

Cytokinin Regulation of Auxin Synthesis in *Arabidopsis* Involves a Homeostatic Feedback Loop Regulated via Auxin and Cytokinin Signal Transduction ^{WJ|OA}

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Together, auxin and cytokinin regulate many of the processes that are critical to plant growth, development, and environmental responsiveness. We have previously shown that exogenous auxin regulates cytokinin biosynthesis in *Arabidopsis thaliana*. In this work, we show that, conversely, the application or induced ectopic biosynthesis of cytokinin leads to a rapid increase in auxin biosynthesis in young, developing root and shoot tissues. We also show that reducing endogenous cytokinin levels, either through the induction of *CYTOKININ OXIDASE* expression or the mutation of one or more of the cytokinin biosynthetic *ISOPENTENYLTRANSFERASE* genes leads to a reduction in auxin biosynthesis. Cytokinin modifies the abundance of transcripts for several putative auxin biosynthetic genes, suggesting a direct induction of auxin biosynthesis by cytokinin. Our data indicate that cytokinin is essential, not only to maintain basal levels of auxin biosynthesis in developing root and shoot tissues but also for the dynamic regulation of auxin biosynthesis in response to changing developmental or environmental conditions. In combination with our previous work, the data suggest that a homeostatic feedback regulatory loop involving both auxin and cytokinin signaling acts to maintain appropriate auxin and cytokinin concentrations in developing root and shoot tissues.

INTRODUCTION

The relationship between auxin and cytokinin has long been recognized as central to normal plant growth and development (Coenen and Lomax, 1997; Swarup et al., 2002; Rashotte et al., 2005). Fundamental multicellular plant processes, such as embryogenesis (Müller and Sheen, 2008), meristem development and maintenance (Hartig and Beck, 2006; Sablowski 2007; Moubayidin et al., 2009), shoot branching (Ongaro and Leyser,

2008; Shimizu-Sato et al., 2009), and lateral root initiation and development (Casimiro et al., 2003; De Smet et al., 2006; Osmont et al., 2007), rely on both hormones. The mechanisms involved in auxin–cytokinin interactions are not yet fully understood, but discoveries during the last few years have been adding pieces to the puzzle. Both hormones act as growth regulators, both can be biosynthesized in many, if not all, parts of the plant, and both can act as long-distance signaling substances and as paracrine signals during development (Baker, 2000; Ljung et al., 2001, 2005; Nordström et al., 2004; Sakakibara, 2006; Robert and Friml, 2009).

A reliance on both hormones suggests an interrelationship between the auxin and cytokinin response pathways. In fact, auxin and cytokinin interact on many levels. For example, strong evidence has emerged over recent years that they interact at the level of hormone perception and signal transduction (Müller and Sheen, 2008; Moubayidin et al., 2009). It has also been shown that polar auxin transport (PAT) can be regulated via cytokinin modulation of the activity of auxin efflux carriers (Dello Ioio et al., 2008; Ruzicka et al., 2009). At the metabolic level, both auxin and cytokinin are able to modify the expression of the key cytokinin biosynthesis *ISOPENTENYLTRANSFERASE (IPT)* genes (Miyawaki et al., 2004). Direct cytokinin biosynthesis measurements in *Arabidopsis thaliana* have shown that auxin can rapidly downregulate cytokinin biosynthesis (Nordström et al., 2004). These authors were the first to demonstrate that cytokinins are

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biosynthesized in both aerial and root tissues and that young, developing leaves have the highest cytokinin biosynthetic capacity. Tanaka et al. (2006) subsequently showed a similar phenomenon exists in pea (*Pisum sativum*), in which apical dominance is maintained at least partially by auxin-induced downregulation of cytokinin biosynthesis in the stem.

Interactions at the metabolic level have also been reported between auxin and ethylene (Ruzicka et al., 2007; Stepanova et al., 2007, 2008; Swarup et al., 2007) and auxin and brassinosteroids (Mouchel et al., 2006). In this article, we present evidence that auxin biosynthesis rates are modulated by cytokinin. In experiments combining inducible cytokinin overproduction and exogenous cytokinin application with stable isotope labeling, we show that an elevation of cytokinin levels leads to a rapid increase in auxin biosynthesis rates in young, developing *Arabidopsis* tissues. We also show that, conversely, a reduction in cytokinin levels leads to lower rates of endogenous auxin biosynthesis, suggesting that cytokinin is an essential regulator of auxin biosynthesis.

Compared with other classic plant hormones, comparatively little is known about the molecular basis of auxin biosynthesis. In part, this may be because of the difficulties arising from the existence of multiple auxin biosynthesis pathways and the high level of redundancy between these pathways (Woodward and Bartel, 2005; Normanly, 2010). Taken together, our data indicate that the regulation of auxin biosynthesis by cytokinin is an important homeostatic control mechanism in developing root and shoot tissues. In combination with previous data that shows that auxin downregulates cytokinin levels (Miyawaki et al., 2004; Nordström et al., 2004; Tanaka et al., 2006; Shimizu-Sato et al., 2009), the data presented here suggest that cellular concentrations of auxin and cytokinin are regulated via an active homeostatic feedback loop, mediated through both auxin and cytokinin signal transduction mechanisms.

RESULTS

Cytokinin Induces de Novo Auxin Biosynthesis in *Arabidopsis*

We have previously shown that IAA is synthesized at a high rate in the *Arabidopsis* root apex (Ljung et al., 2005; Petersson et al., 2009). When we screened different classes of plant hormones for their effects on IAA biosynthesis in the root, we observed that increasing cytokinin levels via the estradiol induction of *IPT8* in the transgenic line *IPT8/plant growth activator 22* (*pga22*) (Sun et al., 2003) caused a rapid increase in the rate of indole-3-acetic acid (IAA) biosynthesis and in the levels of IAA. To test if there were tissue-specific differences in the ability of cytokinin to alter the rate of IAA biosynthesis, we grew *IPT8/pga22* seedlings on vertical plates in long days (LDs; 16 h light/8 h dark cycle) for 10 d and took measurements of separate tissues. Intact seedlings or seedlings divided into root and shoot were incubated for 12 h in a medium containing 17- β -estradiol to induce *IPT8* and cytokinin biosynthesis and then for 24 h in a medium containing 17- β -estradiol and 30% $^2\text{H}_2\text{O}$ (deuterated water). After incubation, the seedlings were divided into (a) apex and young, developing leaves (leaf 3 and smaller), (b) the first two true leaves, and (c) the

root system (Figure 1A). De novo IAA biosynthesis was analyzed in these tissues by gas chromatography–multiple reaction monitoring–mass spectrometry (GC-MRM-MS) (Ljung et al., 2005). A significantly higher rate of IAA biosynthesis was observed in the apex and young leaves and in the root system after the estradiol induction of *IPT8*, both in intact seedlings and in seedlings divided into root and shoot before incubation (Figure 1B, a and c). Interestingly, in contrast with younger tissues, we did not observe an increase in the rate of IAA biosynthesis in older leaves after the estradiol treatment (Figure 1B, b), indicating that elevated cytokinin levels increase the rate of IAA biosynthesis only in young, developing tissues. This may explain the findings of Nordström et al. (2004) who measured the effects of cytokinin on IAA biosynthesis in *Arabidopsis* on a whole plant basis. In their experiments, an increase in cytokinin levels via the induction of a bacterial *IPT* transgene led to a reduction in the IAA pool size and in the rate of IAA biosynthesis at 36 to 48 h of induction. The mass of older tissues in these 3-week-old plants may have masked an increase in the rate of IAA biosynthesis in young, developing tissues. It is also possible that longer term developmental and/or homeostatic effects would have predominated at the time scale used in their experiments, rather than the direct effects that can be seen with shorter treatments used in our experiments.

To test our observations, we incubated wild-type seedlings with two different exogenous cytokinins, 6-benzylamino purine (BAP) and zeatin. Ten-day-old wild-type seedlings were divided into shoot and root and incubated for 24 h in a liquid medium containing 30% $^2\text{H}_2\text{O}$ and BAP or *cis/trans*-zeatin (*c/t-Z*). We observed a significant increase in the rate of IAA biosynthesis in the shoot apex and young leaves and in the root system (Figure 1C, a and c). No increase in IAA biosynthesis was observed in older leaves after the BAP treatment. However, there was an increase after the *c/t-Z* treatment (Figure 1C, b). This difference suggests that very high concentrations of exogenous zeatin (two to three orders of magnitude higher than the *t*-zeatin (*t-Z*) levels observed in older leaves in the *IPT8/pga22* induced seedlings; see Supplemental Table 1 online) are able to overcome the relative insensitivity of older leaves to cytokinins that we observed in the *IPT8/pga22*-inducible line and in the BAP treatment. Mock treatments with 17- β -estradiol in wild-type plants did not induce IAA biosynthesis in either young or old tissues (Figure 1C).

Direct measurements of cytokinin levels in the *IPT8/pga22* line before and after the estradiol treatment showed that the induction of the *IPT8* transgene led to a strong induction of *iP* and *Z*-type cytokinins in all tissues investigated (see Supplemental Table 1 online). In general, before the estradiol treatment, there was a very low concentration of the free bases, *iP* and *Z*, with the highest levels occurring in apex and young, developing leaves. After the induction of the *IPT8* transgene, there was a rapid increase in the cytokinin precursors, *iPMP*, *iPA*, *t-ZMP*, and *t-ZR*, and the free bases *iP* and *t-Z*. Interestingly, the induction of *iP* and its precursors (*iPA* and *iPMP*) was stronger in the shoot than in the root system. The opposite was observed for *t-Z* and its precursors (*t-ZR* and *t-ZMP*) and conjugates (*Z7G*, *Z9G*, and *ZOG*) that were more abundant in the root system than in the shoot. This suggests that the hydroxylation mechanisms that convert *iP* and its precursors to zeatin-type cytokinins

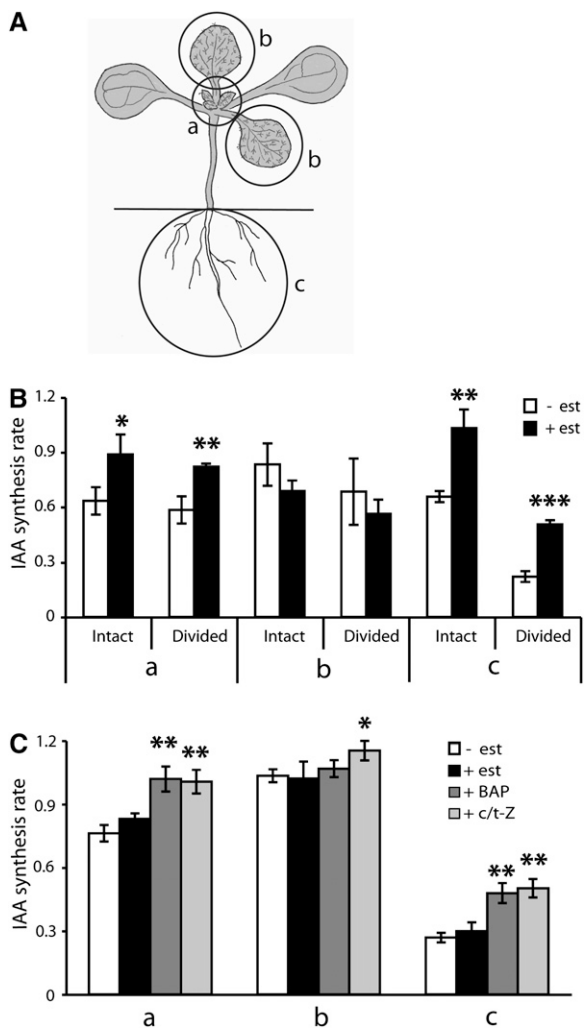


Figure 1. Induction of IAA Biosynthesis by Cytokinin.

(A) The de novo IAA biosynthesis rate was analyzed in specific tissues after incubation of 10 DAG *Arabidopsis* seedlings in a medium containing 30% $^2\text{H}_2\text{O}$. Seedlings were divided into (a) apex and young developing leaves (leaf 3 and smaller), (b) the first two true leaves, and (c) the root system after incubation.

(B) *IPT8/pga22* seedlings (10 DAG; either intact seedlings or seedlings dissected into root and shoot before incubation) were incubated first for 12 h in a medium containing 5 μM 17- β -estradiol and then for 24 h in a medium containing 30% $^2\text{H}_2\text{O}$ and 5 μM 17- β -estradiol (+ est). As a control, seedlings were incubated without estradiol in the medium (- est). The IAA biosynthesis rate was analyzed in the different tissues described in **(A)**.

(C) Wild-type Ws seedlings 10 DAG were divided into root and shoot and incubated for 24 h in liquid medium containing 30% $^2\text{H}_2\text{O}$ alone or with 5 μM 17- β -estradiol, 10 μM BAP, or 10 μM c/t-Z as indicated. The IAA biosynthesis rate was analyzed in the different tissues described in **(A)**. Data are mean \pm SD of three independent experiments ($n = 3$). P values in **(B)** (+ est versus - est) and **(C)** (+ est, + BAP, or + c/t-Z versus - est) were determined by two-tailed Student's *t* test assuming equal variances (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

(Sakakibara, 2006) are more active in the root than in the shoot. This is consistent with the expression pattern of the genes involved in the process, the cytochrome P450 monooxygenase genes *CYP735A1* and *CYP735A2*, which are more highly expressed in roots than in other organs (Takei et al., 2004). We also observed that the induction of *IPT8* led to very high levels of the iP precursor, iPMP, in all tissues. This is in contrast with previous findings in transgenic *Arabidopsis* plants carrying the dexamethasone-inducible *Agrobacterium tumefaciens IPT* gene. In this line, only zeatin-type cytokinins were induced (Ástot et al., 2000).

Cytokinin Induction of Auxin Biosynthesis Is Mediated by Cytokinin and Auxin Signal Transduction

The rapid upregulation of auxin biosynthesis by cytokinin has not been observed previously. Our data indicate that these interactions occur at the metabolic level and are tissue specific. To investigate the mechanisms underlying this cytokinin–auxin interaction, we analyzed root tip–specific cytokinin induction of auxin biosynthesis in different auxin and cytokinin signal perception and signal transduction mutants. Excised roots from 6-d-old wild-type and mutant *Arabidopsis* seedlings were incubated in media containing 30% $^2\text{H}_2\text{O}$ with or without 5 μM t-Z for 24 h. The IAA concentrations and IAA biosynthetic rates were subsequently measured by GC-MRM-MS in 2-mm-long root apex sections. Two cytokinin signaling mutants were investigated, the cytokinin-hypersensitive *ARABIDOPSIS RESPONSE REGULATOR (ARR)* quadruple mutant *arr3 arr4 arr5 arr6* and the cytokinin-insensitive quadruple *ARABIDOPSIS HISTIDINE-PHOSPHOTRANSFER PROTEIN (AHP)* mutant *ahp1 ahp2 ahp3 ahp4*. The *arr3 arr4 arr5 arr6* line has mutations in four of the 10 type A *ARR* genes that are believed to function as negative regulators of cytokinin signaling (To and Kieber, 2008). Mutations in these genes result in plants that are more sensitive to cytokinin. Higher order *ARR* mutants such as this line have been shown to be considerably cytokinin hypersensitive, and several of the type A *ARR* genes (including *ARR3*, *ARR4*, *ARR5*, and *ARR6*) are induced by cytokinin (To et al., 2004). The IAA biosynthetic rate was higher in the *arr3 arr4 arr5 arr6* mutant than in the wild type prior to the cytokinin treatment, and the effect of the treatment was exaggerated in the mutant (Figure 2A). Despite the increased biosynthetic rate, we did not observe a significant difference between the steady state IAA concentrations in the root tips of wild-type and *arr3 arr4 arr5 arr6* plants (Figure 2B).

AHP gene products are believed to be positive elements in cytokinin signal transduction, mediating signaling between the plasma membrane-bound cytokinin receptors and the response regulators within the nucleus (Hutchison et al., 2006). The *ahp1 ahp2 ahp3 ahp4* line has mutations in four of the five *AHP* genes. Surprisingly, similarly to the cytokinin-hypersensitive *arr3 arr4 arr5 arr6* line, the cytokinin-insensitive *ahp1 ahp2 ahp3 ahp4* line had a higher rate of IAA biosynthesis in the roots than did the wild type prior to cytokinin treatment (Figure 2A). In contrast with both the wild-type and *arr3 arr4 arr5 arr6* plants, however, cytokinin treatment led to a significant decrease in the IAA biosynthesis rate in this line, suggesting that the relationship between cytokinin and auxin biosynthesis is fundamentally altered (Figure 2A).

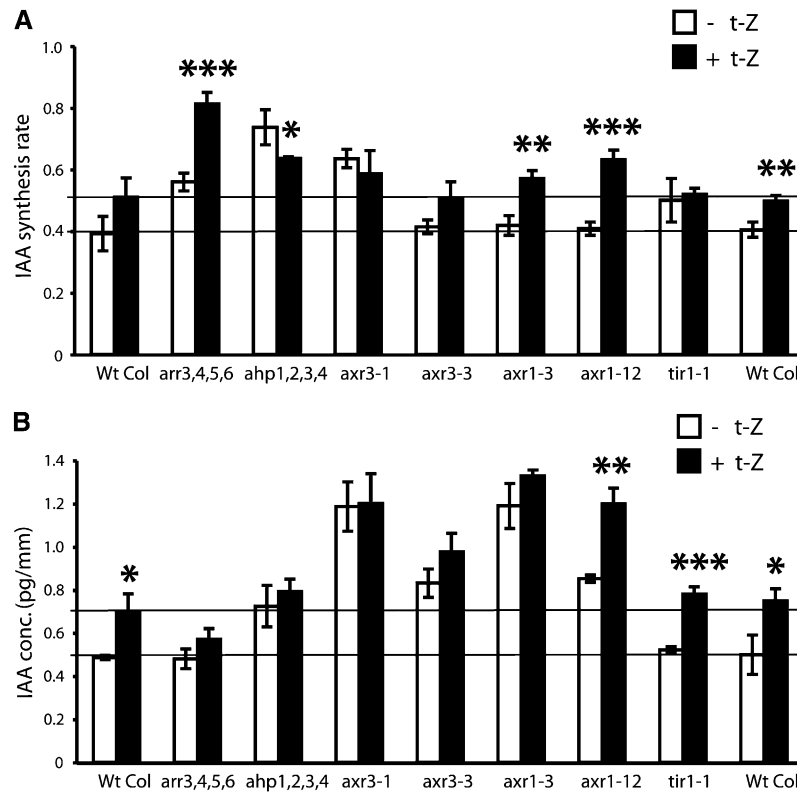


Figure 2. IAA Biosynthesis in Auxin and Cytokinin Signal Transduction Mutants.

De novo IAA biosynthesis rate (**A**) and IAA concentration (**B**) were analyzed in root tips of auxin and cytokinin signal transduction mutants after treatment of 6 DAG seedling roots for 24 h with liquid medium containing 30% $^2\text{H}_2\text{O}$ with or without 5 μM t-Z. Data are mean \pm SD of three independent experiments ($n = 3$). P values (+ t-Z versus - t-Z) were determined by two-tailed Student's *t* test assuming equal variances (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

We also analyzed the effect of cytokinin on the rate of IAA biosynthesis and steady state IAA concentrations in the auxin perception and signal transduction mutants *axr3-1*, *axr3-3*, *axr1-3*, *axr1-12*, and *tir1* (Figures 2A and 2B). The *axr3-1* and *axr3-3* mutants have been described as being hypersensitive to auxin, with the *axr3-1* mutant showing the more severe phenotype of the two (Leyser et al., 1996). *AXR3* is identical to *IAA17*, a member of the auxin-inducible *AUX/IAA* gene family of auxin response transcriptional regulators (Rouse et al., 1998). *axr3-1* and *axr3-3* are both semidominant gain-of-function mutants that increase the stability of the *AXR3/IAA17* protein (Chapman and Estelle, 2009). Leyser et al. (1996) reported that *axr3* plants show striking differences in phenotypic responses to exogenous cytokinin compared with wild-type plants (e.g., in root elongation and adventitious rooting). Prior to cytokinin treatment, we observed that steady state IAA levels were higher in the root tips of the *axr3-1* and *axr3-3* mutants compared with wild-type plants (Figure 2B). The *axr3-1* mutant also had a higher rate of IAA biosynthesis than the wild-type plants (Figure 2A). Interestingly, the *axr3-1* mutant did not respond to exogenous cytokinin. These results suggest that low levels of *AXR3/IAA17* are essential for the maintenance of wild-type basal auxin levels and that cytokinin induction of auxin biosynthesis is the result of increased *AXR3/IAA17* expression or stabilization of its encoded protein.

We next analyzed affect of cytokinin induction on auxin biosynthesis and levels in the auxin-insensitive, *axr1-3*, *axr1-12*, and *tir1-1* loss-of-function mutant lines. The *axr1* lines were discovered in a screen for auxin-resistant mutants (Lincoln et al., 1990). *AXR1* is part of the RUB-activating enzyme required for normal SCF activity and auxin responsiveness (del Pozo et al., 2002; Dharmasiri et al., 2007). The *axr1-12* mutant has a more severe phenotype and a stronger resistance to exogenous IAA than does *axr1-3*. The pretreatment rate of IAA biosynthesis was unchanged from the wild type in both the *axr1-3* and *axr1-12* lines. However, there was an increased response to cytokinin in both mutant lines compared with the wild type (Figure 2A). There was also a considerable increase in steady state IAA levels in the root tips of the mutants compared with the wild type before and after the cytokinin treatment, particularly in the less severe *axr1-3* (Figure 2B). *TIR1* codes for an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). There were no significant differences in IAA levels and biosynthesis rates between the wild type and *tir1-1* before or after the cytokinin treatment (Figures 2A and 2B). As a whole, the data from these mutants indicate that both cytokinin and auxin signal transduction mechanisms are implicated in the cytokinin regulation of the level and biosynthesis rate of IAA in the root apex.

Downregulation of Cytokinin Levels Leads to a Decrease in Auxin Biosynthesis Rates

Having observed that elevated levels of cytokinin lead to an increase in auxin biosynthesis, we next asked whether endogenous basal levels of cytokinin play a role in maintaining the metabolic rate of auxin biosynthesis. We constructed an *Arabidopsis* line carrying an inducible cytokinin oxidase (pMDC7: *AtCKX3*) gene. Six days after germination (DAG), pMDC7: *AtCKX3* and wild-type seedlings were incubated in liquid medium containing 17- β -estradiol, and *CKX3* transcript levels were analyzed after 0, 3, 6, 12, and 24 h of incubation. We observed a rapid induction of *CKX3* expression in pMDC7: *AtCKX3* seedlings, with maximum transcript levels occurring after 12 h of induction (Figure 3A). This coincided with a decrease in cytokinin levels after induction (see Supplemental Table 2 online). Endogenous cytokinin precursor levels were lower after 3 h of induction, and they remained low for 12 h, after which the levels started to rise again (Figure 3B). The concentration of the active cytokinin, iP, was halved between 6 and 24 h of *CKX3* induction (see Supplemental Table 2 online). In 6 DAG pMDC7: *AtCKX3* seedlings, we observed a significant downregulation of IAA biosynthesis rates 12 and 24 h after the onset of incubation (Figure 3C). Tissue-specific measurements of IAA biosynthesis rates in 10 DAG pMDC7: *AtCKX3* seedlings showed that induction of *CKX3* led to a significant downregulation of IAA biosynthesis rates in the shoot apex, in young developing leaves, and in the root system of dissected seedlings (Figure 3D). When we analyzed root tip-specific IAA biosynthesis in estradiol-induced pMDC7: *AtCKX3* seedlings, we observed a small, but not significant, downregulation of IAA synthesis rates (see Supplemental Figure 1 online). To study if constitutive overexpression of cytokinin oxidase was more effective in downregulating IAA biosynthesis, we used the 35S: *AtCKX1* line, which has a *CKX1* transgene under the control of the constitutive cauliflower mosaic virus 35S promoter. The levels of cytokinin precursors, active cytokinins, and IAA have already been shown to be reduced in this line (Werner et al., 2003). In the root tips of this line, we observed a significant downregulation of IAA biosynthesis compared with the wild type (Figure 3E), indicating that the constitutive downregulation of cytokinin leads to lower IAA synthesis rates.

In a second experiment, we analyzed root-specific IAA biosynthesis in different lines carrying mutations in genes encoding IPT proteins. The *Arabidopsis* genes *IPT1*, *IPT3*, *IPT5*, and *IPT7* are all expressed in different cell types of the root tip and are expected to be important in the biosynthesis of cytokinin in those cells (Miyawaki et al., 2004). *IPT1* is expressed in xylem precursor cells in the procambium, *IPT3* in the vasculature (phloem and pericycle), *IPT5* in the columella root cap and in lateral root primordia, and *IPT7* in the endodermis of the elongation zone. *Arabidopsis* wild-type and *ipt* mutant lines were grown for 6 d in LDs and divided into roots and shoots. Isolated roots were then incubated for 24 h in a medium containing 30% $^2\text{H}_2\text{O}$. We observed a downregulation of IAA biosynthesis in the root apex of the *ipt3* and *ipt7* single mutant lines and in the double and triple mutant lines *ipt3 ipt5-2*, *ipt5-2 ipt7*, and *ipt3 ipt5-2 ipt7* (Figure 4). The quadruple mutant line *ipt1 ipt3 ipt5-2 ipt7* showed the strongest reduction in the rate of IAA biosynthesis. These results

provide further indication that the downregulation of endogenous cytokinin levels leads to lower auxin biosynthesis rates and that cytokinin is an essential regulator of auxin homeostasis in *Arabidopsis*.

Cell Type-Specific Analysis of Auxin Biosynthesis in the Root Apex after Cytokinin Treatment

We then analyzed the rates of IAA biosynthesis in *Arabidopsis* lines expressing green fluorescent protein (GFP) in different root cell types to determine if cytokinin affected auxin biosynthesis more in some cell types than in others. Four different GFP-expressing cell lines were chosen so that all cell types in the root apex were sampled (Figure 5B). We have previously shown that the cell types represented in these lines have a high relative IAA synthesis rate compared with the rest of the root (Petersson et al., 2009). Seedlings were grown on vertical plates as described (Petersson et al., 2009), and intact roots were harvested and incubated for 16 h in a liquid medium containing 30% $^2\text{H}_2\text{O}$, 10 μM t-Z, and 10 μM naphthylphthalamic acid (NPA; to block transport of newly biosynthesized IAA between cells). Protoplast isolation, cell sorting, and IAA biosynthesis measurements were performed as described in Methods. There were no significant differences in IAA biosynthesis rates between the four lines, indicating that all responded similarly in terms of the response to an exogenous cytokinin treatment (Figure 5A).

Transcript Profiling of the Root Apex after Cytokinin Induction of Auxin Biosynthesis

A global transcript profiling of *IPT8/pga22* root tips, comparing untreated with estradiol-induced or cytokinin treated seedlings, was conducted to investigate further the mechanisms behind cytokinin regulation of auxin biosynthesis. The profiling was performed on root tip material as used in our previous IAA measurements (Figures 2, 3E, 4, and 5). A time-course experiment was first conducted to identify any differences in the timing of changes in IAA biosynthesis between the estradiol-induced *IPT8/pga22* plants and exogenous t-Z-treated plants. Whole roots of 7 DAG *IPT8/pga22* seedlings were incubated in a medium containing 30% $^2\text{H}_2\text{O}$ and either 5 μM 17- β -estradiol or 5 μM t-Z for 3, 12, and 24 h, and the rate of IAA biosynthesis was analyzed by GC-MRM-MS in the root apex. A significant increase in the rate of IAA biosynthesis was observed in the t-Z-treated roots at 3 h (see Supplemental Figure 2 online). By contrast, the estradiol-induced roots did not show a significant increase in the rate of IAA biosynthesis until 24 h after incubation. Based on this time course, transcript profiling was performed on *IPT8/pga22* seedlings that were first grown for 7 d in LDs and then incubated for 12 and 24 h with medium containing either 0 or 5 μM 17- β -estradiol or 0 or 5 μM t-Z. After incubation, root tips were collected from the seedlings and transcript profiling was performed using the ATH1 Affymetrix array.

As expected, there was a very strong upregulation of *IPT8* in the *IPT8/pga22* line after estradiol treatment but not after the t-Z treatment (see Supplemental Data Set 1 online). This and the upregulation of a number of genes known to be involved in cytokinin signal transduction, including *AHK4/WOL* and several

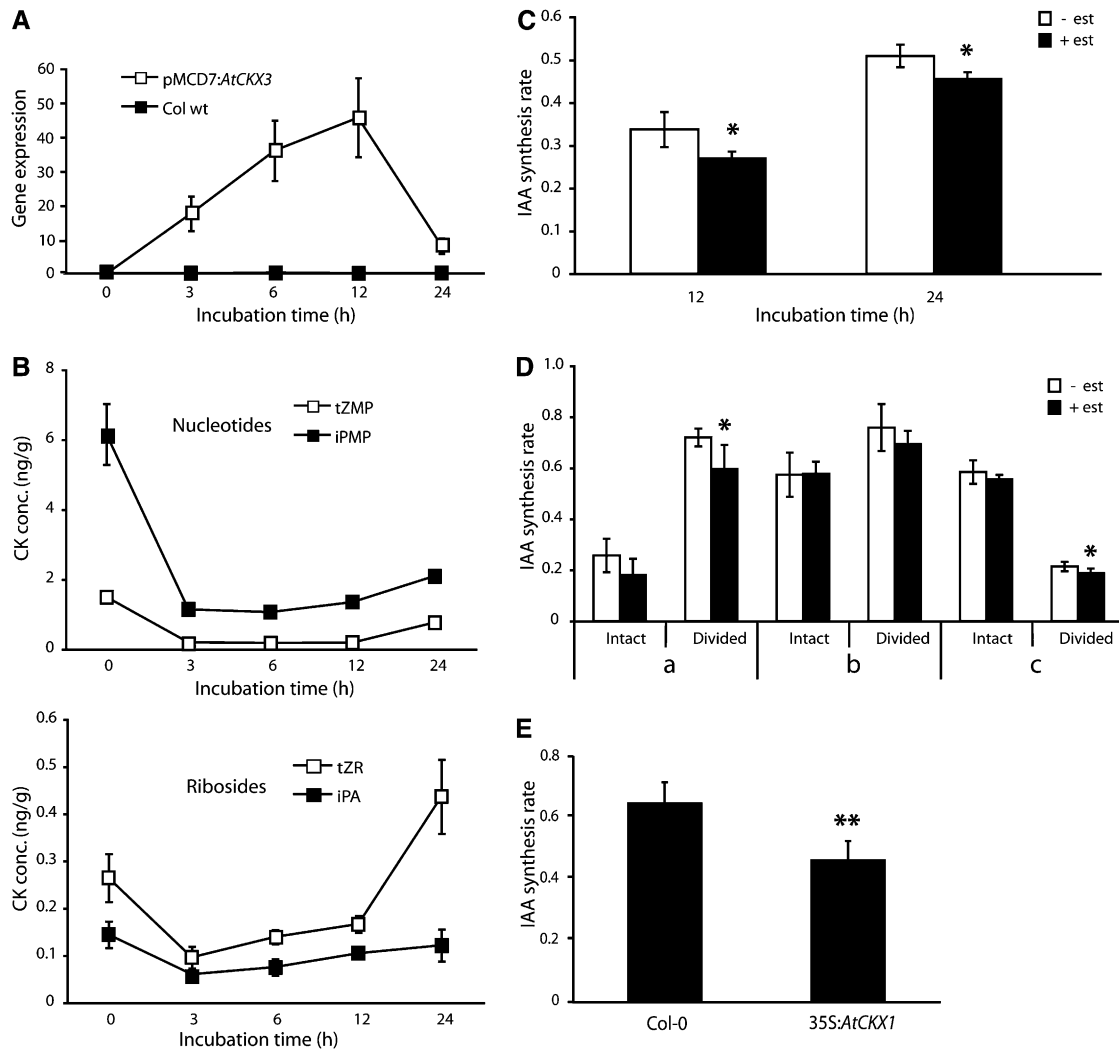


Figure 3. Lowering of Cytokinin Levels Leads to a Downregulation of IAA Biosynthesis.

(A) Analysis of *CKX3* transcript levels by qPCR after incubation of 6 DAG *Arabidopsis* wild-type Columbia and pMDC7:*AtCKX3* seedlings with 5 μ M 17- β -estradiol (est) for 0, 3, 6, 12, and 24 h. Error bars indicate SD ($n = 3$).

(B) Quantification of the nucleotide (tZMP and iPMP) and riboside (tZR and iPA) cytokinin precursors after incubation of 6 DAG pMDC7:*AtCKX3* seedlings with 5 μ M 17- β -estradiol for 0, 3, 6, 12, and 24 h. Error bars indicate SD ($n = 3$).

(C) IAA biosynthesis rate in 6 DAG pMDC7:*AtCKX3* seedlings incubated with medium containing 30% $^2\text{H}_2\text{O}$ with or without 5 μ M 17- β -estradiol for 12 and 24 h. Error bars indicate SD ($n = 3$).

(D) pMDC7:*AtCKX3* seedlings 10 DAG were divided into root and shoot and incubated for 12 h in liquid medium containing 30% $^2\text{H}_2\text{O}$ with or without 5 μ M 17- β -estradiol. The IAA biosynthesis rate was analyzed in the different tissues described in Figure 1A. Error bars indicate SD ($n = 5$).

(E) Col-0 and 35S:*AtCKX1* seedling roots 6 DAG were incubated for 24 h in liquid medium containing 30% $^2\text{H}_2\text{O}$. The IAA biosynthesis rate was analyzed in root tips. Error bars indicate SD ($n = 5$).

Data are mean \pm SD of independent experiments. P values in **(C)** to **(E)** (+ est versus - est) were determined by two-tailed Student's *t* test assuming equal variances (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

cytokinin response regulator genes (*ARRs*), indicated that the treatments were effective. There was also a strong induction of the cytokinin oxidases *CKX3*, *CKX4*, and *CKX5*, suggesting that cytokinin homeostasis is autoregulatory in root tips. Given our previous observations, it was not surprising that the data indicated that several of the genes believed to be involved in IAA metabolism were also affected. The data showed that transcript

levels for the Trp biosynthetic genes *ASA1/WEI2*, *PAT/TRP1*, and *IGPS* were upregulated, as were genes believed to be involved in Trp-dependent IAA biosynthesis, such as *CYP79B2*, *CYP79B3*, *YUCCA6*, and *NIT3*. The data also indicated that transcripts for some putative auxin biosynthesis genes, such as *AMI1*, *YUCCA5*, and a *YUCCA5-like* gene, were downregulated by the treatments (see Supplemental Data Set 1 online).

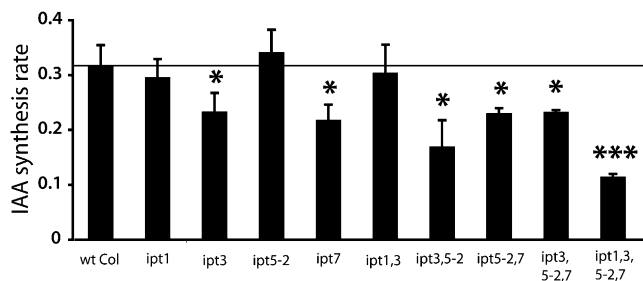


Figure 4. IAA Biosynthesis Is Downregulated in the Root Apex of *ipt* Mutant Lines.

The de novo IAA biosynthesis rate was analyzed in root tips of *ipt* mutant lines after incubation of 6 DAG seedling roots for 24 h with liquid medium containing 30% $^2\text{H}_2\text{O}$. Data are mean \pm SD of three independent experiments ($n = 3$). P values for the mutant lines versus the wild type were determined by two-tailed Student's *t* test assuming equal variances (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

Increased transcript levels were indicated for the IAA conjugating (*GH3.17* and *GH3.9*) and deconjugating (*ILL6*) enzymes, suggesting that a range of IAA homeostatic mechanisms are induced by an increase in cytokinin. The data also indicated upregulation of two genes, *CYP83B1/SUR2* and *SUR1*, that are believed to be involved in indole glucosinolate (IG) biosynthesis. Interestingly, a MYB transcription factor (*ATR1/MYB34*) that has been shown to coregulate several genes in the IAA and IG biosynthetic pathways, including *CYP79B2*, *CYP79B3*, and *CYP83B1/SUR2* (Celenza et al., 2005), was also induced by cytokinin.

The data also indicated that cytokinin regulates the level of transcripts for several genes known to be involved in auxin signal transduction (*ABP1*, *AXR1*, *IAA13*, *IAA17/AXR3*, *IAA29*, *IAA30*, *IAA32*, and *IAA33*) and IAA transport (*PIN2*, *PIN5*, *PIN6*, and *PIN7*), indicating a multilayered interaction between the two hormones. Gene Ontology (GO) analysis was performed for genes with a >1.5 -fold change between treated tissues and control untreated tissues using the GO browser function in Genespring. A range of GO terms was significantly overrepresented (ranked by P value), including cytokinin-mediated signaling pathway, response to cytokinin stimulus, and glucosinolate metabolic process (see Supplemental Data Set 2 online).

Analyses of Auxin-Related Genes by Quantitative PCR

Genes related to auxin biosynthesis, transport, and signaling indicated in the microarray screen as being responsive to cytokinin were analyzed by quantitative PCR (qPCR). To identify genes involved in the primary response to cytokinin, samples were taken earlier after the onset of the treatment than with the microarrays. This shorter time course indicated a rapid response to cytokinin for a number of auxin biosynthesis-related genes. Root tips from 7 DAG wild-type *Arabidopsis* roots were sampled at 0, 0.5, 1, 2, 4, and 6 h after incubation in liquid medium with and without t-Z. *CYP735A2* was used as a positive control, as transcript levels for this gene have previously been shown to increase rapidly in response to elevated cytokinin levels (Takei et al., 2004; see Supplemental Figure 3C online). Transcript levels

for the auxin biosynthesis-related genes *CYP79B2*, *CYP79B3*, *CYP83B1/SUR2*, and *NIT3* were elevated by 2 h after the onset of the cytokinin treatment (Figure 6A). The transcript level of *ATR1/MYB34* was induced by cytokinin treatment even at the earliest sampling, 0.5 h after the onset of the treatment. This supports previous findings (Celenza et al., 2005) and suggests a potential mechanism for the coregulation of genes in the IAA and IG biosynthesis pathways. We also analyzed several other genes believed to be involved in IAA biosynthesis (*ASA1/WEI2*, *PAT1/TRP1*, *IGPS*, *TSA1*, *TAA1*, *AMI1*, *YUCCA3*, *YUCCA5*, *YUCCA6*, *YUCCA7*, *YUCCA8*, *YUCCA9*, *NIT1*, *NIT2*, *NIT4*, and *AAO1* and the *TAA1*-like genes *At4g33070*, *At5g54960*, *At5g01320*, and *At5g01330*) (see Supplemental Figures 3A and 3B online).

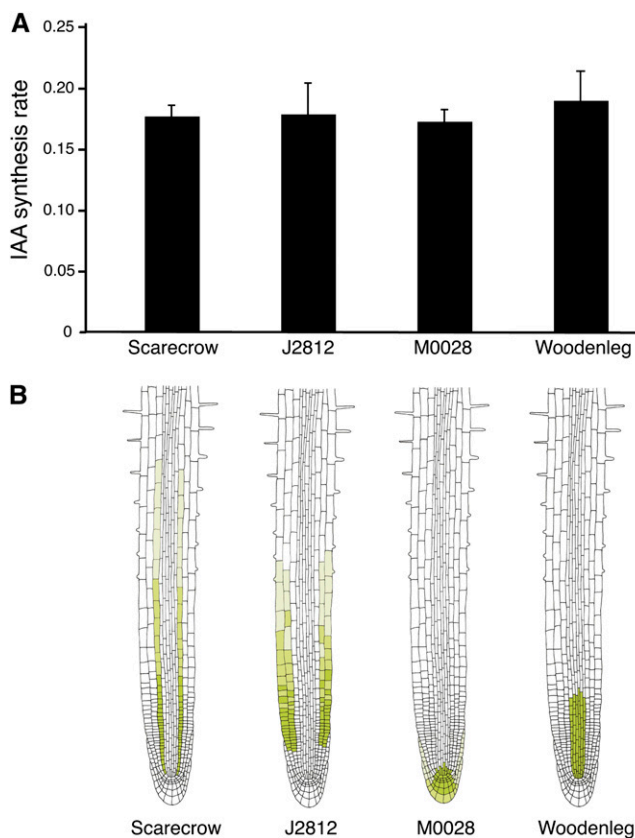


Figure 5. IAA Biosynthesis in Specific Cell Types after Cytokinin Treatment.

(A) Intact roots from 8 DAG *Arabidopsis* seedlings expressing GFP in specific cell types of the root apex were incubated for 16 h in liquid medium (30% $^2\text{H}_2\text{O}$, 30 mM sucrose, 4.4 g MS, and 2.6 mM MES) containing 10 μM NPA and 10 μM t-Z. IAA biosynthesis rates were analyzed in isolated cells from the roots after enzymatic protoplasting and fluorescence-activated cell sorting. A general increase of IAA biosynthesis was observed in all cell types examined, but there were no significant differences between the four GFP-expressing lines. The data were processed in a single factor analysis of variance, which gave a P value of 0.38. Data are mean \pm SD, and the number of biological and technical replicates was between 2 and 4, and 1 and 3, respectively. (B) GFP expression pattern in the *Arabidopsis* lines used in this study.

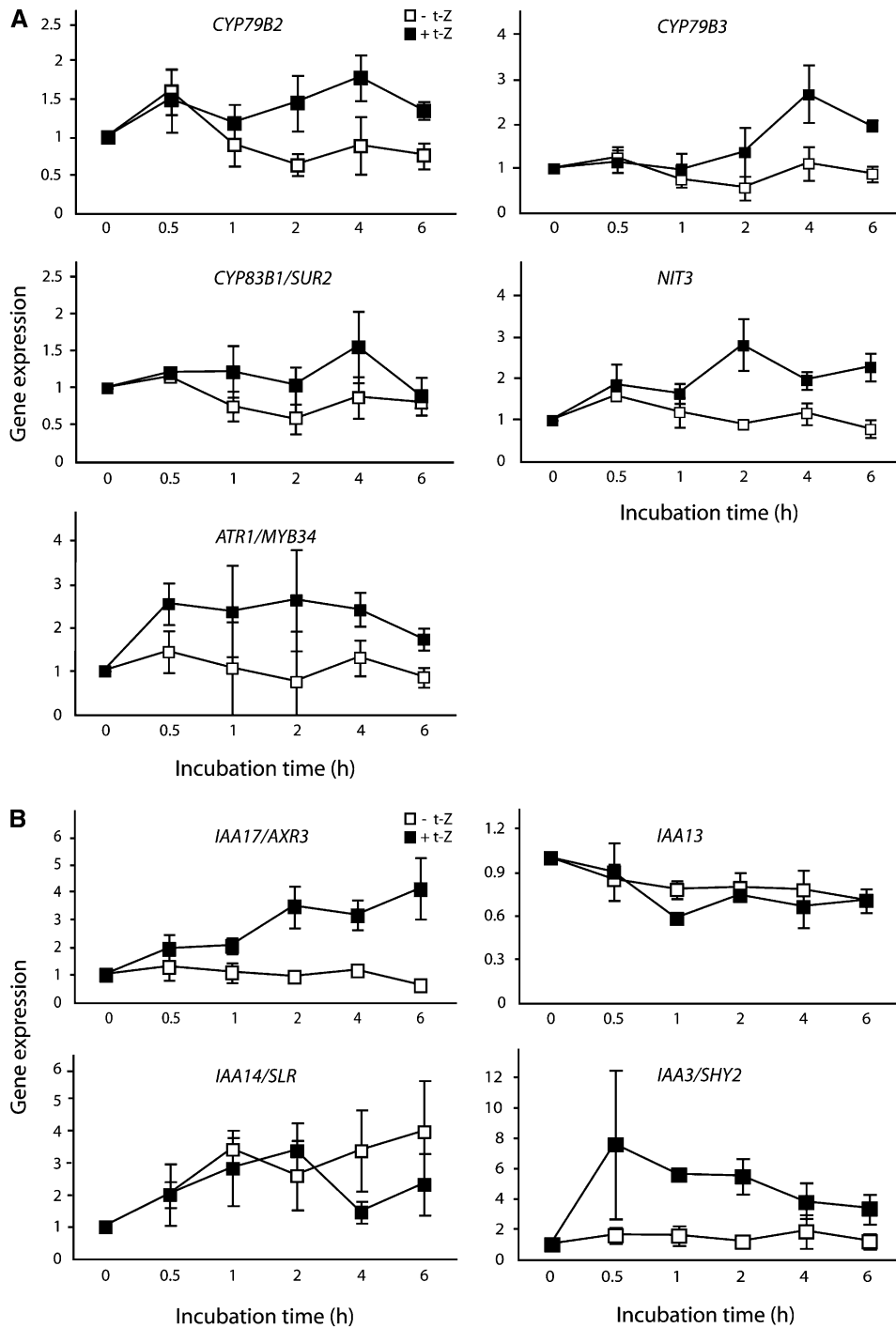


Figure 6. qPCR Analysis of Auxin-Related Genes.

Wild-type *Arabidopsis* (Columbia) seedlings were grown in LD for 7 DAG. The seedlings were then incubated for 0.5, 1, 2, 4, or 6 h in liquid medium with or without 5 μ M t-Z. The root tips were harvested and genes related to auxin biosynthesis (**A**) or auxin signaling (**B**) were analyzed by qPCR. Error bars indicate SE ($n = 3$).

Members of the *YUCCA* and *TAA1*-like gene families were selected for qPCR analysis based on the microarray results and whether or not they were previously shown to be expressed in the roots (Genevestigator). Transcript levels for some genes increased after t-Z treatment (*IGPS*, *NIT1*, and *NIT4*), while others were reduced (*TAA1*, *YUCCA3*, *YUCCA5*, *YUCCA9*, and most of the *TAA1*-like genes).

A rapid increase in transcript abundance was also observed for *AXR3/IAA17* (Figure 6B). Transcript levels for *IAA13*, another *Aux/IAA* indicated by the microarray to be significantly upregulated by cytokinin, did not show this early response (Figure 6B). Transcript abundance for *IAA14/SLR* was downregulated after 4 h. *IAA14/SLR* is involved in lateral root development and has been reported to function as a negative regulator of the DNA binding auxin response element *ARF7/NPH4* (Fukaki et al., 2005). There was no observable early response in *ARF7/NPH4*, *TIR1*, or *IAA30* transcript levels (see Supplemental Figure 3C online). *IAA3/SHY2* and *ARR1* have recently been shown to play a role in root development (Dello Iorio et al., 2008; Moubayidin et al., 2009). We observed a strong and rapid increase in *IAA3/SHY2* transcript levels in response to cytokinin (Figure 6B), but no early response in the *ARR1* transcript levels (see Supplemental Figure 3C online), although the array results indicated that this

gene is downregulated at later time points (see Supplemental Data Set 1 online). Finally, we analyzed the transcript levels for three genes believed to be involved in auxin transport that were indicated by the microarray results to be cytokinin responsive. One of these, *PIN2*, was transiently upregulated, and another, a putative *PIN* gene (*At2g17500*), was shown to be significantly induced in the microarray experiment and rapidly upregulated by cytokinin in the qPCR results, but the third (*PIN5*) did not respond over the time course of the qPCR experiment (see Supplemental Figure 3C online). These data further indicate that auxin transport in the *Arabidopsis* root apex is regulated by cytokinin through the regulation of specific *PIN* genes.

DISCUSSION

Critical plant growth and developmental processes are driven largely by defined changes in hormone levels and responsiveness. Environmental integration is also mediated largely through the biosynthesis and action of hormones. Although an individual hormone may play a predominant role in a particular process, in all processes, elements of the response to one hormone are likely to overlap with elements integral to the response to others.

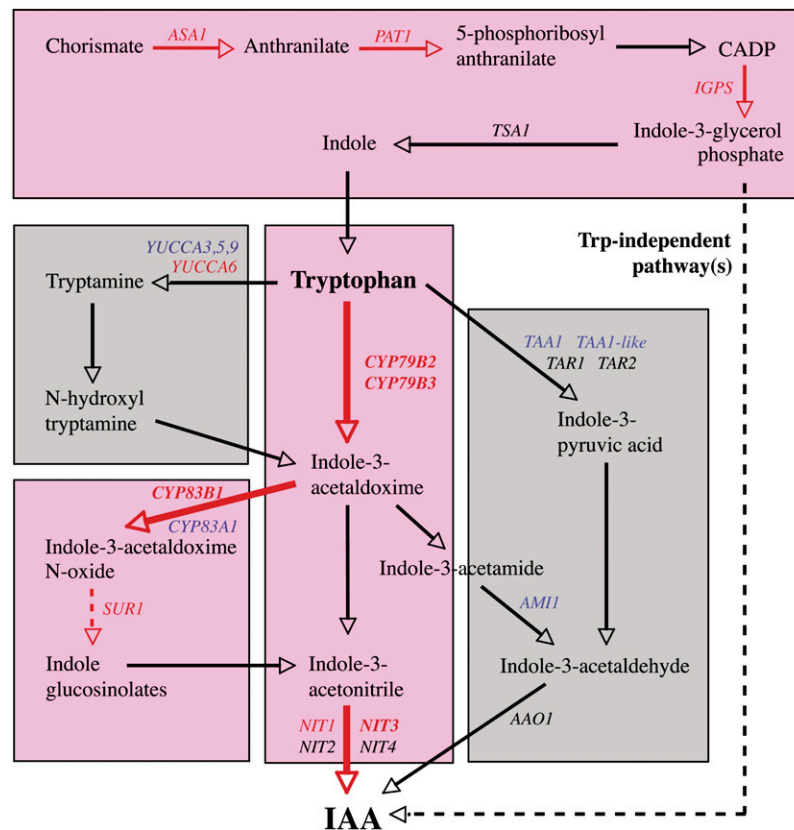


Figure 7. IAA Biosynthesis Pathways in *Arabidopsis* Regulated by Cytokinin.

Putative IAA biosynthesis pathways in *Arabidopsis*, with genes shown to be differentially regulated in the microarray and/or qPCR analysis indicated (up, red; down, blue). Genes and pathways that are more strongly upregulated are indicated with red arrows and a pink background, respectively. A dotted arrow indicates that there is more than one enzymatic step involved in the pathway.

Evidence of interrelationships between hormones has been accumulating over many years. One of the most thoroughly investigated of these is the interplay between auxin and cytokinin (Coenen and Lomax, 1997; Swarup et al., 2002; Moubayidin et al., 2009). Both hormones are key regulators of critical processes, such as organogenesis, embryogenesis, and root and shoot meristem function. We have previously shown that one way that auxin and cytokinin levels are regulated is through the auxin-induced downregulation of cytokinin biosynthesis (Nordström et al., 2004). We show here that, conversely, cytokinin induces auxin biosynthesis and increases steady state auxin levels in young, developing tissues in the root and shoot of *Arabidopsis*. Combined, the data suggest that a feedback homeostasis loop exists to regulate the relative levels of auxin and cytokinin in plants.

Both auxin and cytokinin are involved in the regulation of root growth in response to environmental variables (Osmont et al., 2007). Clearly, mechanisms need to exist to alter the levels and/or responsiveness to the hormones to elicit the appropriate environmental response. Although auxin levels and concentration maxima are maintained partially via the active transport of the hormone (Robert and Friml, 2009), high levels of auxin are produced directly in the root and shoot apices (Ljung et al., 2001, 2005; Petersson et al., 2009). *IPT* gene expression patterns suggest that cytokinin is also biosynthesized throughout the root apex (Miyawaki et al., 2004).

In this study, we have shown that the induction of a transgene encoding the cytokinin biosynthesis protein, IPT8, leads to a rapid upregulation of IAA biosynthesis in the shoot apex, young leaves, and the root system. Older, fully developed leaves did not show a strong cytokinin induction of IAA, except in leaves treated with very high levels of active cytokinins. It remains to be determined if these high levels are physiologically relevant. However, the data suggest that the ability of cytokinin to induce the biosynthesis and increase the levels of IAA is particularly important in actively growing tissues (Figures 1B and 1C). In the cytokinin hypersensitive *arr3 arr4 arr5 arr6* line, the steady state IAA concentration in the root tip remained unchanged from the wild type. However, the rate of IAA biosynthesis was higher in this line than in the wild type prior to the cytokinin treatment, and it had an augmented response to the hormone compared with the wild type (Figure 2). This suggests that a hypersensitivity to both endogenous and exogenous cytokinin increases the ability of cytokinin to induce auxin biosynthesis. Whereas *ahp1 ahp2 ahp3 ahp4* also had an increased rate of auxin biosynthesis compared with the wild type prior to cytokinin treatment, in contrast with the wild type and the *arr3 arr4 arr5 arr6* line, the rate of IAA biosynthesis declined in response to cytokinin in the *ahp1 ahp2 ahp3 ahp4* line. This suggests that the relationship between cytokinin and auxin is fundamentally altered in this mutant. The data indicate clearly that the regulation of auxin biosynthesis is intimately dependent on normal cytokinin responsiveness in root tips.

Many plant hormone signaling components have been shown to be regulated by more than one hormone. To determine whether cytokinin led directly to an increase in the auxin pool size and rate of biosynthesis or achieved this through auxin signaling components, we analyzed IAA biosynthesis and levels

in auxin perception and signal transduction mutants. In the *axr3-1* mutant, both the IAA pool size and auxin biosynthetic rates were increased in comparison to the wild type prior to cytokinin treatment (Figure 2). This suggests that, just as in the cytokinin response mutants, auxin homeostasis is altered in this mutant. As the normal auxin-stimulated degradation of *AXR3/IAA17* is perturbed in the *axr3-1* mutant, it is possible that this protein plays an important role in a feedback loop mechanism regulating IAA levels and biosynthesis rates. Interestingly, cytokinin did not alter the pool size or the rate of auxin biosynthesis in the *axr3-1* mutant, suggesting that an increase in the rate of IAA biosynthesis requires the stabilization and/or increased level of *AXR3/IAA17* expression. Stabilization of the protein in the *axr3-1* mutant may constitutively activate the molecular response mechanism required for cytokinin induction of auxin biosynthesis. Both the microarray and qPCR analyses showed that cytokinin increased the level of *AXR3/IAA17* expression. In contrast with the *axr3-1* mutant, the rate of IAA biosynthesis in the auxin-insensitive mutants *axr1-3* and *axr1-12* was the same as the wild type prior to the cytokinin treatment. However, both had an increased response to cytokinin compared with the wild type. This augmentation of cytokinin-responsive auxin biosynthesis suggests that in the wild type, there is component of feedback inhibition of elevated auxin biosynthesis.

The initial data showing that auxin biosynthesis rapidly increased in response to elevated ectopic or exogenous cytokinin suggested the possibility that endogenous levels of the hormones are similarly interrelated in rapidly growing tissues. In the seedlings with inducible *CKX3* expression, treatment with 17- β -estradiol led to a reduction in cytokinin levels between 3 and 12 h after the onset of the treatment (Figure 3B). This correlated with a downregulation of IAA biosynthesis rates after 12 and 24 h of estradiol incubation (Figures 3C and 3D), providing a first

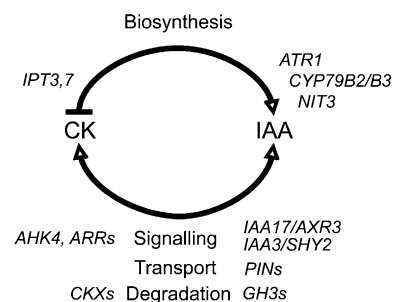


Figure 8. A Model of the Homeostatic Feedback Loop Regulating IAA and Cytokinin Concentrations in the *Arabidopsis* Root Apex.

Cytokinin (CK) acts as a positive regulator of auxin (IAA) biosynthesis in the root apex, possibly by *ATR1* coinduction of the *CYP79B2/B3* and *NIT3* genes. On the other hand, auxin can act as a repressor of cytokinin biosynthesis via downregulation of specific *IPT* genes. Both auxin and cytokinin signaling is important for these interactions, and our data suggest that specific *auxin/IAA* genes (*IAA17/AXR3* and *IAA3/SHY2*) and cytokinin signaling components (*AHK4* and *ARRs*) are involved. Changes in auxin transport (PIN efflux carriers) and in the degradation of auxin (*GH3s*) and cytokinin (*CKXs*) may also play important roles in the modulation of the response.

indication of the involvement of endogenous cytokinin in the regulation of endogenous auxin biosynthesis in young tissues. Further evidence for an interrelationship between endogenous cytokinin and auxin biosynthesis was provided by the observation of lower auxin biosynthesis rates in the root apex of the constitutive *CKX1* overexpressor line (Figure 3E). A third line of evidence was provided by the analysis of IAA levels and biosynthesis rates in single and multiple gene mutants for cytokinin biosynthesis *IPT* genes. All showed reduced rates of endogenous auxin biosynthesis in the root tip (Figure 4) with the reduction varying with the different mutant combinations. As the individual *IPT* genes are expressed in different cell types, we tested whether the different levels of reduction in auxin biosynthesis in the various *ipt* mutant combinations was related to a cell specificity for the cytokinin induction of auxin biosynthesis. An analysis of cell-specific responses revealed that cytokinin had a similar capacity to induce auxin biosynthesis in all cell types tested (Figure 5).

The putative pathways of IAA biosynthesis in *Arabidopsis* (Figure 7) include many steps for which the enzymes responsible are still unknown (Woodward and Bartel, 2005; Normanly, 2010). Our results have shown that several of those that have been clearly identified are regulated by cytokinin. The data suggest that cytokinin is capable of inducing a rapid upregulation of the Trp biosynthesis pathway through an increase in the precursor for Trp-dependent IAA biosynthesis and an upregulation of the pathway from Trp via indole-3-acetaldoxime and indole-3-acetonitrile to IAA (Figure 7). The data also suggest that cytokinin leads to an induction of the related IG pathway. The MYB-type transcriptional regulator ATR1/MYB34 has been shown to co-regulate a number of genes involved in Trp-dependent IAA and IG biosynthesis (Celenza et al., 2005). *ATR1/MYB34* transcript levels were increased at the first time point examined, 0.5 h after the onset of the cytokinin treatment, suggesting that the encoded protein regulates the other IAA and IG biosynthesis genes, *CYP79B2*, *CYP79B3*, *CYP83B1/SUR2*, and *NIT3*, that were also rapidly upregulated by the hormone (Figures 6A and 7).

We provided evidence that cytokinin regulates auxin responses by altering the rate of biosynthesis and steady state levels of auxin. Transcript levels of several auxin-signaling genes were affected by cytokinin treatment. For example, there were strong increases in the levels of *IAA17/AXR3* and *IAA3/SHY2* transcripts in response to cytokinin, and transcripts for both these genes were shown to be increased after only 30-min incubations (Figure 6B). *IAA17/AXR3* and *IAA3/SHY2* are both members of the auxin/IAA family of auxin response regulators that usually work as auxin response repressors (Chapman and Estelle, 2009). Our data show that both auxin and cytokinin are able to induce the expression of *IAA17/AXR3* and *IAA3/SHY2*. Cytokinin induction of both auxin biosynthesis and the expression of putative repressors of the auxin response indicate a complex homeostatic interrelationship between the two hormones. Moubayidin et al. (2009) recently suggested that *IAA3/SHY2* is involved in auxin–cytokinin interactions in the root apical meristem. Our data and that of other groups (Moubayidin et al., 2009; Ruzicka et al., 2009) have shown that cytokinin can also modulate auxin transport in the root via the regulation of *PIN* gene expression. There is strong evidence that this is achieved

through the regulation of *IAA3/SHY2* expression (Dello Iorio et al., 2008).

As might be expected for two hormones with a strong capacity to affect critical plant growth and developmental processes, it is clear from the data presented here that complex mechanisms have evolved for regulating the levels of and the responsiveness to the two hormones. We have shown that the rate of auxin biosynthesis and auxin steady state levels are regulated by cytokinin and that these regulatory mechanisms work through both auxin and cytokinin signaling. This regulation is likely to have evolved to ensure that hormone levels are optimal for cell- and tissue-specific developmental and environmental response processes. We propose a model for auxin/cytokinin interactions in the root apex in which cytokinin acts as a positive regulator of auxin biosynthesis and auxin as a negative regulator of cytokinin biosynthesis (Figure 8). This regulatory loop is mediated via cytokinin and auxin signal transduction and through the transcriptional control of specific cytokinin and auxin biosynthesis genes. Our data have shown that other homeostatic mechanisms, such as catabolism/conjugation and hormone transport, are also likely to participate in the crosstalk that modulates the essential intracellular levels of and relative responses to auxin and cytokinin.

METHODS

Plant Material and Growth Conditions

Seeds of all lines used in this study (wild-type *Arabidopsis thaliana* ecotypes Columbia and Wassilewskija [Ws]; the European Arabidopsis Stock Centre [NASC]; the lines Woodenleg:GFP, Scarecrow:GFP [Philip Benfey], J2812:GFP, M0028:GFP [NASC], and 35S:*AtCKX1* [Thomas Schmülling]; the mutant lines *arr3 arr4 arr5 arr6*, *ahp1 ahp2 ahp3 ahp4* [Joe Kieber], *axr3-1*, *axr3-3*, *axr1-3*, and *axr1-12* [Ottoline Leyser], *tir1-1* [Mark Estelle], *ipt1*, *ipt3*, *ipt5-2*, *ipt7*, *ipt1 ipt3*, *ipt3 ipt5-2*, *ipt5-2 ipt7*, *ipt3 ipt5-2 ipt7*, and *ipt1 ipt3 ipt5-2 ipt7* [Tatsuo Kakimoto], and the inducible lines *IPT8/pga22* [Nam-Hai Chua] and pMDC7:*At-CKX3*) were surface sterilized, plated on solid medium (1× Murashige and Skoog [MS] medium [Duchefa], 1% sucrose, and 1% agar [Merck], pH 5.7), and vernalized at 4°C for 3 d prior to germination. The mutant and transgenic lines are all in the Columbia background except for *IPT8/pga22* (Ws). The GFP lines used in this study were as described by Petersson et al. (2009). For collection of larger amounts of root material for cell sorting, transcript profiling, and qPCR analysis, seeds were sown at high density (~500 seeds per row) on nylon mesh (Sefar Nitex 03-110/47) on solid MS medium. After vernalization, all plates were placed vertically under 150 μE light in LD conditions (16 h light/8 h darkness) at 22°C.

Construction of an Inducible Cytokinin Oxidase Line

For the inducible cytokinin oxidase line, the coding sequence of *CKX3* was amplified with the primers CKX3-F1 (5'-CACCAATGGCGAGTTA-TAATCTTCGTTC-3') and CKX3-R1 (5'-CTACTAACTCGAGTTTATTTTT-TGA-3'). The PCR product was cloned into the estradiol-inducible binary vector pMDC7 (http://www.unizh.ch/botinst/Devo_website/curtisvector/7cassetteB.pdf) via pTOPO TA GATEWAY (Invitrogen). *Arabidopsis* plants were transformed by *Agrobacterium tumefaciens*-mediated floral dip transformation. Analyses of transcript levels by qPCR analysis and quantification of cytokinins were performed on 6 DAG pMDC7:*AtCKX3* and wild-type Columbia seedlings as described. The seedlings were incubated for 0, 3, 6, 12, and 24 h in liquid medium with or without 5 μM 17-β-estradiol (Sigma-Aldrich). For each sample, tissues from 10 to 20

seedlings were pooled, frozen in liquid nitrogen, and stored at -80°C until extraction and purification. Samples were collected in triplicates. The pMDC7:*AtCKX3* line has been deposited at the NASC (<http://Arabidopsis.info/>) under the name N9911.

IAA Biosynthesis Measurements

IPT8/pga22 seedlings were grown on vertical plates in LD for 10 d as earlier described. Intact seedlings or seedlings divided into root and shoot were transferred to Petri dishes and incubated first for 12 h (LD with gentle shaking) in liquid medium ($1\times$ MS and 1% sucrose, pH 5.7) containing $5\ \mu\text{M}$ 17- β -estradiol to induce cytokinin biosynthesis and then for 24 h in medium containing $5\ \mu\text{M}$ 17- β -estradiol and 30% $^2\text{H}_2\text{O}$ (Cambridge Isotope Laboratories). As a control, *IPT8/pga22* seedlings were incubated without estradiol in the medium.

Arabidopsis wild-type Ws seedlings 10 DAG were divided into root and shoot and incubated for 24 h in liquid medium containing 30% $^2\text{H}_2\text{O}$ with or without $5\ \mu\text{M}$ 17- β -estradiol, $10\ \mu\text{M}$ BAP, or $10\ \mu\text{M}$ c/t-Z added to the medium. pMDC7:*AtCKX3* seedlings 6 and 10 DAG were incubated for 12 and 24 h in liquid medium containing 30% $^2\text{H}_2\text{O}$ with or without $5\ \mu\text{M}$ 17- β -estradiol. After incubation, the 10 DAG seedlings were rinsed in distilled water and divided into (a) apex and young, developing leaves (leaf 3 and smaller), (b) the first two true leaves, and (c) the root system (Figure 1A). For each sample, tissues from 10 seedlings were pooled, frozen in liquid nitrogen, and stored at -80°C until extraction and purification.

Arabidopsis wild-type, 35S:*AtCKX1*, and *ipt* mutant lines were grown for 6 d in LDs and divided into root and shoot. Isolated roots were then incubated for 24 h in medium containing 30% $^2\text{H}_2\text{O}$. *Arabidopsis* wild-type and auxin and cytokinin signal transduction mutants were grown for 6 d in LDs and divided into root and shoot. Isolated roots were then incubated for 24 h in medium containing 30% $^2\text{H}_2\text{O}$ with or without $5\ \mu\text{M}$ t-Z. *IPT8/pga22* seedlings were grown for 7 DAG in LD and divided into root and shoot. Isolated roots were incubated for 3, 12, and 24 h in medium containing 30% $^2\text{H}_2\text{O}$ with or without $5\ \mu\text{M}$ 17- β -estradiol or $5\ \mu\text{M}$ t-Z. After incubation, the roots were rinsed in distilled water, and 2 mm of the root apex was collected under the stereomicroscope. Fifty root sections were pooled for each sample, frozen in liquid nitrogen, and stored at -80°C until extraction and purification. Samples were purified according to Andersen et al. (2008) and analyzed by GC-MRM-MS as described (Ljung et al., 2005). The IAA biosynthesis rate was calculated from the measured incorporation of one to three deuterium atoms into the IAA molecule, and corrections were made for the contribution of natural isotopic abundances to a mass-to-charge ratio (m/z) of 203 to 205. The amount of endogenous IAA (labeled + unlabeled) was calculated by combining the corrected areas of the ions from deuterium labeled IAA (m/z 203 to 205) with that of unlabeled IAA (m/z 202).

Cell Type-Specific IAA Biosynthesis Measurements

The *Arabidopsis* lines Woodenleg:GFP, Scarecrow:GFP, J2812:GFP, and M0028:GFP were grown on vertical plates in LDs for 8 d as described by Petersson et al. (2009). Seedling roots were then excised ~ 10 mm from the root/shoot junction and incubated for 16 h in liquid medium containing 30 mM sucrose, 4.4 g MS, 2.6 mM MES, $10\ \mu\text{M}$ NPA, and $10\ \mu\text{M}$ t-Z in 30% $^2\text{H}_2\text{O}$. After incubation, the root tissue was treated with cell wall-degrading enzymes, and protoplasts were isolated as described (Petersson et al., 2009). Cell sorting was performed on a FACSAria equipped with a $100\text{-}\mu\text{m}$ nozzle (Becton Dickinson) as described (Birbaum et al., 2005; Petersson et al., 2009). Intact root protoplasts were identified based on forward and side scatter, and GFP positive and negative cells were then separated according to their GFP emission and auto fluorescence, after excitation by the 488-nm laser. Data were processed using the FACSDiVa 5.0 software. The samples were kept at 4°C during the whole sorting procedure, and 150,000 to 300,000 proto-

plasts were collected for each technical replicate. The protoplasts were suspended in BD FACsFlow buffer (Becton Dickinson) prior to extraction. $^{13}\text{C}_6$ -IAA internal standard (125 pg) was added, and the sample was frozen to rupture the plasma membrane before purification by solid phase extraction, and IAA biosynthesis measurements were performed by GC-MRM-MS as described (Ljung et al., 2005; Petersson et al., 2009). The numbers of biological and technical replicates analyzed were as follows: Woodenleg:GFP (3;3,3,2), Scarecrow:GFP (2;3,3), J2812:GFP (2;2,3), and M0028:GFP. (4;1,1,2,2).

Cytokinin Quantification

IPT8/pga22 seedlings were grown on vertical plates in LDs for 10 d. Intact seedlings were transferred to Petri dishes and incubated for 24 h (LD with gentle shaking) in liquid medium ($1\times$ MS and 1% sucrose, pH 5.7) containing $5\ \mu\text{M}$ 17- β -estradiol to induce cytokinin biosynthesis. As a control, *IPT8/pga22* seedlings were incubated without estradiol in the medium. After incubation, the seedlings were rinsed in distilled water and divided into (a) apex and young, developing leaves, (b) the first two true leaves, and (c) the root system (Figure 1A). For each sample, tissues from 20 seedlings were pooled, frozen in liquid nitrogen, and stored at -80°C until extraction and purification. Samples were purified, derivatized by propionylation, and quantified by liquid chromatography-mass spectrometry analysis in the MRM mode on a Micromass Quattro Ultima mass spectrometer according to Nordström et al. (2004). Chromatographic separation was performed on a reversed phase analytical column ($150\times 1\ \text{mm}$ BetaMax Neutral, $5\text{-}\mu\text{m}$ particle size; Thermo Hypersil). All data were processed by Masslynx software (version 4.0; Waters).

Transcript Profiling

IPT8/pga22 seedlings were grown on vertical plates in LDs for 7 d. The seedlings were then incubated directly on the plate for 12 or 24 h (LD with gentle shaking) in liquid medium ($1\times$ MS and 1% sucrose, pH 5.7) containing either $5\ \mu\text{M}$ 17- β -estradiol or $5\ \mu\text{M}$ t-Z. Control samples were incubated for 12 or 24 h without cytokinins in the medium. Root tips (~ 5 mm long, 10 mg root tissue/sample collected in triplicates) were harvested by cutting the roots with a scalpel on the nylon mesh. Each sample was immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. The methods and statistical analysis are described in detail in Supplemental Methods online.

qPCR Analysis

To quantify the expression of genes related to auxin and cytokinin biosynthesis and signaling in root tips of 7 DAG wild-type *Arabidopsis* (Columbia) seedlings incubated with or without $5\ \mu\text{M}$ t-Z, qPCR analysis was performed with the SYBR-Green fluorochrome technique. We chose *TIP41-like* as the best reference gene for our conditions using GeNorm (Vandesompele et al., 2002). The methods and primer sequences are described in detail in Supplemental Methods and Supplemental Table 3 online. The expression data presented in the figures are means \pm SE of three biological replicates.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ARR3* (At1G59940), *ARR4* (At1G10470), *ARR5* (At3G48100), *ARR6* (At5G62920), *AHP1* (At3G21510), *AHP2* (At3G29350), *AHP3* (At5G39340), *AHP4* (At3G16360), *AXR1* (At1G05180), *AXR3* (At1G04250), *TIR1* (At3G62980), *CKX1* (At2G41510), *CKX3* (At5G56970), *IPT1* (At1G68460), *IPT3* (At3G63110), *IPT5* (At5G19040), *IPT7* (At3G23630), *IPT8* (At3G19160), and *TIP4-like* (At4G34270). The pMDC7:*AtCKX3* line

has been deposited at the NASC (<http://Arabidopsis.info/>) under the name N9911. The NASCArrays experiment reference number is NASCARRAYS-364, and the data have been donated to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/>) with the GEO accession number GSE20231. Accession numbers for the auxin- and cytokinin-related genes identified in the array data and discussed in the text can be found in Supplemental Data Set 1 online. Accession numbers for genes analyzed by qPCR can be found in Supplemental Table 3 online.

Author Contributions

K.L. and G.S. conceived the project. K.L., S.A.G., N.G., and S.M. designed, performed, and analyzed the microarray experiment. B.J., K.L., and S.A.G. designed, performed, and analyzed the qPCR experiments. B.J. constructed the inducible *CKX3* line. K.L. and S.V.P. designed, performed, and analyzed the IAA biosynthesis measurements. P.T. and K.D. performed the cytokinin analyses. K.L. and B.J. wrote the manuscript. All authors contributed to the interpretation of results and edited the manuscript.

Supplemental Data

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

Supplemental Figure 1. Root Tip-Specific IAA Biosynthesis after Induction of the *CKX3* Gene.

Supplemental Figure 2. Timing in the Induction of IAA Biosynthesis by Cytokinins.

Supplemental Figure 3. qPCR Analysis of Genes Related to Auxin and Cytokinin Biosynthesis, Signaling, and Transport after Cytokinin Treatment.

Supplemental Table 1. Cytokinin Concentration in Uninduced and Induced *IPT8/pga22* Seedlings.

Supplemental Table 2. Cytokinin Concentration in Uninduced and Induced *pMCD7:AtCKX3* Seedlings.

Supplemental Table 3. Oligonucleotide Primers for Genes Analyzed by qPCR.

Supplemental Data Set 1. Expression of Auxin- and Cytokinin-Related Genes in the ATH1 Affymetrix Microarray.

Supplemental Data Set 2. Gene Ontology Analysis.

Supplemental Methods. Microarray Analysis and qPCR Analysis.

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