The Plant Cell, Vol. 15, 2489–2492, November 2003, www.plantcell.org © 2003 American Society of Plant Biologists

IN THIS ISSUE

A New Classic of Cytokinin Research: Cytokinin-Deficient Arabidopsis Plants Provide New Insights into Cytokinin Biology

Cytokinins are a structurally diverse group of N^6-substituted purine derivatives capable of inducing plant cell division. The discovery of cytokinins by Folke Skoog and colleagues in the 1950s initially focused on kinetin, a synthetic compound derived from autoclaved salmon sperm DNA (Miller et al., 1955). Miller, Skoog, and others subsequently found that cytokinins are naturally occurring plant hormones that are essential for normal plant development. In a classic series of experiments, Skoog and Miller (1957) showed that the ratio of cytokinin to auxin in nutrient media profoundly influences the morphogenesis of roots and shoots in plant tissue culture. Numerous plant developmental processes have been found to be influenced by cytokinin, including cell expansion, inhibition of leaf senescence, chloroplast development, mobilization of nutrients, and root and shoot branching (reviewed by Mok, 1994).

Despite a wealth of knowledge on the effects of cytokinins on plant growth and development, a lack of specific mutants and specific inhibitors has retarded attempts to determine their in planta function(s). Rather, cytokinin function has been inferred from the effects of exogenous application or from increased accumulation of the hormone in transgenic plants expressing the bacterial cytokinin biosynthesis enzyme isopentenyl transferase. Because the artificial enhancement of hormone levels may cause artifactual, nonphysiological effects, hormone-deficient mutants are considered essential to determine in vivo function conclusively (Faure and Howell, 1999).

In this issue of *The Plant Cell*, **Werner et al. (pages 2532–2550)** contribute significant insights into cytokinin function and metabolism with an in-depth study of cytokinin-deficient transgenic Arabidopsis plants that overexpress members of the Arabidopsis cytokinin oxidase/dehydrogenase (*CKX*) gene family. CKX enzymes inactivate cytokinins irreversibly in a single enzymatic step. Cytokinin levels in plant tissues are determined by the rate (and location) of biosynthesis, the formation of inactive conjugates (mainly glycosides), and the rate of catabolism. Rapid turnover of cytokinin has been reported, and catabolism by CKX enzymes is believed to play a critical role in regulating cytokinin activity in vivo (Schmülling et al., 2003). The approach of Werner et al. overcomes the lack of cytokinin biosynthesis mutants and offers the opportunity for loss-of-function analysis. In addition, the authors present localization and expression analyses of different members of the CKX family in Arabidopsis (Figure 1).

THE *CKX* GENE FAMILY IN ARABIDOPSIS

CKX genes are found in plants and in bacteria. Phylogenetic analysis has indicated

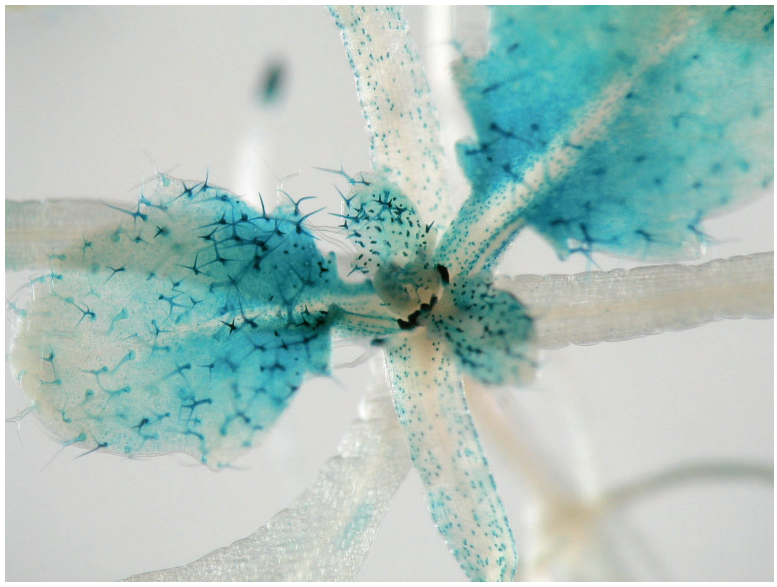

Figure 1. Expression Analysis of *CKX* Gene Promoters Fused to the Reporter Gene β-Glucuronidase Revealed Highly Specific and Individual Gene Expression Patterns for Each of the Six *CKX* Genes Analyzed.

Gene expression often was concentrated in regions of high mitotic activity, such as shoot apices, young leaves, developing stomata and trichomes, and developing floral organs, consistent with a primary role for cytokinin in regulating aspects of cell division and differentiation. *CKX* gene expression also was noted in tissues in which a functional requirement for cytokinin degradation was not anticipated. The localization of *CKX4-GUS* in a young Arabidopsis plant is shown. Expression is marked in dividing and endoreduplicating cells (e.g., developing trichomes and stipules).

IN THIS ISSUE

that the plant genes might have been acquired through a lateral transfer from bacteria to plants via the chloroplast, which is of cyanobacterial origin (Schmülling et al., 2003). There are seven *CKX* genes in Arabidopsis (described by Schmülling et al., 2003). Werner et al. characterized the expression of six of these (*CKX1* to *CKX6*) and constructed transgenic plants individually overexpressing each gene under the control of the strong 35S promoter of *Cauliflower mosaic virus*. The seventh member of the family, *CKX7*, was identified as a cDNA but until recently it was in an unsequenced region of the genome (Schmülling et al., 2003) and was not included in the study. The results were consistent with previous work by Werner et al. (2001) that examined cytokinin deficiency in tobacco plants overexpressing (individually) the Arabidopsis genes *CKX1* to *CKX4*. The current work broadens the scope of the investigation and presents new information on the *CKX* gene family and cytokinin function.

EFFECTS OF CYTOKININ DEFICIENCY

The authors obtained at least 10 independent transgenic lines for each of the six gene constructs, and all lines showed increased CKX activity compared with the wild type. Total cytokinin content measured in plants from two of the lines (overexpressing CKX1 and CKX2) was between 30 and 45% of wild-type concentrations, and all of the lines showed phenotypic traits, such as retarded shoot development and enhanced root growth, consistent with cytokinin deficiency, indicating that all six Arabidopsis genes encode functional CKX enzymes.

The cytokinin response system in the transgenic plants was analyzed by introgression of a cytokinin reporter gene, consisting of the cytokinin-sensitive promoter of the response regulator ARR5 and the β-glucuronidase gene. The significantly lower expression of the reporter gene in the transgenic background supported the analytical data showing reduced cytokinin content and revealed that this was not compensated for by enhanced sensitivity of the signaling system. This finding suggests that the signaling system is autonomous and not governed, at least in this case, by the hormone concentration. Therefore, the control of hormone metabolism itself is an important parameter in plant morphogenesis.

The major effects of cytokinin deficiency were reduced shoot development, leading to dwarfed late-flowering plants, enhanced root growth, and altered reproductive development. Light microscope examinations of plant tissues suggested that most of these effects resulted from the effects of cytokinin on shoot and root meristem activity. The size of the shoot apical meristem was reduced compared with that in the wild type. In the strongest CKX overexpressers, the growth of the shoot was arrested completely after germination, indicating that cytokinins are indispensable for the meristem. Evidently, cytokinins are required in this tissue to maintain the cell division cycle, but the change in shoot meristem size indicates that they also might be involved in promoting the transition from undifferentiated stem cells to differentiation. Similarly, the formation of leaf primordia was slower in cytokinin-deficient plants, and the number of leaf cells was reduced strongly. This underscores the positive regulatory role of the hormone in the shoot and suggests that cytokinins limit the number of leaf cell divisions. These results are not unexpected in light of the known ability of cytokinins to induce shoot formation in tissue culture. However, it was previously unknown whether cytokinins have a critical function during further shoot growth. Targeted expression of *CKX* genes should reveal whether cytokinin is required in specific subdomains and whether it has a role in cellular differentiation in addition to its function in regulating meristem activity quantitatively.

In contrast to the inhibitory consequences of cytokinin deficiency on shoot growth, root growth was enhanced in *CKX*-overexpressing plants. Changes in organ growth were traced back to changes at the cellular level. The root apical meristem was enlarged in cytokinin-deficient lines, and the induction of lateral roots occurred more frequently compared with that in the wild type. This was a surprising result and indicates that physiological levels of cytokinins limit root growth. By analyzing the expression of a cell cycle marker gene in the transgenic background, Werner et al. demonstrated that the control of the exit of cycling cells from the root meristem is a primary function of cytokinins in roots. Moreover, the results suggested that the hormone plays a significant role in the formation of adventitious roots and in the control of cell number in some cell layers of the radial axis. Root growth and branching are complex traits that are influenced by many factors. The demonstration of enhanced root growth by the introduction of a single dominant gene offers the opportunity to investigate the beneficial contributions of improved root growth for the acquisition of soil minerals and water, factors that limit growth and yield in most agricultural ecosystems (Lynch, 1995).

OBSERVATIONS ON CYTOKININ AND AUXIN INTERACTIONS, SENESCENCE, AND FERTILITY

The authors made a number of somewhat unexpected observations, some of which also were consistent with the previous results of Werner et al. (2001) in tobacco. First, in contrast to the inhibitory effect of reduced cytokinin on the shoot apical meristem, cytokinin-deficient plants showing severe phenotypic changes exhibited earlier growth of axillary branches compared with wild-type plants. This finding indicates decreased apical dominance, and indeed, auxin content, the major factor controlling apical dominance, was found to be significantly lower in shoots of cytokinin-deficient lines relative to wild-type lines. As demonstrated by the early work of Skoog and Miller (1957), interactions between cytokinin and auxin (indole 3-acetic acid and related compounds) are crucial in the control of plant morphogenesis. Exogenous application of these two hormones frequently causes antagonistic ef-

fects (e.g., shoot formation is inhibited by auxin and enhanced by cytokinin). Based largely on hormone overexpression studies, it has been suggested that cytokinin acts to reduce the pool of active auxin and vice versa (Coenen and Lomax, 1997; Eklöf et al., 1997, 2000). The mutual regulation of cytokinin and auxin metabolism could provide a mechanistic basis for their antagonistic activities. However, in this model, a reduction in auxin content in the shoots of cytokinin-deficient plants is unexpected. Werner et al. hypothesize that auxin content is reduced in these plants as a result of the reduction in the size of the shoot apical meristem and young leaves, which are the major sites of auxin biosynthesis. This suggests that more complicated mechanisms than mutual regulation of metabolism are necessary to explain the interactions of the two hormones.

A second unexpected observation was that leaves of cytokinin-deficient plants showed signs of delayed rather than accelerated senescence. Cytokinin has been reported to delay leaf senescence (Gan and Amasino, 1996), so it was predicted that cytokinin deficiency might promote senescence. Instead, chlorophyll was retained in cytokinin-deficient leaves longer than in wild-type leaves, particularly in regions adjacent to major veins. The authors concluded that decreasing the cytokinin content of leaves may be a prerequisite for senescence but not a signal that triggers its onset. The prolonged lifespan of leaves could be attributable to their retarded development or an improved nutritional status. Normal leaf senescence is marked by a number of characteristics in addition to the loss of chlorophyll, including the mobilization of nutrients and the enhanced expression of various senescence-associated genes (Quirino et al., 2000). Further investigation of these characteristics, as well as the introduction of cytokinin deficiency into known senescence mutants, might reveal more about the putative function of cytokinin in the control of senescence.

Finally, cytokinin-deficient plants showed a marked reduction in fertility. Flowers of transgenic plants appeared similar to wild-type flowers, but on closer examination were found to have fewer cells that were 80% larger than normal. The first flowers to develop produced no pollen, and later flowers yielded only a small amount of pollen relative to wild-type flowers. The resulting siliques of cytokinin-deficient plants carried only 8 to 20 viable seeds, whereas wild-type seeds typically produce as many as 60 seeds. Surprisingly, embryos of CKX1- and CKX3-expressing plants were twice the size of wild-type embryos, as a result of the enhanced number of cells and the increased cell size. These observations suggest that cytokinin plays a critical role in floral meristem activity as well as during gamete and embryo formation.

SUBCELLULAR LOCALIZATION OF CKX PROTEINS

Some of the most interesting results reported by Werner et al. focused on tissue and subcellular localizations of the six CKX proteins in Arabidopsis. The subcellular localization of three of the proteins (CKX1 to CKX3) was examined by fusing the C terminus of each protein to green fluorescent protein (GFP) and expressing the individual CKX-GFP constructs in a wild-type background under the control of the 35S promoter of *Cauliflower mosaic virus*. Patterns of GFP fluorescence together with amino acid sequence analyses suggested that all six proteins are targeted to the endoplasmic reticulum (ER) and the secretory pathway. GFP fluorescence suggested that CKX1 and CKX3 ultimately are targeted to the vacuole, whereas CKX2-GFP was detected in the ER. However, the CKX2 amino acid sequence does not include a consensus ER-retrieval motif, so it is likely that the protein normally is secreted, and the authors hypothesized that it may be released to the apoplast. The remaining proteins, CKX4 to CKX6, were predicted with high reliability scores to be targeted to the ER and the secretory pathway. Interestingly, CKX7, which was not examined in the current work, is different from the six other CKX proteins in that it does not contain a hydrophobic N-terminal region indicative of an N-terminal subcellular target sequence.

Different subcellular localizations of the CKX enzymes were correlated with differences in the expressivity of the cytokinin deficiency syndrome. Transgenic lines overexpressing enzymes with a vacuolar localization, such as CKX1 and CKX3, exhibited more severe phenotypic changes than those overexpressing enzymes with a predicted extracellular localization, similar to the results obtained in tobacco by Werner et al. (2001). This difference suggests that the vacuole is a stronger sink for cytokinin or that it is the sink for more active cytokinin metabolites.

The subcellular compartmentation of the enzymes and the prediction of extracellular and vacuolar cytokinin degradation sites were surprising. Virtually nothing was known previously about the subcellular location of cytokinin catabolism, and the specific functions of vacuolar versus extracellular CKX enzymes are not known. Functions in the control of cytokinin import and/or export in a given tissue, in the degradation of cell cycle–derived cytokinins, and in resetting the cytokinin-sensing system to a basal level have been suggested (Schmülling et al., 2003). A future challenge will be to dissect the frequently underscored role of subcellular compartmentation in cytokinin biology and in hormone biology in general.

DIFFERENTIAL TISSUE EXPRESSION OF *CKX* FAMILY MEMBERS

Analysis of gene expression using fusion constructs of *CKX* promoter regions and β-glucuronidase (GUS) showed that each of the six *CKX* genes exhibits specific and highly individual patterns of expression in Arabidopsis. For example, *CKX1* showed prominent expression in the vascular cylinder of lateral roots, whereas *CKX2* showed strongest expression in the shoot apex, and *CKX4* showed an interesting pattern of expression in developing trichomes, stomata, and stipules, as well as the root cap—mostly regions of high mitotic activity (Figure 1). In addition to differential pat-

IN THIS ISSUE

terns of expression in developing shoots and roots, *CKX5* showed strong expression in stamen primordia and developing pollen, whereas *CKX6* was expressed in the gynoecium at various stages of development. Although highly intriguing, the GUS expression analyses are preliminary and ultimately will need to be tested, for example by in situ hybridization techniques.

CONCLUSIONS

Together, the results presented by Werner et al. show that cytokinin deficiency leads to complex pleiotropic phenotypic alterations that cannot be explained completely with our current knowledge of cytokinin activities. A positive regulatory role for cytokinins in the shoot and a negative regulatory role in the root are firmly established. However, the mechanistic basis of the divergent functions in roots and shoots is not known. Comparisons with mutants that are altered in the expression of cell cycle genes indicate that cell cycle components might be a primary target of cytokinin in these tissues. Further studies that combine cytokinin deficiency with known mutations in key genes controlling meristem activity should reveal more about this critical process in plant growth and development in roots and shoots.

The cell- and tissue-specific expression of *CKX* genes may help clarify the specific functions of cytokinins. The highly specific tissue localization patterns and subcellular localization experiments suggested that the differential expression and subcellular localization of CKX family members play important roles in the fine control of cytokinin levels in shoot and root meristems and other mitotically active cells in which cytokinin likely functions in the control of cell division and differentiation. Overall, the work of Werner et al. represents a major advance in defining the in vivo role of cytokinin in plant growth and development.

Nancy A. Eckardt
News and Reviews Editor
neckardt@aspb.org

REFERENCES

Coenen, C., and Lomax, T.L. (1997). Auxin-cytokinin interaction in higher plants: Old problems and new tools. Trends Plant Sci. **2,** 351–356.

Eklöf, S., Åstot, C., Moritz, T., Blackwell, J., Olsson, O., and Sandberg, G. (1997). Auxin-cytokinin interactions in wild type and transgenic tobacco. Plant Cell Physiol. **33,** 225–235.

Eklöf, S., Åstot, C., Sitbon, F., Moritz, T., Olsson, O., and Sandberg, G. (2000). Transgenic tobacco plants co-expressing *Agrobacterium iaa* and *ipt* genes have wild-type hormone levels but display both auxin- and cytokinin-overexpressing phenotypes. Plant J. **23,** 279–284.

Faure, J.-D., and Howell, S.H. (1999). Cytokinin perception and signal transduction. In Biochemistry and Molecular Biology of Plant Hormones, P.J.J. Hooykaas, M.A. Hall, and K.R. Libbenga, eds (Amsterdam: Elsevier Science), pp. 461–474.

Gan, S.S., and Amasino, R.M. (1996). Cytokinins in plant senescence: From spray and pray to clone and play. Bioessays **18,** 557–565.

Lynch, J. (1995). Root architecture and plant productivity. Plant Physiol. **109,** 7–13.

Miller, C.O., Skoog, F., von Saltza, M.H., and Strong, M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. J. Am. Chem. Soc. **77,** 1329–1334.

Mok, M.C. (1994). Cytokinins and plant development: An overview. In Cytokinins: Chemistry, Activity and Function, D.W.S Mok and M.C. Mok, eds (Boca Raton, FL: CRC Press), pp. 155–156.

Quirino, B.F., Noh, Y.S., Himelblau, E., and Amasino, R.M. (2000). Molecular aspects of leaf senescence. Trends Plant Sci. **5,** 278–282.

Schmülling, T., Werner, T., Riefler, M., Kruplová, E., and Bartina y Manns, I. (2003). Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, *Arabidopsis* and other species. J. Plant Res. **116,** 241–252.

Skoog, F., and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp. Soc. Exp. Biol. **11,** 118–131.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in regulating shoot and root meristem activity. Plant Cell **15,** 2532–2550.

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA **98,** 10487–10492.

A New Classic of Cytokinin Research: Cytokinin-Deficient Arabidopsis Plants Provide New Insights into Cytokinin Biology

Nancy A. Eckardt

Plant Cell 2003;15;2489-2492
DOI 10.1105/tpc.151110