

Cytokinin signaling regulates cambial development in poplar

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Although a substantial proportion of plant biomass originates from the activity of vascular cambium, the molecular basis of radial plant growth is still largely unknown. To address whether cytokinins are required for cambial activity, we studied cytokinin signaling across the cambial zones of 2 tree species, poplar (*Populus trichocarpa*) and birch (*Betula pendula*). We observed an expression peak for genes encoding cytokinin receptors in the dividing cambial cells. We reduced cytokinin levels endogenously by engineering transgenic poplar trees (*P. tremula* × *tremuloides*) to express a cytokinin catabolic gene, *Arabidopsis* *CYTOKININ OXIDASE 2*, under the promoter of a birch *CYTOKININ RECEPTOR 1* gene. Transgenic trees showed reduced concentration of a biologically active cytokinin, correlating with impaired cytokinin responsiveness. In these trees, both apical and radial growth was compromised. However, radial growth was more affected, as illustrated by a thinner stem diameter than in WT at same height. To dissect radial from apical growth inhibition, we performed a reciprocal grafting experiment. WT scion outgrew the diameter of transgenic stock, implicating cytokinin activity as a direct determinant of radial growth. The reduced radial growth correlated with a reduced number of cambial cell layers. Moreover, expression of a cytokinin primary response gene was dramatically reduced in the thin-stemmed transgenic trees. Thus, a reduced level of cytokinin signaling is the primary basis for the impaired cambial growth observed. Together, our results show that cytokinins are major hormonal regulators required for cambial development.

cambial activity | cambium | secondary development | *Populus* | *CYTOKININ OXIDASE*

In plants, development of vascular tissues is unique because of its dynamic nature. During embryogenesis, a continuum of provascular tissue is evident between the shoot and root apical meristems. Soon after germination, a subset of these provascular cells differentiates into 2 conductive tissue types, xylem and phloem. Between the xylem and phloem, however, some meristematic cells persist through primary development. On initiation of secondary development, a lateral meristem, vascular cambium, is derived from these procambial cells, together with interfascicular cells in shoots and pericycle cells in root. Secondary vascular xylem and phloem are subsequently produced via cell divisions taking place in the cambium.

Compared with apical meristems, our knowledge about the genetic control of cambium is much less complete. Previously, a radially oriented gradient of basipetal auxin transport has been shown to be present across the cambial zone (1, 2) in accordance with specific expression patterns of auxin signaling-related genes in the region (3). In classic hormone treatment studies, apically applied exogenous auxin was able to reactivate cambium in decapitated shoots (4, 5). Recently, using a transgenic approach based on down-regulating auxin signaling, it was demonstrated that auxin is required for cell proliferation and cell differentiation during cam-

bial development (6). Aside from auxin, several other hormones, including cytokinin (7, 8), gibberellin (9, 5), and ethylene (10), have been implicated in control of cambial activity because of their stimulatory effect on cell division upon hormone treatment. However, there is no indication as to whether these hormones are normally required for cambial activity.

Cytokinin responses in plants are mediated by a signal transduction pathway consisting of components characteristic of bacterial 2-component molecules (11, 12). They have been shown to function in opposite modes, depending on the developmental context, in regulation of apical meristem activity. Based on various genetic and molecular studies (13–17), including systemic overexpression of a catabolic *CYTOKININ OXIDASE* (*CKX*) gene, it has become evident that in the shoot, apical meristem cytokinins appear to promote cell proliferation. In contrast, in the root, apical meristem cytokinins appear to inhibit root elongation (14, 16, 18). The negative effect of cytokinins on root development has recently been connected to their function in cell differentiation. Reduction in root cytokinin level delays cell differentiation, leading to a longer proximal meristem zone, and thus enhancement of the root growth (19).

Considering vascular development, cytokinin signaling is required for the pluripotent identity of the procambial cell files during the primary phase of *Arabidopsis* root development (20–22). Furthermore, cytokinins appear to be required for proliferation of the vascular cell files during primary vascular development in both the root and shoot (14, 20, 21).

To address the mode of cytokinin function in the secondary meristem, vascular cambium, we studied cytokinin signaling during cambial development in the trunks of 2 hardwood tree species, poplar (*Populus trichocarpa*) and silver birch (*Betula pendula*). For functional studies, we engineered transgenic poplar trees (*P. tremula* × *tremuloides*) to ectopically express a cytokinin-degrading enzyme in the cambial zone for the purpose of repressing cambial

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [*Betula pendula* *CYTOKININ RECEPTOR 1* (*BpCRE1*) genomic EU583454, cDNA EU583455; *HISTIDINE KINASE 2* (*BpHK2*) cDNA EU583456; *HISTIDINE KINASE 3* (*BpHK3*) cDNA EU583457; and *TUBULIN ALPHA* (*BpTUA*) cDNA FJ228477].

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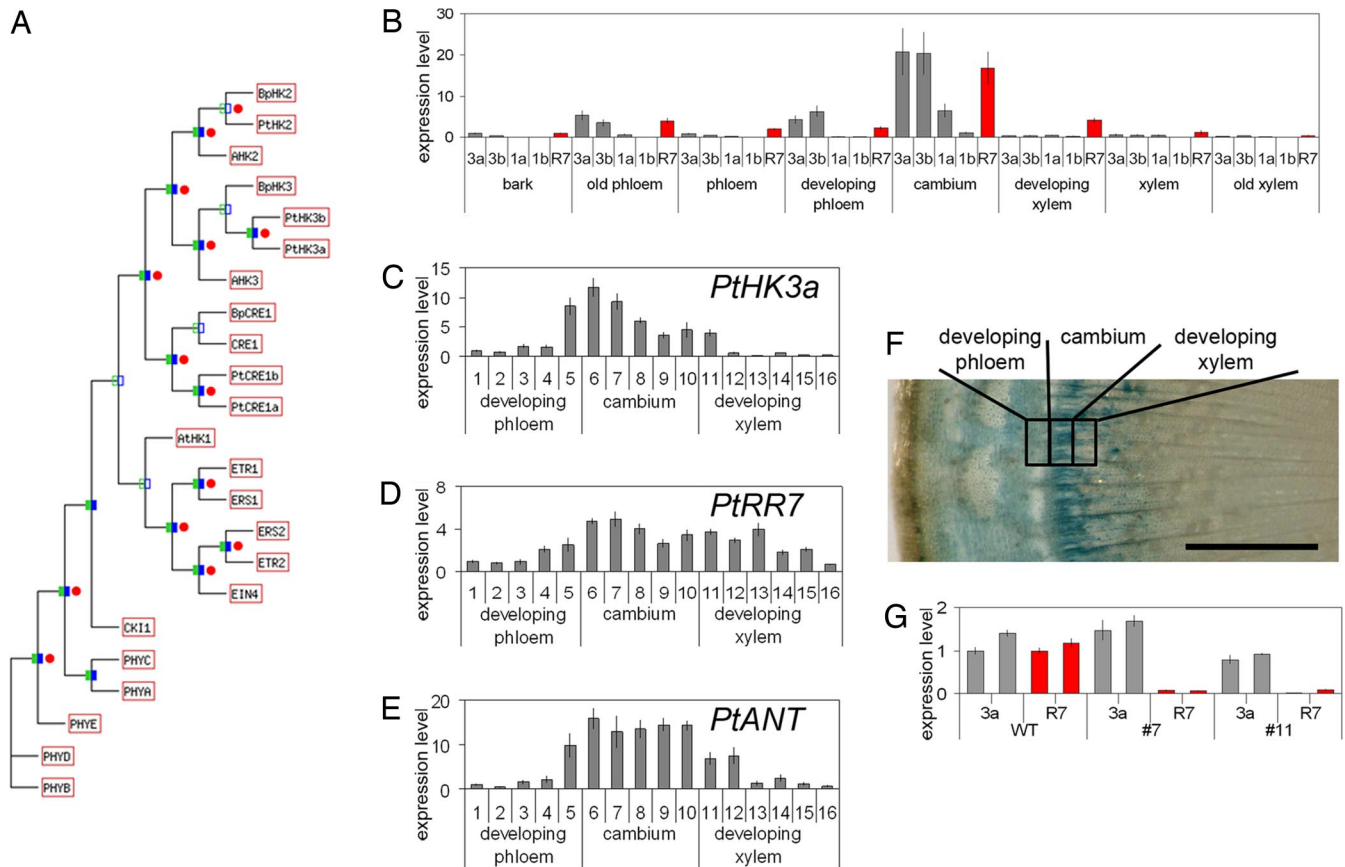


Fig. 1. Expression of poplar and birch cytokinin receptor genes peaks in the cambial zone of tree trunk. (A) Phylogenetic tree of *Arabidopsis* 2-component regulators, including CRE family genes *WOL/CRE1*, *AHK2* and *AHK3*, together with CRE family receptors from *B. pendula* (*BpCRE1*, *BpHK2*, and *BpHK3*) and *P. trichocarpa* (*PtCRE1a*, *PtCRE1b*, *PtHK2*, *PtHK3a*, and *PtHK3b*). The maximum-likelihood tree is based on an alignment of amino acid sites. Filled green/blue boxes indicate groups with bootstrap support >75%, and open boxes indicate groups with less. Where tree topologies agree, red circles indicate >75% bootstrap support from parsimony analysis of the same alignment. (B) Expression of poplar cytokinin receptor genes *PtCRE1a* (1a), *PtCRE1b* (1b), *PtHK3a* (3a), and *PtHK3b* (3b), together with an A-type response regulator *PtRR7* (R7), across the *P. trichocarpa* trunk by qRT-PCR. Receptor expression is given relative to the *PtHK3a* level in bark and *PtRR7* relative to itself in bark (error bars = SE). Expression of *PtHK3a* (C), *PtRR7* (D), and *PtANT* (E) across the *P. trichocarpa* cambial zone in 16 24- μ m sections. Expression is given relative to the developing phloem section 1. (F) Expression of *pBpCRE1::GUS* peaks in the cambial zone of *P. tremula* \times *tremuloides* stem. (Scale bar: 1 mm.) (G) Expression of *PtHK3a* and *PtRR7* in whole-stem samples of WT and *pBpCRE1::AtCKX2* transgenic *P. tremula* \times *tremuloides* lines 7 and 11. Expression is given relative to the WT. Two trees were analyzed per line.

cytokinin signaling. Our results indicate that cytokinins are major hormonal regulators required for cambial development.

Results

The CRE Cytokinin Receptor Gene Family Is Conserved Between Herbaceous and Woody Plant Species. Cytokinins are perceived by histidine kinase receptors, which initiate the cytokinin signal transduction phosphorelay (11, 20, 23). The *Arabidopsis* genome harbors 3 loci for the cytokinin receptors, *CRE1/WOL*, *AHK2*, and *AHK3*, all of which belong to the superfamily of 2-component regulators (12, 24). The CRE cytokinin receptor gene family has been shown to be conserved between the non-woody plant species *Arabidopsis*, rice, and maize (25, 26).

To compare cytokinin receptors between herbaceous plants and hardwood trees, we identified genes belonging to this gene family from 2 hardwood tree species, silver birch and poplar. We were able to isolate birch mRNAs for 3 CRE family genes (*BpCRE1*, *BpHK2*, and *BpHK3*). From the sequenced *P. trichocarpa* genome, we identified 5 genes (*PtCRE1a*, *PtCRE1b*, *PtHK2*, *PtHK3a*, and *PtHK3b*) orthologous to the CRE gene family members. One gene (*PtHK2*) was orthologous to *AHK2*, 2 (*PtHK3a* and *PtHK3b*) to *AHK3*, and 2 (*PtCRE1a* and *PtCRE1b*) to *CRE1*. From phylogenetic analysis, it is apparent that these genes show high sequence similarity to the 3 *Arabidopsis* CRE family histidine kinases (Fig.

1A). We were also able to verify that the birch ortholog for *Arabidopsis* *CRE1*, *BpCRE1*, encodes a functional cytokinin receptor. When expressed under the *Arabidopsis* *CRE1* promoter, *BpCRE1* was able to complement the phenotype of an *Arabidopsis* mutant lacking all 3 CRE family genes [supporting information (SI) Fig. S1]. These results further support the view that all flowering plants perceive cytokinin through members of the CRE cytokinin receptor family.

Cytokinin Signaling Genes Are Expressed in the Cambial Zone. To explore the potential role of cytokinins during cambial development, we studied the expression of cytokinin signal transduction components across the radius of the tree trunk. In both silver birch and poplar, members of the cytokinin receptor gene family are expressed across the cambial zone (Fig. 1B and C and Fig. S2H). In poplar, *PtHK3a* and *PtHK3b* show the highest cambial expression, peaking in the same zone as the marker gene for cambial cell identity, *PtANT* (27) (Fig. 1B, C, and E and Fig. S3). A similar pattern was seen through the expression of GUS under the *BpCRE1* promoter in both transgenic birch (Fig. S2G) and poplar (Fig. 1F). Also, the expression of a cytokinin primary response gene from poplar, encoding an A-type response regulator *PtRR7* (28), peaked in the cambial zone along with the receptor genes (Fig. 1B and D and Fig. S3). The expression of cytokinin receptors in vascular

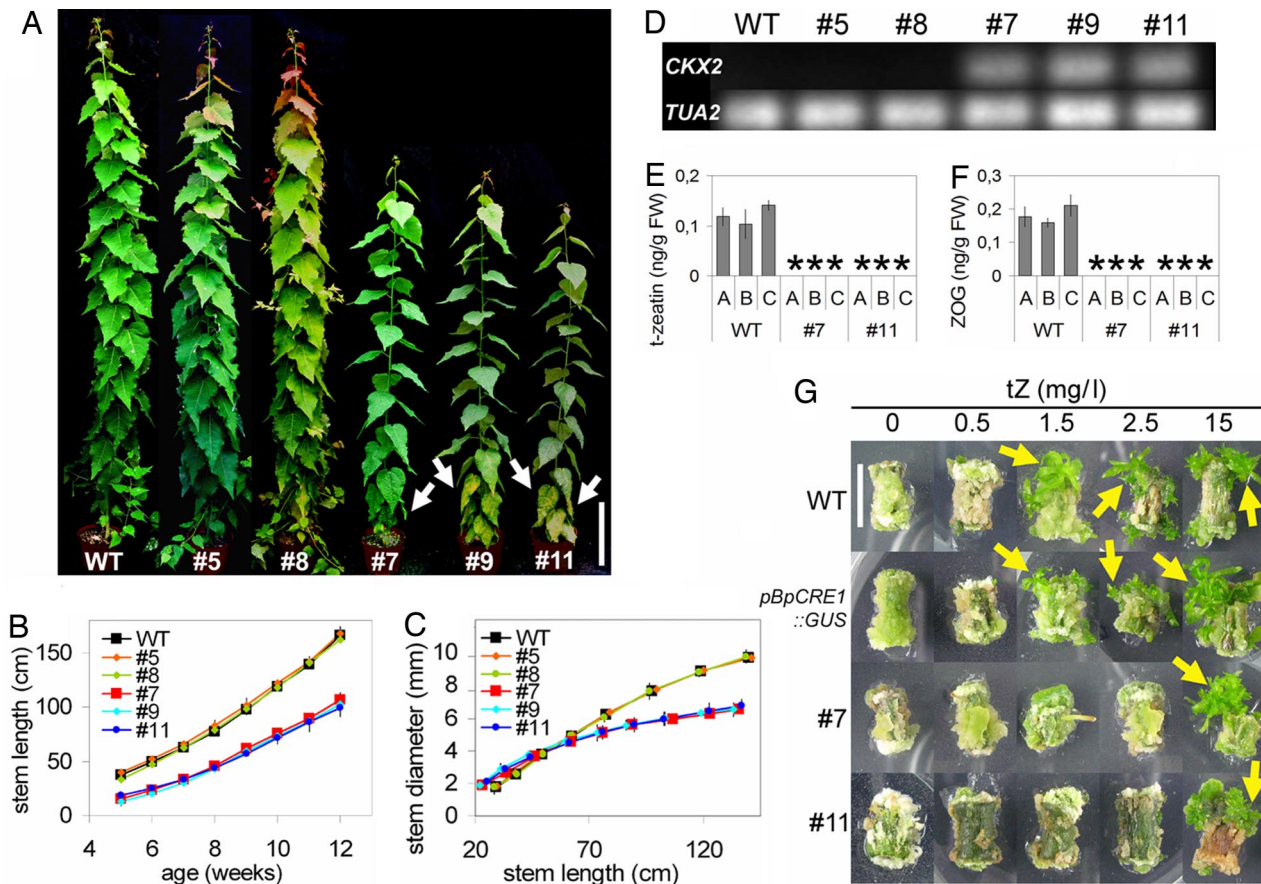


Fig. 2. *pBpCRE1::AtCKX2* poplar lines with a high expression level of the transgene display reduced cytokinin content and responsiveness, together with a thin-stemmed phenotype. (A) WT and *pBpCRE1::AtCKX2* poplar lines 5, 8, 7, 9, and 11 (4-month-old trees). Lines 7, 9, and 11 with a strong transgene expression have elongated internodes and show premature leaf senescence (indicated by white arrows). (Scale bar: 20 cm.) Height vs. age ratio (B) and width vs. height ratio (C) in WT and *pBpCRE1::AtCKX2* poplar lines 5, 8, 7, 9, and 11 ($n = 3$). *pBpCRE1::AtCKX2* poplar lines 5 and 8 have a WT-like phenotype, and lines 7, 9, and 11 display a thin-stemmed phenotype (error bars = SD). (D) *AtCKX2* and *PtTUA2* expression in WT and *pBpCRE1::AtCKX2* poplar lines 5, 8, 7, 9, and 11 by qRT-PCR shown in a gel. Both t-zeatin (E) and ZOG (F) are below the detection limit (*) in the shoot of lines 7 and 11. Three biological replicates (A–C) are shown per line (error bars = SD). (G) Cytokinin responsiveness assay. Medium with indole acetic acid (IAA) at 0.5 mg/L IAA and 0, 0.5, 1.5, 2.5, or 15 mg/L t-zeatin. A low cytokinin-to-auxin ratio induces root regeneration, and a high cytokinin-to-auxin ratio enhances shoot regeneration. The following lines were analyzed: WT, *pBpCRE1::GUS*, and *pBpCRE1::AtCKX2* lines 7 and 11. WT and *pBpCRE1::GUS* regenerate shoots from medium to high cytokinin concentrations (1.5–15 mg/L), whereas lines 7 and 11 regenerate shoots only in a high (15 mg/L) cytokinin concentration. Emerging shoots are indicated by yellow arrows. (Scale bar: 1 cm.)

cambium suggests that cytokinin signaling participates in a regulation function in this meristem.

In addition to the cambial meristem, CRE family genes are expressed in apical meristems. By in situ hybridization, *BpCRE1* was shown to be expressed in both birch shoot and root apical meristems (Fig. S2A, B, E, and F). This pattern was reproduced by expressing GUS under the *BpCRE1* promoter in transgenic birch (Fig. S2C and D) and poplar (data not shown). The observed *BpCRE1* expression pattern resembles *CRE1* expression in *Arabidopsis* apical meristems (20, 21), further indicating that the function of cytokinin receptors is highly conserved among different plant species.

***pBpCRE1::AtCKX2* Poplars Display Several Phenotypes Diagnostic for Reduced Cytokinin Responsiveness.** We engineered transgenic poplar (*P. tremula* × *tremuloides*) trees that were compromised in cytokinin signaling during cambial development. These trees expressed a cytokinin catabolic gene from *Arabidopsis cytokinin oxidase 2*, under the promoter of a birch cytokinin receptor, *BpCRE1*. CKX family enzymes irreversibly degrade active cytokinin species (29), and they have been successfully expressed under the systemic 35S promoter to reduce the general cytokinin levels in several plant species (13, 14, 30, 31). The *BpCRE1* promoter was chosen for its high cambial expression (Fig. 1F); as a promoter

driving the expression of a cytokinin receptor gene, it would presumably direct the expression of the cytokinin-degrading enzyme to the location of cytokinin perception.

The regeneration capacity of *pBpCRE1::AtCKX2* transgenic plants was partially compromised, apparently indicating the importance of cytokinin action for plant development. Fewer transgenic lines were obtained from the same number of stem segments transformed with the *pBpCRE1::AtCKX2* construct than with the *pBpCRE1::GUS* construct (data not shown). We were able to obtain 11 *pBpCRE1::AtCKX2* lines. Four were WT-like, and 7 showed a distinct phenotype during in vitro growth; they had stunted shoots with short internodes and small dark-green leaves. Both the apical growth of the shoots and internode elongation were severely retarded, whereas root growth was extensive (data not shown). These observed phenotypic alterations in vitro resemble those reported for the *p35S::CKX* plants in tobacco and *Arabidopsis* (13, 14).

We were able to further propagate 3 (lines 7, 9, and 11) of the 7 lines with the stunted in vitro phenotype. When these 3 lines were grown in soil, their growth improved, although not to the WT level (Fig. 2A). Both the apical and radial growth of lines 7, 9, and 11 was reduced compared with the WT or with the WT-like lines 5 and 8 (Fig. 2B and C). However, the radial growth of lines 7, 9, and 11

was more compromised than apical growth; these trees had thinner trunks than WT trees of similar height (Fig. 2C). We next analyzed the status of *AtCKX2* expression in the greenhouse-grown *pBpCRE1::AtCKX2* lines. Strong *AtCKX2* expression correlated with the phenotype of reduced growth in the thin-stemmed lines 7, 9, and 11, whereas no *AtCKX2* expression was detected in either of the analyzed WT-like lines 5 and 8 (Fig. 2A, C, and D).

The greenhouse-grown thin-stemmed lines 7, 9, and 11 also had longer internodes than WT and displayed premature leaf senescence, an indication of impaired cytokinin action (Fig. 2A). To study if ectopic *AtCKX2* expression affects cytokinin levels, we measured the cytokinin content of the thin-stemmed lines 7 and 11. Compared with WT, the levels of trans-zeatin (t-zeatin), one of the biologically active cytokinin species, and its storage form, zeatin-*O*-glucoside (ZOG), were reduced below detection limit in the stem of these lines (Fig. 2E and F). The level of another biologically active cytokinin, isopentenyladenine, was reduced only in line 11 (Fig. S4). Levels of other metabolic forms of cytokinin were not reduced from WT levels in either line (Fig. S4). Free bases are preferable substrates of *AtCKX2* (32) and the reduction of either isopentenyladenine and/or t-zeatin is consistent with earlier *p35S::CKX* transgenic tobacco and *Arabidopsis* studies (13, 14). However, high levels of other metabolic forms have not been observed, which may be attributable to the cell type-specific promoter used in our study. The reduced t-zeatin and ZOG content indicates that ectopic *AtCKX2* expression leads to a reduced content of active cytokinin species, which results in the observed thin-stemmed phenotype.

To further evaluate the effect of ectopic *AtCKX2* expression on cytokinin signaling in the thin-stemmed lines, we tested their cytokinin responsiveness. In the classic cytokinin responsiveness assay (33), a low cytokinin-to-auxin ratio induces root regeneration from plant segments and a high cytokinin-to-auxin ratio promotes shoot regeneration instead. In this assay, lines 7 and 11 showed reduced cytokinin responsiveness compared with WT and a *pBpCRE1::GUS* line (Fig. 2G). A low cytokinin-to-auxin ratio is also known to support apical dominance by inhibiting axillary bud outgrowth, whereas a high ratio reduces it by facilitating bud outgrowth (34). Decapitated *pBpCRE1::AtCKX2* trees produced fewer new shoots from the axillary buds than the control trees, indicating enhanced apical dominance (data not shown). Taken together, our data indicate that the *pBpCRE1::AtCKX2* trees are compromised in cytokinin-regulated developmental processes.

Dissecting the Effects of Cytokinins on Radial Versus Apical Growth.

We further examined the apical and radial growth of several *pBpCRE1::AtCKX2* lines in greenhouse conditions. In the shoot apex of the transgenic lines 7 and 11, 0.36 ± 0.02 leaf primordia per day were produced compared with 0.59 ± 0.05 in WT (average \pm SD, 4- to 12-week-old plants, $n = 3$), indicating somewhat reduced activity of the shoot apical meristem. To address the impact of reduced apical growth on the radial growth in the thin-stemmed *pBpCRE1::AtCKX2* trees, we studied the relation between apical and radial growth by means of a grafting experiment. One transgenic thin-stemmed line (line 7) was reciprocally grafted to WT ($n = 3$) (Fig. 3A and B and Fig. S5). In the WT/WT and line 7/7 grafts, the stock and scion reached the same diameter (9.0 ± 0.1 SD vs. 8.8 ± 0.1 mm and 5.9 ± 0.2 SD vs. 5.7 ± 0.2 mm, respectively) during 4 months of linear growth. In contrast, the thin-stemmed scion did not reach the diameter of the WT stock (7.2 ± 0.3 SD vs. 8.9 ± 0.4 mm), whereas the WT scion outgrew the diameter of the thin-stemmed stock (8.1 ± 0.5 SD vs. 6.7 ± 0.4 mm). The data show that the WT stem was not transformed to the thin-stemmed phenotype by a *pBpCRE1::AtCKX2* apex, nor was the thin-stemmed phenotype rescued to WT by a WT apex. Thus, compromised cytokinin activity during radial growth rather than the reduced activity of apical meristem appears to be the major determinant of the thin-stemmed phenotype.

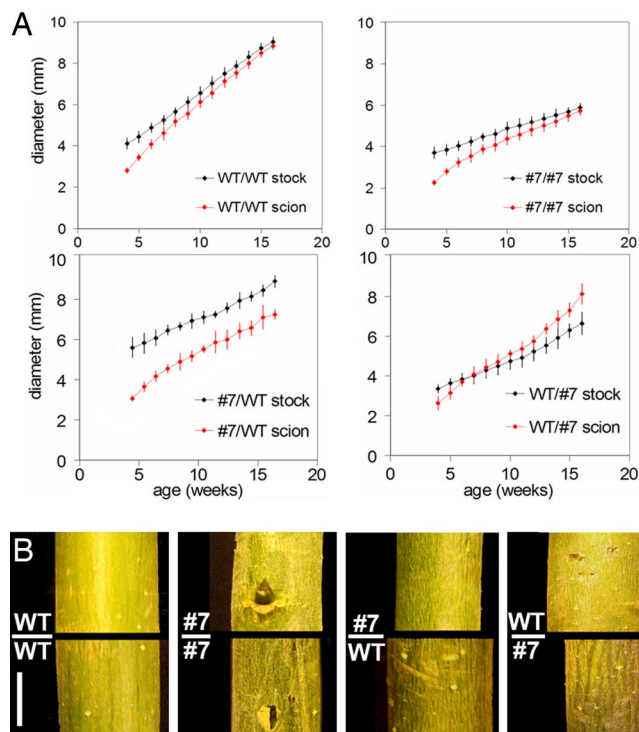


Fig. 3. Relation between the apical and radial growth in *pBpCRE1::AtCKX2* poplars. (A) Reciprocal grafts with WT and *pBpCRE1::AtCKX2* line 7. Graphs show scion and stock diameter vs. age. Diameter was measured 5 cm above and below the scion/stock junction. WT/WT (self-grafting): both the WT scion and stock part reach the same diameter. Line 7/7 (self-grafting): both the line 7 scion and stock part reach same diameter. Line 7/WT (line 7 scion, WT stock): line 7 scion part does not reach diameter of the WT stock. WT/line 7 (WT scion, line 7 stock): WT scion outgrows the line 7 stock. Average from 3 individual grafted trees is shown in each graph. (B) Grafted trees shown 5 cm above and below the scion/stock junction 6 months after the grafting. (Scale bar: 5 mm.)

Reduced Cambial Activity in *pBpCRE1::AtCKX2* Poplars. To study the effect of reduced cytokinin signaling on cambial growth-related cell division and differentiation, we analyzed the vascular anatomy of the thin-stemmed lines. The cambial cells were seen as undifferentiated, thin-walled, flat cells localized between differentiating xylem and phloem cells. In the *pBpCRE1::AtCKX2* trees, the vascular cambium consists of fewer cell layers than in WT. The reduction was evident when comparing internodes of similar position or similar diameter. The 20th WT internode with a diameter of 7.3 mm contained 16.4 ± 0.11 (average \pm SE) apparently undifferentiated cells per 1 cambial cell file ($n = 100$ cambial cell files, data from 50 files from 2 separate trees combined). Line 7 contained, on average, 10.5 ± 0.1 apparently undifferentiated cells in the 20th internode, and line 11 contained 10.7 ± 0.1 cells. When comparing internodes of similar diameter (7.3 mm) with WT, line 7 contained 11.0 ± 0.1 cells and line 11 contained 11.4 ± 0.1 cells (Fig. 4). The significantly ($P < 0.001$) reduced number of undifferentiated cells in the cambial cell files indicates that fewer cell divisions occur in the cambial layer of the transgenic trees than in WT trees.

We also analyzed wood anatomy of the *pBpCRE1::AtCKX2* trees. Thin-stemmed lines had slightly shorter xylem fibers than WT ($P < 0.05$), whereas the fiber width was not significantly different from WT (Fig. S6A). The opposite was observed with vessel dimensions: vessels were slightly wider in WT than in the thin-stemmed lines ($P < 0.05$; Fig. S6B), whereas no significant difference was observed in vessel length.

Reduced Cambial Cytokinin Signaling in *pBpCRE1::AtCKX2* Poplars. To associate reduced cambial activity in the *pBpCRE1::AtCKX2* pop-

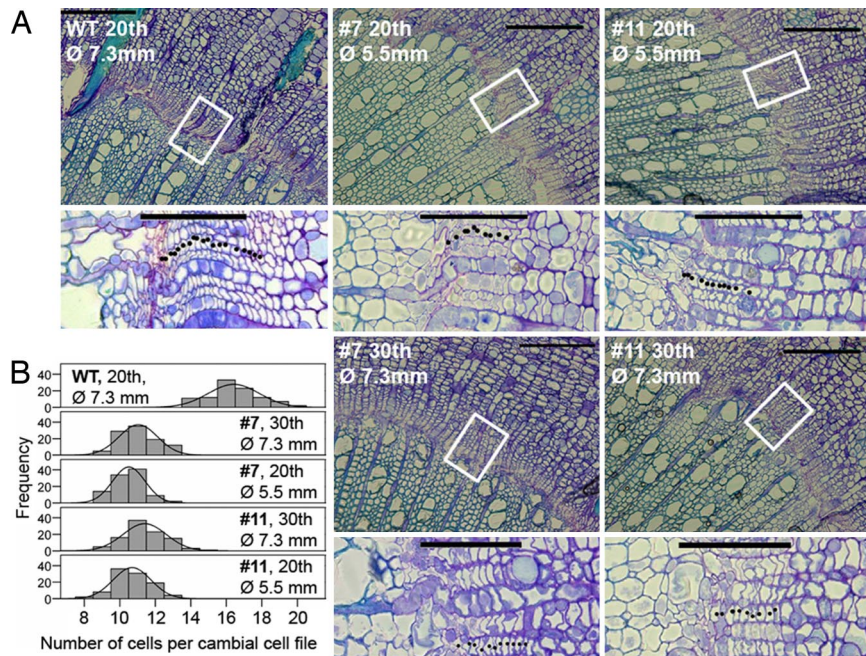


Fig. 4. *pBpCRE1::AtCKX2* poplars have a reduced number of cambial cells. (A) Cross sections of WT, line 7, and line 11 stem. The stem thickness and internode number at the section site are indicated. The cambial cell file is indicated with dots. The number of undifferentiated cambial cells (marked between asterisks) per cell file is reduced in lines 7 and 11 compared with WT (see text for details). (Scale bar: 0.2 mm [0.1 mm in *Insets*].) (B) Number of undifferentiated cambial cells was reduced in the thin-stemmed *pBpCRE1::AtCKX2* lines. Frequency distributions of cell numbers in cambial cell files in the 20th WT internode (\varnothing 7.3 mm) and in internodes of *pBpCRE1::AtCKX2* lines 7 and 11 of the same position (20th from the apex) or the same diameter (\varnothing 7.3 mm) are shown ($n = 100$ cambial files, data from 50 files from 2 separate trees combined). The difference in distributions was statistically significant ($P < 0.001$) between WT and *pBpCRE1::AtCKX2* lines.

lars with cytokinin function, we analyzed the status of cambial cytokinin signaling. In the trunks of thin-stemmed lines 7 and 11, the expression level of *PttRR7* was dramatically reduced compared with WT, whereas the expression of cytokinin receptor *PttHK3a* was essentially the same as in WT (Fig. 1G). This indicates that a reduced level rather than a restricted spatial domain of cytokinin signaling is the primary basis for the impaired cambial growth of *pBpCRE1::AtCKX2* trees.

Discussion

Understanding the regulation of the radial growth that underlies wood development is of great importance for the future use of tree products as a renewable resource. To understand the role of various phytohormones in regulation of wood development, it is important to investigate the consequences of their reduced action. Here, we have taken a transgenic approach to reduce cytokinin levels in *P. tremula* \times *tremuloides* by driving the expression of a cytokinin catabolic gene (*AtCKX2*) using the promoter of a birch gene (*BpCRE1*) that encodes a cytokinin receptor. As a consequence, we were able to show that reduced levels of cytokinins and their storage forms (t-zeatin and ZOG) are produced in selected transgenic lines that strongly express the transgene. Furthermore, these lines display various symptoms indicative of reduced cytokinin action: impaired shoot regeneration in tissue culture, enhanced apical dominance, enhanced leaf senescence, and impaired apical growth of shoot. Concerning the longer internodes and enhanced leaf senescence, the affected lines differ from the earlier reported tobacco and *Arabidopsis p35S::CKX* lines, which display short internodes and nonaccelerated leaf senescence (13, 14).

Our experimental focus here has been whether cytokinins are required for the secondary phase of vascular development characterized by the activity of vascular cambium, a stem cell population that orchestrates plant radial growth. The transgenic trees with high *AtCKX2* expression have significantly impaired radial growth. Radial growth in these lines appears to be more affected than apical growth. Furthermore, by grafting, we have shown that the defects in the apical growth cannot explain the reduced radial growth. A detailed anatomical characterization of the transgenic lines reveals that the number of undifferentiated cell files in the cambial zone is reduced in the transgenic plants with high *AtCKX2* expression. Taken together, our data show that cytokinins are required for

vascular cambium function in controlling radial growth. In this respect, the cambial meristem function resembles the shoot apical meristem, which is also positively regulated by cytokinin signaling (14–17), and differs from the root apical meristem, which is characterized by negative cytokinin regulation (14, 16, 18, 19).

Using transgenic approaches to impair auxin signaling during cambial development, it has recently been shown that auxin is required for both normal cambial cell proliferation and differentiation of xylem cells during secondary growth (6). In our transgenic trees with high *AtCKX2* expression levels, we also observed slight differences in wood anatomy: fiber lengths and vessel widths were slightly reduced. It remains to be studied, however, whether or not these differences can be attributed to the altered rate of cell proliferation in the cambial zone.

During primary vascular development, cytokinins appear to be required for both cell proliferation and cell specification (19–22). We have shown here that during secondary development, their major function is the regulation of cell proliferation. Thus, cytokinins appear to have diverse roles during vascular and meristem development, perhaps dependent on how they interact with other growth regulators. Our finding of the proliferative role of cytokinins in regulating cambial development in radial stem growth may contribute to the development of more efficient plant biomass production systems in the future.

Materials and Methods

Cloning of Birch Genes. Short cDNA fragments corresponding to *BpCRE1* and *BpHK2* genes were amplified by PCR using degenerate primers. A partial *BpHK3* 1.4-kb fragment was identified from a birch leaf cDNA library (J. Kangasjärvi, personal communication). A birch genomic library (Y.H., unpublished data) was screened with *BpCRE1* cDNA fragment as a probe. The genomic clone for *BpCRE1* was isolated and subcloned for sequencing. A genomic fragment containing the *BpCRE1* gene was concatenated from 2 different clones with 5 kb of 5' upstream sequence and a coding region with 11 exons (GenBank EU583454). cDNAs representing *BpCRE1* (EU583455), *BpHK2* (EU583456), and *BpHK3* (EU583457) were cloned using RT-PCR and 5' RACE techniques. The sizes of predicted proteins encoded by the cDNAs were 1,004, 1,260, and 1,066 amino acids, respectively.

Phylogenetic Analyses. Phylogenetic analyses were performed on an amino acid alignment of selected 2-component receptor genes. Two phylogeny reconstruction methods were used: parsimony and maximum likelihood. Details are provided in *SI Materials and Methods*.

Constructs. To generate the *pBpCRE1::GUS* construct, a 4,723-kb fragment containing the *BpCRE1* promoter was isolated from a genomic DNA clone and cloned into *pBI101* GUS-reporter vector. For the *pBpCRE1::AtCKX2* construct, *Arabidopsis CKX2* gene (*At2g19500*) was amplified by PCR from genomic DNA and cloned into *pGEM-T Easy* vector downstream of the *BpCRE1* promoter. Details are provided in *SI Materials and Methods*.

Transgenic Plants. *B. pendula* clone BPM5 was transformed with *pBpCRE1::GUS* and *pBpCRE1::AtCKX2* using an *Agrobacterium*-mediated method (35). *P. tremula* × *tremuloides* clone T89 was transformed with *pBpCRE1::GUS* and *pBpCRE1::AtCKX2* by an *Agrobacterium*-based method (36). Details of the tissue culture are provided in *SI Materials and Methods*.

Quantitative RT-PCR. For poplar quantitative PCR analysis, samples were collected from two 8-month-old *P. trichocarpa* "Nisqually-1" trees (data shown from 1 tree). Birch quantitative RT-PCR (qRT-PCR) analysis is described in *SI Materials and Methods*. Expression of putative poplar cytokinin receptors *PtHK2*, *PtHK3a*, *PtHK3b*, *PtCRE1a*, and *PtCRE1b* and of a putative A-type response regulator, *PtRR7* (28), was analyzed across the trunk. A tangential cryosectioning protocol (27) was used to section the stem into fractions representing "bark," "old phloem," "phloem," "developing phloem," "cambium," "developing xylem," "xylem," and "old xylem." Anatomical cross sections representing each fraction were cut with a razor blade. No detectable expression of *PtHK2* was observed in any of the fractions by the 2 primer pairs used (data not shown). Expression of the other receptors was very weak outside the cambial zone. The cambial zone of 2 trees was further divided into 16 24- μ m cryosections. Details are provided in *SI Materials and Methods*. The *P. trichocarpa TUBULIN ALPHA 2 (PtTUA2)* gene was used as a reference for relative quantification. The identity of cambial cells was verified by a marker diagnostic for cambium (*AINTEGUMENTA*, *PtANT*) (27). Details about markers for cambial zone tissues are provided in *SI Materials and Methods*. Expression of the transgene in 6-month-old *P. tremula* × *tremuloides* lines (WT and *pBpCRE1::AtCKX2* lines 5, 7, 8, 9, and 11) was verified by qPCR analysis for *AtCKX2*, and reaction products for *AtCKX2* and *PtTUA2* were run into 2% (w/v) agarose gel for visualization. In qPCR analysis of *PtRR7/PtTHK3a* expression from the stem of WT and lines 7 and 11, the primers for *PtTUA2*, *PtRR7*, and *PtHK3a* were used. Expression studies of *PtRR7/PtTHK3a* and cytokinin analyses were conducted from the same sample material.

Grafting Experiment. A poplar (*P. tremula* × *tremuloides*) reciprocal grafting experiment with WT and *pBpCRE1::AtCKX2* line 7 was done essentially as described elsewhere (37). Details are provided in *SI Materials and Methods*.

Histological Techniques and a Physiological Assay. Plastic sectioning for anatomical samples (*Arabidopsis* and poplar) was performed as described elsewhere (20). Histochemical staining for GUS activity was carried out according to ref. 38, except that 20 mM ascorbic acid was added to the assay solution to prevent browning of the tissues. For the cytokinin responsiveness assay, 20 stem segments (\varnothing 0.5 cm, 1 cm in length) were cut from greenhouse poplars per line (WT, *pBpCRE1::AtCKX2* lines 7 and 11, *pBpCRE1::GUS*), surface sterilized, and grown for 2 months on a 0.5 Murashige and Skoog Basal Medium with 0.5 mg/L IAA and 0, 0.5, 1.5, 2.5, or 15 mg/L t-zeatin.

Cytokinin Analysis. Cytokinins were analyzed from stem tissue representing the apical part of greenhouse-grown poplar shoot. The 10 topmost internodes were used for analysis, and the shoot apical meristem, leaf nodes, and leaves were removed. Three individual trees were analyzed per line. Cytokinins were extracted, purified, and analyzed by liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) as described elsewhere (39).

Statistical Analysis of Cambial Cell Numbers and Xylem Cell Dimensions. The number of undifferentiated cambial cells was calculated from 50 cambial cell files from 2 trees per line (\varnothing 5.5 mm in lines 7 and 11 and \varnothing 7.3 mm in WT and lines 7 and 11). Xylem cells were macerated according to the method described in ref. 6 from stem samples with \varnothing 7.3 mm. Two hundred fiber cells and 100 vessel cells were measured from 2 trees per line (WT, line 7, and line 11). The data from 2 trees were combined for statistical analysis. Details of statistical analysis are provided in *SI Materials and Methods*.

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