Regulation of plant growth by cytokinin

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Cytokinins are a class of plant-specific hormones that play a central role during the cell cycle and influence numerous developmental programs. Because of the lack of biosynthetic and signaling mutants, the regulatory roles of cytokinins are not well understood. We genetically engineered cytokinin oxidase expression in transgenic tobacco plants to reduce their endogenous cytokinin content. Cytokinin-deficient plants developed stunted shoots with smaller apical meristems. The plastochrone was prolonged, and leaf cell production was only 3–4% that of wild type, indicating an absolute requirement of cytokinins for leaf growth. In contrast, root meristems of transgenic plants were enlarged and gave rise to faster growing and more branched roots. These results suggest that cytokinins are an important regulatory factor of plant meristem activity and morphogenesis, with opposing roles in shoots and roots.

Cytokinins were discovered during the 1950s because of their ability to induce plant cell division (1). Shortly after their discovery, Skoog and Miller coined the auxin–cytokinin hypothesis of plant morphogenesis (2). The hypothesis predicted that cytokinin, together with auxin, plays an essential role in plant morphogenesis, having a profound influence on the formation of roots and shoots and their relative growth.

Chemically, natural cytokinins are N^6-substituted purine derivatives. Isopentenyladenine (iP), zeatin (Z), and dihydrozeatin (DZ) are the predominant cytokinins found in higher plants. The free bases and their ribosides (iPR, ZR, DZR) are thought to be the biologically active compounds. Glycosidic conjugates play a role in cytokinin transport, protection from degradation, and reversible and irreversible inactivation (3).

Numerous reports ascribe a stimulatory or inhibitory function to cytokinins in different developmental processes such as root growth and branching, control of apical dominance in the shoot, chloroplast development, and leaf senescence (4). Conclusions about the biological functions of cytokinins have mainly been derived from studies on the consequences of exogenous cytokinin application or endogenously enhanced cytokinin levels (5, 6). Up to now, it has not been possible to address the reverse question: what are the consequences for plant growth and development if the endogenous cytokinin concentration is decreased? Plants with a reduced cytokinin content are expected to yield more precise information about processes cytokinins limit and, therefore, might regulate. Unlike other plant hormones such as abscisic acid, gibberellins, and ethylene, no cytokinin biosynthetic mutants have been isolated (7).

The catabolic enzyme cytokinin oxidase (CKX, ref. 8) plays possibly the principal role in controlling cytokinin levels in plant tissues. CKX activity has been found in a great number of higher plants and in different plant tissues (8). The enzyme is a FAD-containing oxidoreductase that catalyzes the degradation of cytokinins bearing unsaturated isoprenoid side chains. The free bases, iP and Z, and their respective ribosides are the preferred substrates. The reaction products of iP catabolism are adenine and the unsaturated aldehyde 3-methyl-2-butenal (8). Recently, a cytokinin oxidase gene from *Zea mays* has been isolated (9, 10). The manipulation of *CKX* gene expression could partially overcome the lack of cytokinin biosynthetic mutants and might be used as a powerful tool to study the relevance of iP- and Z-type cytokinins during the whole life cycle of higher plants. In this article, we report the cloning of four putative *CKX* genes from *Arabidopsis thaliana* and the results of their systemic overexpression in transgenic tobacco plants. Our data indicate an important role for cytokinins in plant growth regulation via a differential influence on the number and/or duration of cell division cycles in the root and shoot meristems.

Materials and Methods

Gene Cloning. The genomic sequences of the *AtCKX1*, *AtCKX2*, *AtCKX3*, and *AtCKX4* genes were amplified by PCR from DNA of *A. thaliana* accession Col-0. Oligonucleotide primers were designed according to the published genomic sequences of *AtCKX* genes [GenBank accession nos. AC002510 (*AtCKX1*), AC005917 (*AtCKX2*), AB024035 (*AtCKX3*), and AL079344 (*AtCKX4*)] and had 5′ and 3′ overhangs with *Sal*I or *Kpn*I restriction sites, which permitted subcloning in the vector pUC19. The length of the amplified sequences were 2,235 bp (*AtCKX1*), 3,104 bp (*AtCKX2*), 3,397 bp (*AtCKX3*), and 2,890 bp (*AtCKX4*). Genes were sequenced and inserted into vector pBINHygTx under the transcriptional control of a constitutively expressed 35S promoter (11). The cDNA of *AtCKX2* was cloned by reverse transcription–PCR from total RNA of *AtCKX2* transgenic plant tissue with the OneStep reverse transcription–PCR kit (Qiagen, Chatsworth, CA). The PCR products were sequenced and positioned under control of the *GAL1* promoter in the yeast expression vector pYES2. The control strain harbored only the empty vector. Induction of gene expression by galactose was carried out for 6 h as suggested by Invitrogen.

Plant Transformation and Plant Culture. *Nicotiana tabacum* L. cv. Samsun NN leaf explants were transformed and regenerated as described (12). At least 15 independent transformants showing very similar phenotypes were obtained for each of the four genes. Plants were cultured *in vitro* on MS medium or in a glass house with 15-h light/9-h dark cycles, 20°C during the dark period and 24°C during the light period. Characterizations of the transgenic tobacco were carried out on T_2 progeny obtained by selfing. Phenotypic changes noted for the independent transformants were very similar and differed only gradually. Independent transformants, confirmed by Northern blot analysis and/or by measuring the cytokinin oxidase activity, looked similar to the transformants shown in Fig. 2*B*. Quantitative growth parameters were obtained from at least ten individuals of two independent clones (AtCKX1-28, AtCKX1-50 and AtCKX2-38, AtCKX2-40, respectively). Segregation analyses of the hygromycin resistance

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Abbreviations: CKX, cytokinin oxidase; iP, isopentenyladenine; Z, *trans*-zeatin; SAM, shoot apical meristem.

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gene indicated one or two (AtCKX2-40) chromosomal T-DNA insertion loci. For the sake of clarity, the results for only one clone are shown in Figs. 2 (*C* and *D*) and 3 (*C* and *D*).

RNA Preparation and Blot Analysis. Total RNA extraction from leaf tissue and Northern blot analysis, with 50 μg of total RNA, was carried out essentially as described (13).

Histological Analysis. Plant tissue was fixed and embedded in LR White (Plano, Wetzlar, Germany) according to ref. 14, and 2.5 μM thin sections were stained with 0.1% toluidine blue. For DNA staining, roots were fixed in ethanol:acetic acid (6:1), incubated for 15 min in a solution of 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml H_2O), and washed three times with water. Scanning electron microscopy was carried out according to ref. 15.

Quantitative Analysis of Cytokinin Oxidase Activity. The standard assay for CKX activity was based on the conversion of [2-^{3}H]iP to adenine as described (16).

Quantitative Analysis of Cytokinin Content. Cytokinin extraction, immunopurification, HPLC separation, and quantification by ELISA methods were carried out as described (13).

Results

The Structure of the *AtCKX* Genes. A BLAST search of the GenBank database identified six cytokinin oxidase-like genes in *Arabidopsis* that code for enzymes with 32–41% amino acid identity to the maize protein and 33–66% amino acid identity between individual family members. The gene structure is partially conserved between maize and *Arabidopsis*. The predicted *Arabidopsis* genes have five exons and four introns, whereas the maize gene has only two introns that are at identical positions as two of the *Arabidopsis* introns. Common motifs of these *CKX* genes are putative N-terminal signal peptides, which indicate for most of the corresponding proteins transport to the secretory pathway, and a ≈70-aa-long FAD-binding domain in the N-terminal region (data not shown; ref. 17).

Transgenic Plants Show Increased Cytokinin Oxidase Activity. We cloned the *AtCKX1*, *AtCKX2*, *AtCKX3*, and *AtCKX4* genes, positioned them under the control of a constitutive 35S promoter, transformed tobacco plants individually with these genes, and selected overexpressing transgenic clones by Northern blot analysis (Fig. 1*A*). The leaves of expressing transgenic tobacco lines showed a 2.6-fold to 10.4-fold increase in cytokinin oxidase activity (Fig. 1*B*). Likewise, cells of *Saccharomyces cerevisiae* expressing *AtCKX2* showed a higher cytokinin oxidase activity than the control strain (Fig. 1*C*). The majority of the enzyme activity accumulated in the yeast culture medium (Fig. 1*C*). This result, and similar observations in fission yeast and *Physcomitrella patens* cells expressing the cytokinin oxidase of maize (9, 10), indicates that the cytokinin degradation pathway might be, at least partially, located extracellularly. The apparent K_m values for the cytokinin oxidases analyzed in this study are in the range of 0.3 to 9.5 μM with iP as a substrate. This is similar to or even lower than the maize enzyme, which has an apparent K_m of 19.2 μM in kernels (18) and a native K_m of 2.8 μM (17) with iP as a substrate. These results demonstrate that the proteins encoded by these four *AtCKX* genes do indeed have cytokinin oxidase activity and could be used as a tool to study the relevance of cytokinins during the whole life cycle of higher plants.

Transgenic Plants Have a Reduced Cytokinin Content. The endogenous concentrations of different cytokinin metabolites was significantly reduced in *AtCKX1*- and *AtCKX2*-expressing transgenic seedlings. The total content of iP and Z metabolites in

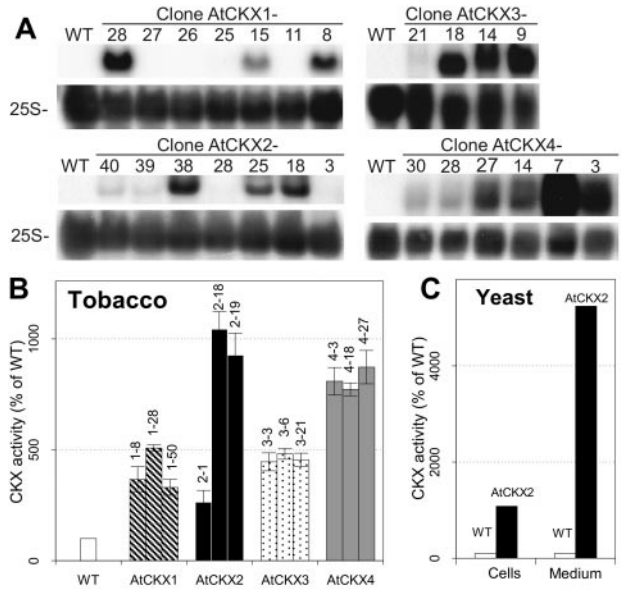

Fig. 1. *AtCKX* gene expression and enzyme activity in transgenic tobacco plants and yeast. (*A*) Northern blots (50 μg total RNA) of individual transformants were probed with gene-specific probes that covered the whole genomic sequences. Only clones with detectable *AtCKX* transcripts showed a phenotype, and no cross-hybridization with untransformed tobacco (WT) was detected. 25S, hybridization with 25S rRNA as control for loading. (*B*) Cytokinin oxidase activity in leaves of tobacco plants. Specific activity in extracts of wild type (100%) was 8 ± 0.9 pmol adenine × mg protein^{-1} × h^{-1}. Bars show SD; n = 3. (*C*) Cytokinin oxidase activity in yeast cells and medium. Specific activity of the control strain (100%) was 1.16 nmol adenine × mg protein^{-1} × h^{-1}.

individual transgenic clones was between 31% and 63% that of wild type (Table 1). Among the 16 different cytokinin metabolites that were measured, the greatest change occurred in the iP-type cytokinins in *AtCKX2* overexpressers. Smaller alterations were noted for Z-type cytokinins, which could be due to different accessibility of the substrate or a lower substrate specificity of the protein. Interestingly, the cytokinin reserve pool of *O*-glucosides was also lowered in the transgenics (Table 1). The concentrations of *N*-glucosides and dihydrozeatin-type cytokinins were very low in wild-type plants and were not, or only marginally, altered in transgenic seedlings (data not shown). It is noteworthy that the overall decrease in the content of iP-type cytokinins is more pronounced in *AtCKX2*-expressing plants than in *AtCKX1* transgenics, which show a stronger phenotype in the shoot. The changes in concentration of Z and ZR were similar in both cases. It is not known which cytokinin metabolite is relevant for the different traits that were analyzed in the transgenic plants. It may be that the different cytokinin forms have differing roles to play in the various developmental processes.

Transgenic Plants Have an Altered Phenotype. *AtCKX* gene overexpression caused striking developmental alterations in the plant shoot and root system. The alterations were very similar, but not identical, for the different genes. *AtCKX1* and *AtCKX3* overexpressers were alike as were *AtCKX2* and *AtCKX4* transgenics. Generally, the two former showed higher expression of the traits, particularly in the shoot. Because of these similarities, the phenotypic changes of two independent clones expressing *AtCKX1* or *AtCKX2* were analyzed in greater detail. Most data shown refer to the stronger phenotype of the *AtCKX1* transgenics.

The most noticeable changes in the shoot were a severely retarded development with shorter internodes leading to a

Table 1. Cytokinin content of *AtCKX* transgenic plants

Line	Wild type	AtCKX1-2		AtCKX1-28		AtCKX2-38		AtCKX2-40	
Cytokinin metabolite	Concentration	Concentration	% of WT	Concentration	% of WT	Concentration	% of WT	Concentration	% of WT
iP	5.90 ± 1.80	4.76 ± 0.82	81	4.94 ± 2.62	84	1.82 ± 0.44	31	2.85 ± 0.62	48
iPR	2.36 ± 0.74	1.53 ± 0.14	65	0.75 ± 0.27	32	0.55 ± 0.39	23	0.89 ± 0.07	38
iPRP	3.32 ± 0.73	0.87 ± 0.26	28	1.12 ± 0.13	34	0.80 ± 0.48	24	1.68 ± 0.45	51
Z	0.24 ± 0.06	0.17 ± 0.02	71	0.22 ± 0.03	92	0.21 ± 0.06	88	0.22 ± 0.02	92
ZR	0.60 ± 0.13	0.32 ± 0.12	53	0.34 ± 0.03	57	0.34 ± 0.15	57	0.32 ± 0.05	53
ZRP	0.39 ± 0.17	0.42 ± 0.11	107	0.28 ± 0.15	72	0.06 ± 0.01	15	0.17 ± 0.06	44
ZOG	0.46 ± 0.20	0.32 ± 0.09	70	0.26 ± 0.13	57	0.20 ± 0.07	43	0.12 ± 0.02	26
ZROG	0.48 ± 0.17	0.30 ± 0.06	63	0.47 ± 0.02	98	0.23 ± 0.05	48	0.30 ± 0.13	63
Total	13.75	8.69	63	8.38	61	4.21	31	6.55	48

Three independently pooled samples of approximately 100 2-week-old seedlings (2.5 g/sample) were analyzed for each clone. Concentrations are in pmol × g fresh weight^{-1}. iP, N^6-(Δ^2isopentenyl)adenine; iPR, N^6-(Δ^2isopentenyl)adenine riboside; iPRP, N^6-(Δ^2isopentenyl)adenine riboside 5′-monophosphate; ZR, zeatin riboside; ZRP, zeatin riboside 5′-monophosphate; ZOG, zeatin *O*-glucoside; ZROG, zeatin riboside *O*-glucoside.

dwarfed growth habit, the formation of lanceolate epinastic leaves, and the formation of a reduced number of flowers (Fig. 2 *A* and *B*). The time between the initiation of new leaves (plastochrone) at the borders of the shoot meristem was on average 2.6 ± 0.1 days in wild type and 4.4 ± 0.1 days in *AtCKX1* transgenics (Fig. 2*C*). The surface area of leaves formed by the transgenics during a defined time period was ≈15% that of wild type (Fig. 2*D*). The width-to-length ratio of mature leaves was lowered from 1:2 in wild type to 1:3 in *AtCKX1* transgenics. The vasculature of *AtCKX1* transgenic leaves was less developed, the spacing between veins was larger, and the veins were flat and not raised as in wild type. In contrast to wild-type leaves, leaf parenchyma cells continued to expand in transgenic clones in the transverse direction, resulting in thicker and rigid old leaves. A prominent difference was also noted for progression of leaf senescence. In tobacco, leaf senescence starts in the most basal leaves and leads to a uniform reduction of leaf pigment content. In contrast, aging leaves of *AtCKX1* transgenic plants developed chlorotic intercostal regions but retained chlorophyll along the leaf veins (Fig. 2*E*). Leaf aging was similar in *AtCKX2*-expressing plants, but chlorosis was less pronounced. Transgenic plants started to flower up to 3 months later than wild-type plants (Fig. 2*C*) and produced only 5–10 normal-sized flowers compared with >100 flowers in the wild types. The final leaf number at the onset of flowering was similar in wild type and the transgenic clones, supporting the notion that leaf number is a determinant for flower induction in day-neutral tobacco (Fig. 2*C*). Lateral buds in the leaf axils of transgenic plants developed two to three tiny leaves early during vegetative development, in contrast to lateral buds of wild type, which remained completely inhibited. This indicates incomplete apical dominance in the transgenic plants.

In contrast to the inhibited shoot development of *AtCKX* transgenic tobacco, their root growth was enhanced (Fig. 3*A* and

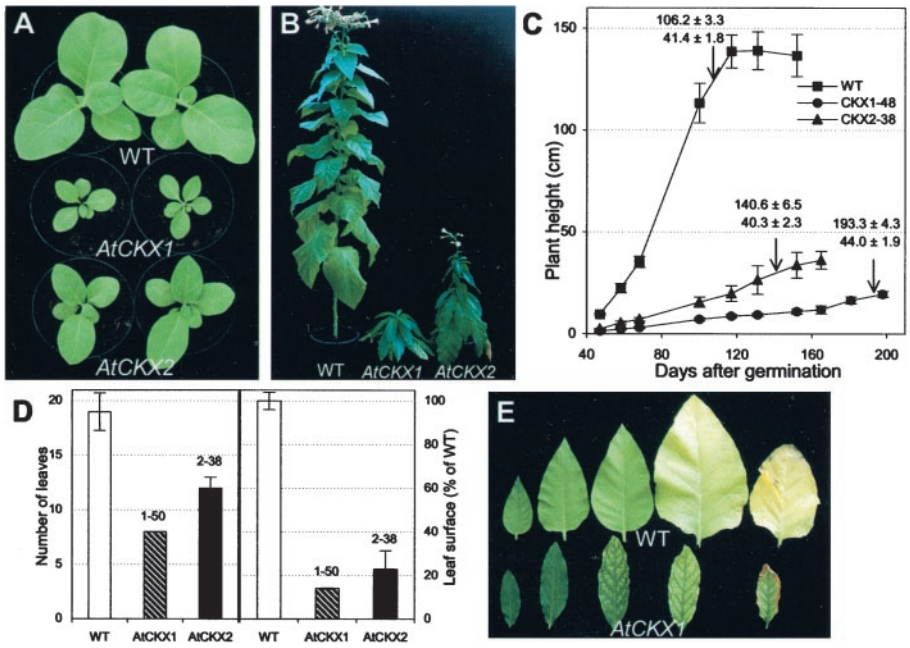

Fig. 2. Shoot phenotype of *AtCKX1*-expressing tobacco plants. (*A*) Top view of 6-week-old plants. (*B*) Tobacco plants at the flowering stage. (*C*) Kinetics of stem elongation. Arrows mark the onset of flowering. Age of plants (days after germination) and leaf number at that stage are indicated above the arrows. Bars indicate SD; $n = 12$. (*D*) Number of leaves ($n = 12$) formed between day 68 and day 100 after germination and final surface area of these leaves (100% of wild type is 3646 ± 144 cm^2; $n = 3$). (*E*) Comparison of leaf size and senescence. Leaves were from nodes number 4, 9, 12, 16, and 20 from the top (from left to right).

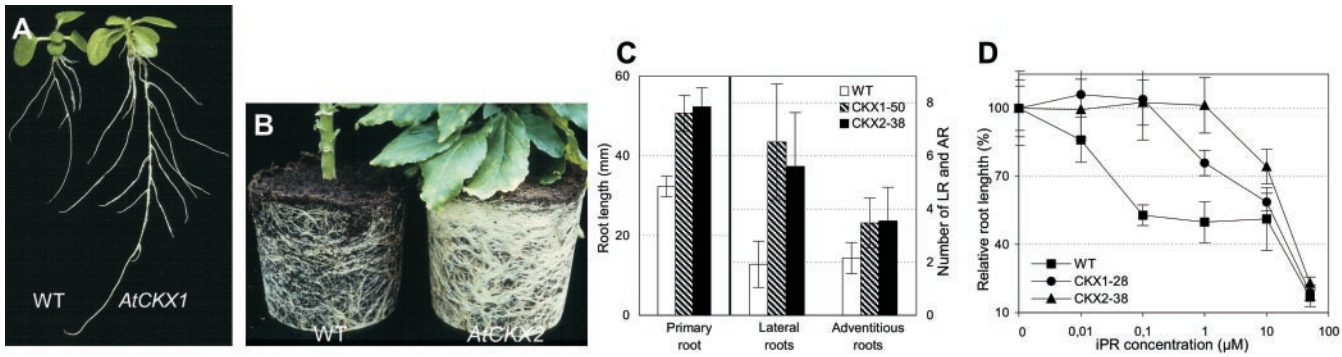

Fig. 3. Root phenotype of *AtCKX*-expressing transgenic tobacco plants. (*A*) Seedlings 17 days after germination. (*B*) Root system of soil-grown plants at the flowering stage. (*C*) Root length, number of lateral roots (LR), and adventitious roots (AR) on day 10 after germination. (*D*) Dose-response curve of root growth inhibition by exogenous cytokinin. Seeds were sown on MS medium containing 3% sucrose and the indicated concentration of iPR. The length of primary roots was determined after 10 days of cultivation in the dark on vertically positioned plates. Bars indicate ± SD; $n = 30$.

B). Elongation of the primary root was more rapid, primordia of lateral roots were noted closer to the root apex than in wild-type plants, and the number of lateral branches, as well as adventitious roots, increased (Fig. 3*C*). Enhanced root growth led to a 60% increase in root dry weight in transgenic plants grown in hydroponic solution (data not shown). These results suggest that cytokinins are involved in controlling both root growth rate and the generation of new root meristems. The dose-response curve of root growth inhibition by exogenous cytokinin showed the transgenic roots to have cytokinin resistance (Fig. 3*D*). Interestingly, the resistance of *AtCKX1* transgenics to iPR was less marked than for *AtCKX2*, which is consistent with the smaller changes in iP-type cytokinins in the latter (Table 1).

Histology of the Shoot Meristem, Shoot Organs, and Root Meristems. A decreased or increased organ growth rate as a consequence of a reduced cytokinin content could be due to a changed cell division rate in the meristematic regions, a different population size of dividing cells, or altered cell growth. In the *AtCKX* transgenics, the final length of cells in the stem was not reduced, and the final length of root cells was slightly decreased (149.7 ± 31.7 μM in clone AtCKX1-50 versus 167.0 ± 32.0 μM in wild type; $n = 100$), indicating that differences in cell growth did not contribute to, or even partially compensate for, altered growth of stem and roots. However, microscopic inspection of the shoot apical meristem (SAM), leaf, and the root meristem revealed that the morphological changes described above were reflected in distinct changes in cell number and rate of cell formation in the *AtCKX* transgenics.

The SAM of *AtCKX1* transgenic plants was smaller than in wild-type plants and fewer cells occupied the space between the central zone and the peripheral zone of lateral organ formation, but the cells were of the same size and no obvious changes of the differentiation pattern occurred (Fig. 4*A*). Also, the overall tissue pattern of leaves in cytokinin oxidase overexpressers was unchanged. However, the sizes of both phloem and xylem were significantly reduced (Fig. 4*B*). In contrast, the average cell size of leaf parenchyma and epidermal cells was increased 4- to 5-fold (Fig. 4 *C* and *D*). New cells of *AtCKX1* transgenic leaves are formed at 3–4% of the rate of wild-type leaves, and final leaf cell number is estimated to be in the range of 5–6% that of wild type. Similar but less pronounced changes occurred in the shoot of *AtCKX2*-expressing plants (data not shown). In contrast to leaves, neither cell size nor cell form of floral organs was altered in the transgenic lines. Also, seed weight was similar in wild type and *AtCKX1* and *AtCKX2* transgenic plants (data not shown).

The cell population in root meristems in the *AtCKX1* and *AtCKX2* transgenic plants was enlarged approximately 4-fold, and the cell numbers in both the central and lateral columnella were increased (Fig. 4 *E* and *F*). Final root diameter was increased by 60% due to the increased diameter of all root cell types and an increased number of cells in each cell file. The radial root pattern was identical in wild type and transgenics, with the exception that frequently a fourth layer of cortex cells was noted in transgenic roots (Fig. 4*G*).

Discussion

This analysis of the consequences of reduced endogenous cytokinin content strongly indicates in which plant processes cytokinins are limiting and might, therefore, have a regulatory function. The slowed formation of new cells in the SAM, as well as of leaf primordia, and the reduced size of the SAM indicates that cytokinins have a dual function in the control of SAM proliferation. They are required to maintain the cell division cycle but might also be involved in promoting the transition from undifferentiated stem cells to differentiation. Earlier work has shown that in unorganized growing cells, cytokinins induce the formation of shoot meristems, demonstrating that they have a function beyond maintaining the cell cycle (2). Known coordinating factors of cell proliferation and differentiation in the SAM are transmembrane receptor proteins (e.g., CLV1) and transcription factors of the homeodomain class (e.g., WUS, STM, KNAT1), which interact in regulatory loops (19). Recent data indicate that a reciprocal interaction between cytokinins and some of these transcription factors exists (20–22). A role for cytokinins in the regulation of SAM differentiation could be realized through local gradients of the hormone or differences in the distribution of different cytokinin metabolites. This might alter effector gene expression quantitatively, which could in turn influence cellular fate. Developmental changes in the concentration and localization of different cytokinin metabolites have been reported for the SAM of tobacco (23). The reduced activity of the SAM could also be the cause of the incomplete apical dominance, which was noted in transgenic plants, as the amount of auxin produced for the maintenance of apical dominance might be lowered.

The slowed formation of leaf cells and their reduced number indicates an absolute requirement for cytokinins during leaf formation, both to drive the cell division cycle at normal speed and to obtain the required number of divisions for a normal leaf size. That cytokinins function as a regulatory factor in leaf cell formation is supported by the fact that transgenic *Arabidopsis* plants with an enhanced cytokinin content produced more leaf cells than control plants (20). Moreover, cytokinins appear to restrict leaf cell size as the cells of transgenic leaves are larger than in control plants. Alternatively, a compensatory mechanism

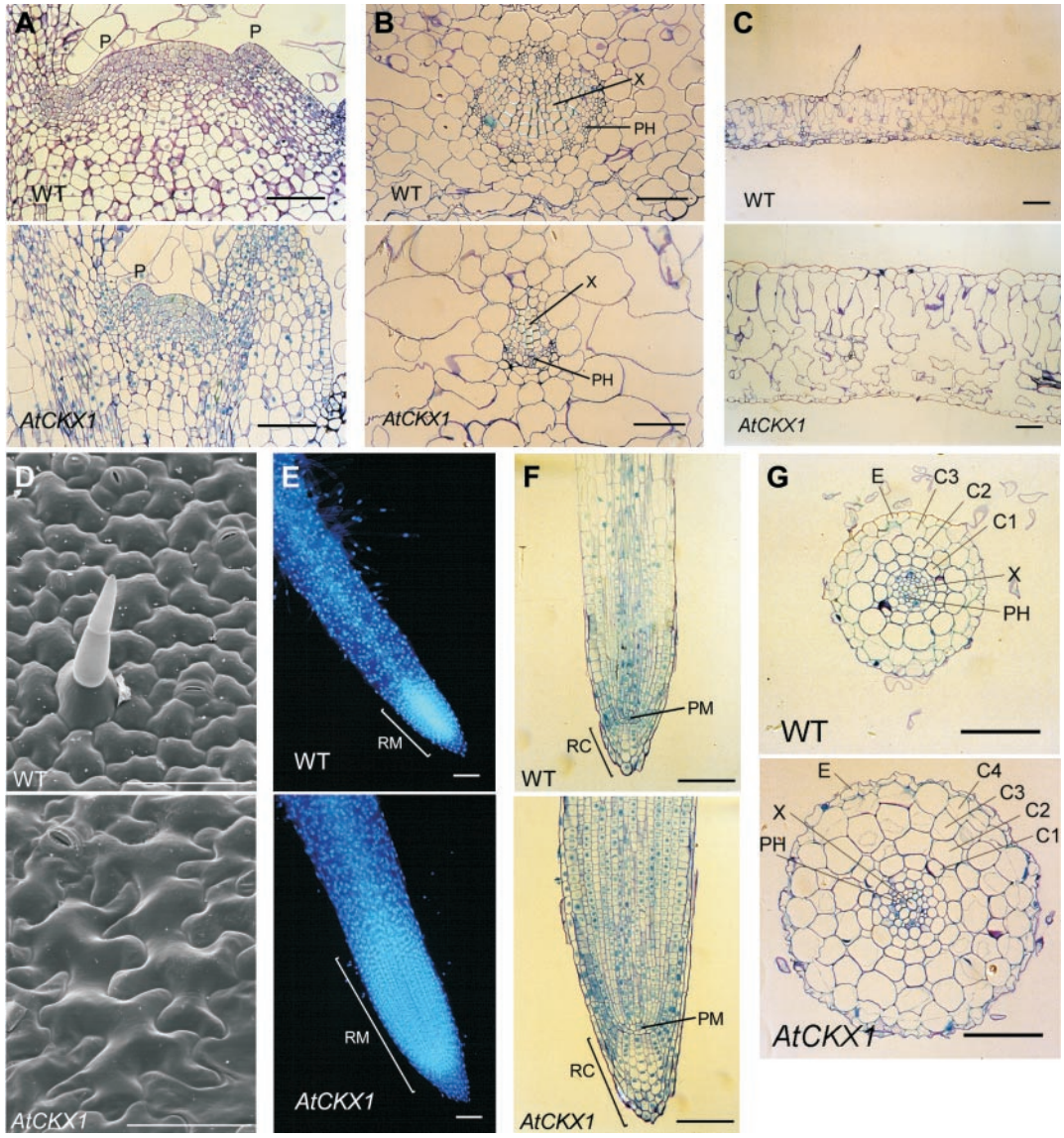

Fig. 4. Histology of shoot meristems, leaves, and root meristems. (*A*) Longitudinal median section through the vegetative SAM. P, leaf primordia. (*B*) Vascular tissue in second order veins of leaves. X, xylem, PH, a phloem bundle. (*C*) Cross sections of fully developed leaves. (*D*) Scanning electron microscopy of the upper leaf epidermis. (*E*) Root apices stained with 4′,6-diamidino-2-phenylindole. RM, root meristem. (*F*) Longitudinal median sections of root meristems 10 days after germination. RC, root cap; PM, promeristem. (*G*) Transverse root sections 10 mm from the apex. E, epidermis, C1–C4, cortical cell layer; X, xylem; PH, phloem. The material for the analysis of the SAM and the mature fully expanded leaves was from 38- and 100-day-old plants (clone AtCKX1-50), respectively, which were cultivated in a green house. Root analysis was performed with primary roots of seedlings 10 days after germination. Bars, 100 μm.

may be activated in transgenic plants to reach a genetically determined organ size, as has been reported for plants expressing a dominant-negative form of cdc2 (24). In either case, the leaf phenotype of *AtCKX* overexpressers supports the view that cell proliferation and growth in tobacco leaves are not coupled.

Interestingly, the flower phenotype of the transgenic plants was unaltered. This suggests that the role of cytokinins in the regulation of development of reproductive organs might be less important than it is during the vegetative phase. It may be that once the plant has entered the reproductive cycle, a more stringent mechanism operates in the meristem to ensure the proper course of the developmental program.

Contrasting with the promotive role in the SAM, cytokinins have a negative regulatory function in root growth. The increased cell number in the transgenic root meristems and the slightly reduced final cell length in transgenic roots indicate that the enhanced root growth is because of an enhanced cycling of cells rather than increased cell growth. In the presence of lowered cytokinin content, root meristem cells have a prolonged meristematic phase and eventually undergo additional rounds of mitosis before they leave the meristem and start to elongate. We conclude that the activity of the initials and/or the exit of cells from the root meristem is regulated by a mechanism that is sensitive to cytokinins.

Taken together, the investigation of cytokinin-deficient plants has shown that the influence of cytokinins on morphogenesis is primarily achieved through cell cycle regulation. Multiple functions and several molecular targets of cytokinins during different phases of the cell cycle are known. The hormone is required for S-phase entry in leaf mesophyll protoplasts and tobacco pith explants, and S-phase progression is accelerated in the presence of cytokinins (25, 26). Several cell cycle genes are regulated by cytokinins, including *cdc2*, *CycD3*, and different B-type cyclins (27–29). There is evidence that regulatory genes of the cell cycle

are expressed in a tissue-specific fashion and that cytokinin effects on the cell cycle vary between different cell types (30, 31). Distinct expression patterns of cytokinin targets could be a reason for the opposite effects seen in shoot and root meristems. The enhanced organ growth in plants overexpressing D- and B-type cyclins (32, 33) is consistent with the hypothesis that cytokinins act through the regulation of cell cycle progression.

What could the role of CKX proteins during plant growth and development be? A likely role is the degradation of cytokinins that accumulate transiently during the G_2/M transition of cycling cells to a level that is several orders of magnitude higher than during the other cell cycle phases (34). It is not known how these cytokinin levels are rapidly readjusted to normal levels. CKX enzymes could have a role in recycling this cell division-derived cytokinin. The expression of *AtCKX* genes in regions of active growth is consistent with the proposed function in cycling cells (unpublished results). Additional roles for the enzymes could be the maintenance of an optimal level of cytokinins for growth and/or resetting a cytokinin signaling system to a basal level.

To summarize, in this work we have obtained proof of the function of four cytokinin oxidases from *A. thaliana* and used these genes as tools to generate plants with a reduced cytokinin content. The data lend support to the auxin–cytokinin hypothesis for quantitative growth parameters and organ ratio in plants (2). However, we note that some, but not all, phenotypic changes can be explained by an altered ratio of these two hormones, as several aspects of auxin-overproducing plants are distinct from plants with a reduced cytokinin content (35, 36). This indicates that the auxin–cytokinin balance determines only a subset of morphogenetic parameters. Natural cytokinin levels are inhibitory to the development of a maximal root system, and fine adjustment of cytokinin levels is needed to achieve the optimal growth of shoots (20, 37). Evidently, the targeted manipulation of *CKX* gene expression can be an important and novel tool to modulate growth characteristics and yield parameters of crop plants.

We dedicate this article to C. O. Miller and the late F. S. Skoog, who discovered cytokinins almost 50 years ago and coined the auxin–cytokinin hypothesis of plant growth. We are indebted to K. Lemcke for initial help with gene cloning and sequence analyses, M. Riefler for support with structural gene analysis, M. Lenhard and Y. Stierhof for advise for microscopic analyses, and M. Kamínek for helpful comments on the manuscript. We thank H. Martínková and V. Lacmanová for excellent technical assistance and M. J. Beech and C. Scott-Taggart for proofreading. We acknowledge financial support of the Deutsche Forschungsgemeinschaft (Schm 814/13-1), the Volkswagen-Stiftung (I/72076), the Grant Agency of the Czech Republic (522/00/1346), and the Czech Ministry of Education (MSM 153100008).

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