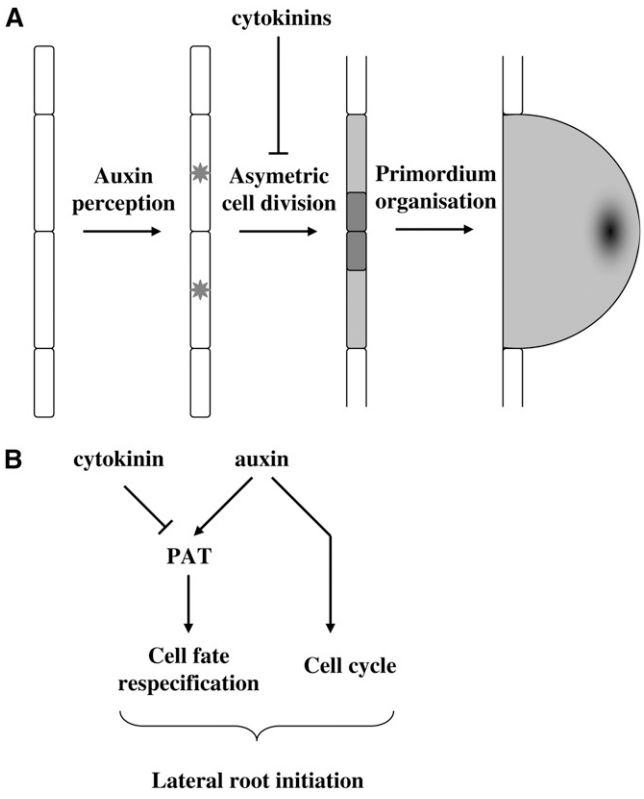

Figure 7. Model of Cytokinin–Auxin Interaction during Root Development.

(A) Lateral root initiation is triggered by auxin perception by some xylem-pole pericycle cells leading to an asymmetric cell division. Cytokinins do not block auxin perception or response in lateral root founder cells but act downstream to perturb the asymmetric cell division.

(B) Lateral root initiation requires auxin-induced cell fate respecification and cell cycle progression (Vanneste et al., 2005). Cell fate respecification depends on the expression of *PIN* genes to create an auxin gradient responsible for asymmetric cell division and acquisition of LRP identity. Cytokinins inhibit this step by downregulating *PIN* gene expression.

et al., 2005; Figure 7A). However, we have demonstrated in this study that cytokinins did not change the perception of auxin in lateral root founder cells (Figure 7A). Instead, we observed that auxin was still able to induce cell divisions in xylem-pole pericycle cells expressing the *IPT* gene but that this led to disorganized primordia. Interestingly, this phenotype is reminiscent of plants perturbed in PIN-mediated polar auxin transport (Benková et al., 2003; Geldner et al., 2004). The organization of LRP is known to be dependent on the coordinated expression of multiple PIN genes to create an auxin maximum (Benková et al., 2003). We observed that cytokinin treatment disrupted the induction of some *PIN* genes during lateral root initiation (i.e., the very first division of lateral root founder cells). Moreover, the early establishment of an auxin gradient was perturbed by cytokinins. This suggests that cytokinins interfere with the initial asymmetric division, and the resulting perturbation could explain the subsequent changes in the pattern of cell division and cell fate in cytokinin-treated plants as revealed by altered expression of *PIN6* or *CUC3* markers that define the LRP flanks.

In conclusion, we propose the following model to explain cytokinin–auxin interaction during lateral root initiation (Figure 7B). Lateral root initiation requires the coordinated action of cell cycle progression and cell fate respecification in lateral root founder cells both induced by auxin (Vanneste et al., 2005). These two processes can be uncoupled by mutation in the *Diageotropica* gene in tomato (*Solanum lycopersicum*) that results in proliferative cell divisions but no lateral root initiation in the pericycle in reponse to auxin (Ivanchenko et al., 2006). Similarly, overexpression of a D-type cyclin (CYCD3;1) in the *Arabidopsis solitary root/iaa14* mutant leads to proliferative cell divisions but is not sufficient to rescue lateral root initiation (Vanneste et al., 2005). Thus, cell cycle activation in xylem-pole pericycle cells is not sufficient for lateral root initiation. It also requires auxin to induce the expression of *PIN* genes during lateral root initiation (Vanneste et al., 2005; Vieten et al., 2005; this study) to create an auxin gradient responsible for asymmetric cell division leading to cell fate respecification and acquisition of LRP identity as shown by the phenotype of *Arabidopsis* plants perturbed in polar auxin transport (Benková et al., 2003; Geldner et al., 2004). Our results indicate that cytokinins do not block lateral root initiation in lateral root founder cells by acting on auxin-induced cell division but rather by inhibiting auxin-induced cell fate respecification by downregulating *PIN* gene expression. The endogenous source of cytokinins responsible for lateral root development inhibition in planta remains to be defined. Cytokinins are produced in a wide range of tissues and organs. Interestingly, the root cap has been proposed as a site of cytokinin production (Miyawaki et al., 2004) and genetic ablation of the root cap stimulate lateral root initiation (Tsugeki and Fedoroff, 1999). Future work will focus on the identification of the source of cytokinins that control lateral root initiation and how cytokinin production is regulated by environmental factors, such as nutrient concentrations.

Cytokinins and auxin have antagonistic effects on many aspects of plant development, including lateral root formation. It was shown that auxin can directly downregulate cytokinin biosynthesis, while cytokinins had little effect on auxin biosynthesis (Nordström et al., 2004). Our results suggest that cytokinins act on auxin homeostasis by changing auxin transport (via the downregulation of *PIN* gene expression) rather than changing auxin biosynthesis or perception. This illustrates that a complex network of interacting hormones, including auxin and cytokinins, regulates root architecture. An exciting challenge in the years to come will be to understand this network of interacting hormonal signals that controls lateral root formation.

METHODS

Plant Lines and Growth Conditions

C24, Col-0, and *etr1-1* seeds were obtained from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/).

J0121 (C24) belongs to a collection of GAL4-GFP enhancer trap lines available through the stock centers (http://www.plantsci.cam.ac.uk/Haseloff). J0192 (C24) was isolated from a collection of 401 GAL4-GFP enhancer trap lines generated by root transformation of C24 wild-type plants during a screen for lateral root expressed lines (Laplaze et al., 2005).

The *ProUAS:IPT* construct was created by cloning the *Agrobacterium tumefaciens* IPT coding sequence (Akiyoshi et al., 1984) between the 6xUAS promoter and the *NOS* terminator in pSDM7022 (Weijers et al., 2003) and subsequent transfer of the *ProUAS:IPT:tNOS* gene into pSDM7006 (Weijers et al., 2003). The construct was transformed into Col-0 wild-type plants, and transgenic lines were selected based on T-DNA number, wild-type phenotype, and GAL4-dependent *GUS* expression as described (Weijers et al., 2003). The *ProUAS-CKX1* transgenic line was described previously (Ioio et al., 2007)

Lines carrying *ProPIN1:GUS*, *ProPIN1:PIN1-GFP*, *ProPIN3:GUS*, *ProPIN6:GUS*, *ProDR5:GUS*, and *ProCUC3:GUS* for analysis of expression in LRPs were described previously (Benková et al., 2003).

Plants were grown at 23°C, 60% humidity, in 60 μE constant light on vertical half-strength MS 1.2% phytagel plates under long-day conditions (16 h light/8 h dark). Seeds were surface sterilized and cold treated for 2 d at 4°C in the dark before transfer to the growth chamber. Plants in soil were grown in a 1:1 (v/v) compost/vermiculite mix in a growth room at 21°C in a 16-h-light/8-h-dark cycle.

Lateral root induction in the whole pericycle was performed according to Himanen et al. (2002).

Root length was measured from digital images of the plates using the NIH Image 1.62 software. Emerged lateral roots were counted using a binocular. Data were analyzed using the Excel statistical package. Experiments were repeated at least two times independently.

Microscopy

GUS activity was assayed by immersing seedlings in a staining solution (Svistoonoff et al., 2003) at 37°C. To limit the diffusion of the blue staining, 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were added. Tissues were cleared in 70% ethanol for 2 d. Tissues were then immersed in 50% (v/v) ethanol/10% (v/v) glycerol for 2 h, 30% (v/v) ethanol/30% (v/v) glycerol for 2 h, and in 50% (v/v) glycerol for 2 h. Seedlings were then mounted in 50% (v/v) glycerol and visualized on a DMRB microscope (Leica).

Quantitative RT-PCR

Col-0 seeds were germinated on medium containing 10 μM NPA and transferred 3 d after germination under continuous light to 10 μM NAA or 10 μM NAA + 10 μM BAP for 6 h. The root apical meristems were cut off, and the shoots were removed by cutting below the adventitious root primordia. Only the differentiated part of the root was used for RNA

extraction using the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 1 μg of total RNA with Superscript III reverse transcriptase (Invitrogen) and quantified on an I cycler apparatus (Bio-Rad) with the qPCR core kit for SYBR green I (Eurogentec). PCR was performed in 96-well optical reaction plates heated for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 50 cycles of denaturation for 60 s at 95°C and annealing extension for 60 s at 58°C. Target quantifications were performed with specific primer pairs designed with Beacon Designer 4.0 (Premier Biosoft International). PCR experiments were performed in triplicate. Expression levels were first normalized to *ACTIN2* expression levels that did not show clear systematic changes in Ct value and then to the respective expression levels in the NPA-grown roots.

The primers used to quantify gene expression levels were as follows: At3g18780/*ACTIN2*, 5′-TTGACTACGAGCAGGAGATGG-3′ and 5′-ACAAACGAGGGCTGGAACAAG-3′; At1g73590/*PIN1*, 5′-TACTCCGAGACCTTCCAACTACG-3′ and 5′-TCCACCGCCACCACTTCC-3′; At5g57090/*PIN2*, 5′-GGCGAAGAAAGCAGGAAGA-3′ and 5′-GGTGGGTACGACGGAACA-3′; At1g70940/*PIN3*, 5′-GAGGGAGAAGGAAGAAAGGGAAAC-3′ and 5′-CTTGGCTTGTAATGTTGGCATCAG-3′; At2g01420/*PIN4*, 5′-ATGCTGGTCTTGGAATGGCTATG-3′ and 5′- CTGAACGATGGCTATACGGAGAAG-3′; At1g77110/*PIN6*, 5′-CCACGGCGGAGGAGGAAG-3′ and 5′-AGTAAGCATCGGAGGAAGCATAAC-3′; At1g23080/*PIN7*, 5′-ACTCCTCGTCCGTCTAATCTCAC-3′ and 5′-GAAGCCATAGCACAACTCTCCTC-3′.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. BAP Inhibits Root Growth and Lateral Root Formation.

Supplemental Figure 2. The Inhibitor of Ethylene Biosynthesis AVG Prevents Cytokinin Inhibition of Primary Root Growth but Does Not Change the Effect of Cytokinins on Lateral Root Density.

Supplemental Figure 3. L1-Specific IPT Expression Recovers Cytokinin Phenotypes.

Supplemental Figure 4. IPT Transactivation in Xylem-Pole Pericycle Cells Does Not Change Cell Specification.

Supplemental Figure 5. IPT Transactivation in Young Lateral Root Primordia Does Not Change Root Growth or Branching.

Supplemental Figure 6. IPT Transactivation in Xylem-Pole Pericycle Cells Leads to the Formation of Disorganized Primordia.

Supplemental Figure 7. Cytokinin Treatment Does Not Change Auxin Sensitivity in Root Xylem-Pole Pericycle Cells.

Supplemental Figure 8. PIN Gene Expression Is Strongly Reduced in the Shoot of Cytokinin-Treated Plants.

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