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New Insights into the Functions of Cytokinins in Plant Development

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Abstract

Recent breakthroughs in cytokinin research have shed new light on the role of cytokinin in plant development. Loss-of-function mutants of a cytokinin receptor reveal a role for the hormone in establishment of the vasculature during embryonic development. Cytokinin controls the number of early cell divisions via a two-component signaling system. Genetically engineered plants that have a reduced cytokinin content demonstrate the regulatory role of the hormone in control of meristem activity and organ growth during postembryonic development, with opposite roles in roots and shoots. There is increasing evidence from work with transgenic plants and mutant analysis that cytokinins do not perform the previously proposed function as a root-derived signal for the regulation of shoot branching. Root-borne cytokinins might serve as a long-range signal controling other processes at distant sites, such as responding to nutritional status, particularly nitrogen availability.

Key words: Cytokinin; Cytokinin Oxidase; Cytokinin receptor; Plant Development; Hormone signaling; Apical dominance

Introduction

Major advances have been made recently in cytokinin biology that have provided us with new insights into the role of this hormone in plant development. The identification of cytokinin receptors (Inoue and others 2001; Suzuki and others 2001), early response genes (Brandstatter and Kieber 1998; Taniguchi and others 1998), biosynthetic genes (Kakimoto 2001a; Takei and others 2001a), genes of cytokinin catabolism (Houba–Hérin and others 1999; Morris and others 1999; Bilyeu and others 2001; Werner and others 2001), and cytokinin glycosyltransferases (Martin and others 1999a, 1999b, 2001) has provided new tools to analyze the role of cytokinins in plant development. This review article summarizes recent results pertaining to classic questions in cytokinin biology that have been obtained using these novel tools.

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Loss of Cytokinin Receptor Function Reveals a Role for Cytokinins in the Formation of the Embryonic Vasculature

The first cytokinin receptor gene identified was *CRE1/AHK4/WOL* (Mähonen and others 2000; Inoue and others 2001; Suzuki and others 2001), which will be referred to hereafter as *CRE1/WOL*. This gene encodes a histidine kinase typical of two-component regulatory systems, a type of signal transduction machinery found in prokaryotes, lower eukaryotes, and plants. CRE1/WOL has structural features characteristic of eukaryotic sensor kinases: an extracellular input domain and cytoplasmic

kinase transmitter and receiver domains. The functionality of these domains has been shown by mutational analysis (Mähönen and others: 2000; Inoue and others 2001; Suzuki and others 2001). Meanwhile it was shown that *AHK3* and possibly *AHK2,* two other members of this His-kinase gene family, also encode cytokinin receptors (Yamada and others 2001; T. Kakimoto personal communication). This links the cytokinin-signaling pathway to the two-component signaling network in plants (for detailed reviews of these aspects see Haberer and Kieber 2002; Schmülling 2001).

The different members of the cytokinin receptor gene family are differentially expressed (Mähönen and others 2000; Inoue and others 2001; Ueguchi and others 2001) The earliest detectable expression of *CRE1/WOL* is in the four innermost cells of the globular embryo, which are the precursors to the vascular tissue (Mähönen and others 2000). Its expression remains associated with the provascular tissue throughout later stages of embryogenesis (Figure 1A). In the primary root, the gene is expressed in the vascular cylinder and in the pericycle. *CRE1/WOL* mRNA is also detectable in shoot tissue during postembryonic development, although in lower abundance than in the roots (Mähönen and others 2000; Ueguchi and others 2001). Other members of the cytokinin receptor gene family, *AHK2* and in particular *AHK3,* have greater expression in the aerial parts of *Arabidopsis* wild-type plants (Ueguchi and others 2001).

CRE1/WOL is the only member of the cytokinin receptor gene family for which a loss of function study has been performed. Consistent with its expression pattern, the phenotype of *cre1/wol* mutants affects mainly the vasculature. The first visible defect in *wol/crel* mutant plants is the absence of cell divisions in the embryonic axis during the late stages of embryogenesis. The embryonic axis produces vascular tissue from the vascular initials. Reduced cell divisions in the embryonic axis of *cre1/wol* mutants lead to fewer vascular initials, which subsequently form only protoxylem cells but no metaxylem or phloem cells (Scheres and others 1995; Mähönen and others 2000) (Figure 1B). In turn, this leads to the formation of primary roots with a narrower vascular cylinder composed only of protoxylem cell files. The primary root meristem of *wol* mutants lacks periclinal divisions of the procambium. The hypocotyl, which is also derived from the embryonic axis, shows the same radial pattern defect as roots. It was shown that introgression of the *fass* mutation in the *cre1/wol* background enhances the cell number in the embryonic axis and restores the wild-type phenotype (Scheres and others 1995).

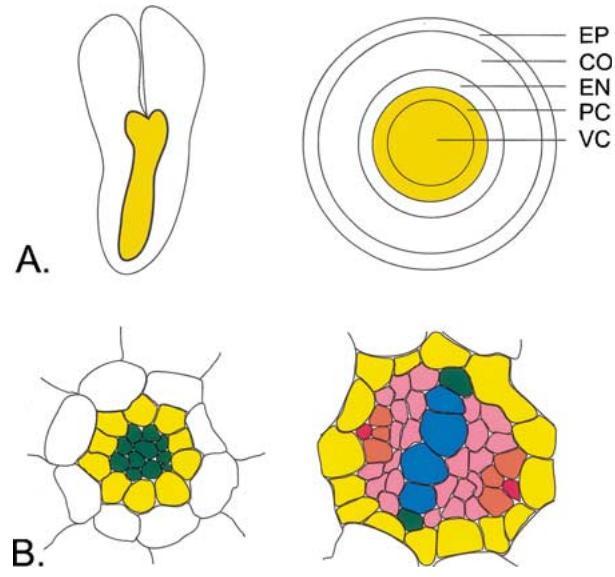

Figure 1. Expression of the cytokinin receptor gene *CRE1/WOL* and phenotype of receptor mutants. (**A**) Expression domains of the *CRE1/WOL* gene in *Arabidopsis* embryos at the heart-shape stage. The expression domain is indicated by the dark yellow area. (**B**) Transverse section of a primary root of wild-type *Arabidopsis* (left) and the *cre1/wol* mutant. The data are from Mähönen and others (2000). Abbreviations: CO-cortex cells; EN-endodermis; ER-epidermis; PC-pericycle; VC-vascular cylinder. Color code: White-endodermis; yellow-pericycle; red-protophloem; orange-metaphloem; green-protoxylem; blue-metaxylem; Pink-procambium.

This suggests that the lack of early cell divisions rather than the lack of specification of metaxylem and phloem cells causes the phenotype, although it is surprising that the final number of protoxylem files in *wol* mutants is even higher than in wild type (Figure 1B). According to this model, the reduced number of vascular initials that are formed in *cre1/wol* embryos are used up for protoxylem formation and none are left over to form metaxylem and phloem (Scheres and others 1995; Mähönen and others 2000). These findings establish an important role for cytokinins in the formation of embryogenic pattern and, more specifically, in vascular morphogenesis, *wol/cre1* mutant seedlings are resistant to cytokinin in a root elongation assay (Inoue and others 2001). They also demonstrate a role for the receptor in mediating cytokinin signals to the root meristem, whose activity is indeed controlled by cytokinin (see below).

How does cytokinin signaling mediate cell cycle control and how is this integrated with other cytokinin-regulated developmental processes? There is now compelling evidence that cytokinin signaling

in *Arabidopsis* occurs via a receptor-phosphotransfer protein-response regulator phosphorelay that is typical of eukaryotic two-component systems (see Deruère and Kieber, this issue). The various components of such a system are encoded in the *Arabidopsis* genome and include five putative AHP histidine phosphotransfer proteins (AHP1–AHP5) and 22 genes encoding at least two different types of two-component response regulators (type-A and type-B ARR genes) (D'Agostino and Kieber 1999; Imamura and others 1999). The expression of the type-B ARR transcripts are not regulated by cytokinins. The type-B ARRs have a receiver domain and a C-terminal output domain that contain putative nuclear localization signals and a GARP domain that is related to the DNA binding motives of the bHLH and MYB classes of transcription factors (Riechmann and others 2000). The type-B ARRs are localized in the nucleus and act as transcriptional activators (Lohrmann and others 1999; Sakai and others 2000, 2001). The type-A ARRs lack the transcriptional activator domain and their RNA levels are rapidly upregulated by cytokinins (Brandstatter and Kieber 1998; Taniguchi and others 1998; Kiba and others 1999; D'Agostino and others 2000).

Models for cytokinin signaling that involve these elements are shown in Hwang and Sheen (2001) and Deruère and Kieber (this issue). A cytokinin signal initiates a phosphorylation cascade involving a receptor and a histidine phosphotransfer protein (AHP) that results in the activation of type-B response regulators (Hwang and Sheen 2001). Subcellular localization studies revealed that AHP1 and AHP2, but not AHP5, are transiently translocated from the cytoplasm to the nucleus in response to exogenous cytokinin, and once in the nucleus they are postulated to activate type-B ARRs (for example, ARR1, ARR2, ARR10) (Hwang and Sheen 2001). In response to cytokinin, activated type-B ARRs are thought to bind to *cis* elements in the promoters of target genes and activate transcription. Overexpression of type-B ARR resulted in direct activation of a reporter target gene, suggesting that they are a rate-limiting factor for the cytokinin response (Hwang and Sheen 2001). Interestingly, mutation of the phosphorylation site of type-B ARRs did not abolish their activating capability. This indicated that phosphorylation is not required for transcriptional activation but has a different function, for example, releasing sequestered type-B ARRs from a negative regulator protein (Hwang and Sheen 2001). ARR2 Overexpression promoted cytokinin-independent cell proliferation and shoot and leaf formation, indicating that this type-B response regulator might be a central regulator of cytokinin responses (Hwang and Sheen 2001).

Likely targets for transcriptional activation by cytokinin-activated type-B ARR proteins are genes encoding type-A ARRs (Hwang and Sheen 2001; Sakai and others 2001). Type-A ARR proteins may in turn modulate the output of cytokinin signalling via interactions with other members of the signaling chain or, alternatively, may by themselves regulate other downstream targets. Indeed, overexpression of several type-A ARRs in transgenic *Arabidopsis* represses certain cytokinin responses. For example, overexpression of ARR4, ARR5, ARR6, and ARR7 repressed cytokinin induction of a *ARR6* reporter gene in *Arabidopsis* mesophyll protoplasts (Hwang and Sheen 2001) and overexpression of ARR8 decreased shoot formation from stem explants and the level of expression of two genes (*cycDS, cab*) that are known to be under cytokinin control (Osakabe and others 2001). This result is compatible with a negative regulatory function of type-A ARRs in cytokinin signal transduction. However, ARR4 has been reported to enhance shoot formation, indicating that negative feedback regulation might not be the only function of type-A ARRs (Osakabe and others 2001). Other targets of type-B ARRs have been reported as well. ARR2 was shown to be highly expressed in pollen grains and to bind specifically a pollen-box DNA element in the promoter of nuclear genes that code for components of the mitochondrial respiratory complex (so-called *nCI* genes) (Lohrmann and others 2001). Certainly there are additional regulatory targets to be identified among the numerous known cytokinin-regulated genes (Schmülling and others 1997).

In conclusion, the multiple elements of the plant two-component signaling system may offer the diversity required to integrate a simple hormone signal into the diverse developmental processes that are regulated by cytokinins. For example, differences in signal interpretation and output can be reached by combining different parts of the signaling chains in a cell-specific fashion. This system may also crosstalk with other signaling pathways such as those of known co-actors of cytokinin, ethylene and light (Cary and Howell 1995; Sweere and others 2001). For example, activated ARR4 interacts with the photoreceptor phytochrome B and stabilizes its active form, which suggests that ARR4 may link cytokinin and light signaling (Sweere and others 2001). Using forward and reverse genetics, the pathways and biological processes governed by cytokinins, either through individual receptors and/or receptor combinations, should be eludicated. Such studies should shed light on which other

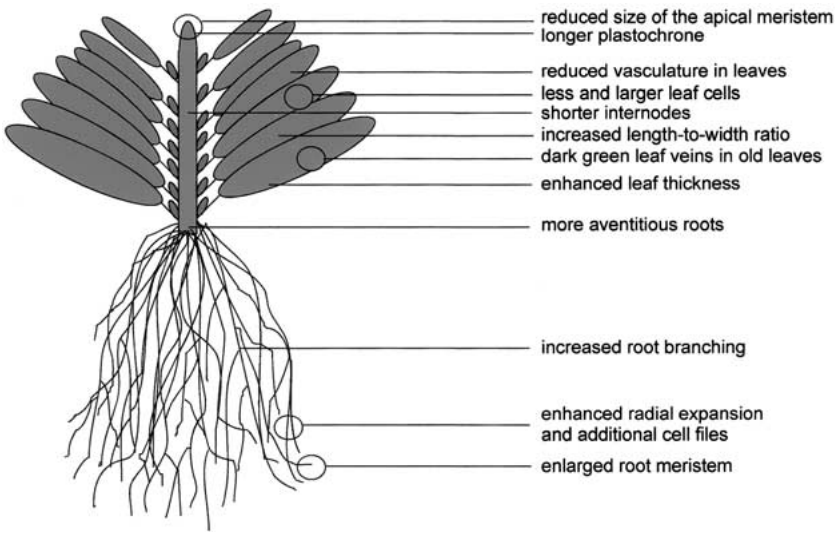

Figure 2. Schematic representation of phenotypic changes observed in transgenic tobacco plants that have a reduced cytokinin content due to expression of *AtCKX* genes.

molecular components participate in the fine tuning of classical cytokinin functions, like leaf greening and shoot meristem activity, that might be controlled by the two-component system.

Plants with Reduced Cytokinin Content Confirm Multiple Role for Cytokinins in Plant Development

Most, if not all, of our previous knowledge about the role of cytokinins in plant development was derived from experiments that involved the exogenous addition of cytokinins or an endogenously enhanced cytokinin synthesis and the analyses of its phenotypic consequences. Additional data of correlative nature were obtained from observing the changes in endogenous cytokinin content during developmental changes. However, neither biosynthetic mutants nor well-defined cytokinin signaling mutants have been available until recently, and specific inhibitors of cytokinin synthesis or activity have not been widely used. These types of gain-of-function experiments are not fully conclusive. It could be that additional cytokinin affects processes that are normally not under cytokinin control. For example, the inhibitory effect of exogenous cytokinin on root elongation does not mean necessarily that cytokinins have a physiological role in control of root elongation.

A recent study on plants that were genetically engineered to contain reduced cytokinin concentrations confirmed many but not all of the previous assumptions (Werner and others 2001). The study was made possible by the identification and cloning of a cytokinin oxidase/dehydrogenase gene of *Zea mays* (*ZmCKX1*) (Houba–Hérin and others 1999; Morris and others 1999). Distantly related sequences were identified in *Arabidopsis* and shown to encode functional CKX enzymes (Bilyeu and others 2001; Werner and others 2001). The *Arabidopsis* cytokinin oxidase/dehydrogenase gene family currently comprises seven members: *AtCKX1–AtCKX7* (for recent results indicating that the cytokinin-degrading enzyme is a dehydrogenase rather than an oxidase, see Galuszka and others 2001). Four *AtCKX*s contain N-terminal signal peptides that possibly direct the proteins to the secretory pathway; two signal peptides predict transport to mitochondria; and a single gene family member has a predicted cytosolic location (Bilyeu and others 2001; Werner and others 2001; K. Bilyeu and T. Werner personal communications).

Transgenic tobacco plants expressing any one of four *Arabidopsis AtCKX* genes displayed reduced cytokinin content and showed distinct developmental alterations of the shoot and root (Figure 2). *AtCKX* transgenic plants were dwarfed with shorter internodes; they flowered later, formed fewer flowers, and had a reduced leaf surface with a smaller vasculature. Histological analyses showed that the shoot apical meristem was smaller, but cell size was not altered. The formation of new leaf primordia (plastochrone) and the formation of new leaf cells was slowed significantly. In total, only about 5% of leaf cells of wild-type plants were formed in *AtCKX*-expressing plants indicating a stringent requirement of cytokinins for leaf cell formation (Werner and others 2001). The reduced number of cell divisions could involve the AINTEGUMENTA (ANT) protein, a member of the APETALA2-like family of transcription factors. Overexpression of the *ANT* gene

leads to an additional round of cell division in the *Arabidopsis* leaf (Mizukami and Fischer 2000). Whether *ANT* is regulated by cytokinins is currently not known.

The shoots of transgenic plants were also characterized by the slow growth of all lateral buds, which led to the formation of two tiny leaves and no outgrowth of buds (Werner and others 2001). After release of apical dominance, for example, at the onset of flowering, numerous side branches were formed. In contrast, buds remained completely dormant in wild type throughout the vegetative growth period and only one or two apical branches started growing after release of apical dominance. The altered growth pattern is somewhat surprising, as the auxin/cytokinin balance hypothesis would have predicted enhanced apical dominance in plants with a lowered cytokinin content. The altered pattern was interpreted to be the consequence of the reduced activity of the dominant apical bud, which could lead to a reduced auxin formation and, subsequently, to reduced apical dominance (Werner and others 2001). Cytokinin can retard leaf senescence (see, for example, Gan and Amasino 1995) and it has been suggested that a reduction in the cytokinin concentration below a threshold level could serve as a signal to trigger leaf senescence. However, visual leaf senescence did not occur early in *AtCKX* overexpressers. In contrast, older leaves remained green (with the exception of intercostal regions) leading to a prolonged lifespan of individual leaves. This phenotype suggests that cytokinins are not a physiological signal that triggers the onset of senescence.

In contrast to shoot growth, root growth was enhanced in the *AtCKX* expressers (Werner and others 2001). This was due to a more rapid elongation of the primary root and lateral roots, an increased formation of lateral roots, and an increased number of adventitious roots. Changes in organ growth were traced back to changes at the cellular level. The root meristems of *AtCKX* transgenics were larger than in wild-type plants. The number of columnella tiers and the number of cells in individual cell files of the cell division zone were higher than in wild type. This suggested that cytokinins may regulate the numbers of divisions of cells before they leave the meristem. In summary, this result suggests that cytokinins may have a physiological role as a negative regulator of root growth. An increased meristematic cell population could be a primary reason for the increased root growth, as it enhances cell production even if cell division rates remain constant. The final cell length of root epidermal cells was similar in *AtCKX* expressers and wild type (Werner and others 2001). Whether the rate of expansion of cells differs between wild type and transgenics was not analyzed. The observation that the exit of a cell from the meristem is sensitive to the level of cytokinins raises the possibility that the cytokinin level is involved in the regulation of the poorly understood process of cell cycle counting, a prerequisite to controling meristem size.

Molecular elements that link cytokinin and root meristem activity are currently not known. Candidate proteins are cyclin B1 and STP1. The root growth of *cycB1* overexpressers (Doerner and others 1996) shares some features with *AtCKX*-expressing roots as they grow faster than wild-type roots. However, a direct comparison is not possible as information about cell size and number in these roots is lacking. Root expansion of the *Arabidopsis stunted plant 1* (*stp1*) mutant is resistant to cytokinins, while the callus growth response of the mutant towards cytokinin was similar to wild type (Baskin and others 1995). This indicated that STP1 is specifically required to mediate cytokinin effects on root expansion. Cytokinin treatment of wild-type roots phenocopied the *stp1* mutation (Beemster and Baskin 2000). It has been proposed that STP1 is an elongation-promoting protein and that cytokinin-dependent inhibition of root growth requires down-regulation of STP1 (Baskin and others 1995). According to this model, less cytokinin in *CKX* overexpressers would lead to an increased level of STP1 and, in turn, to an increase of root elongation.

Taken together, these data lend support for the earlier idea that the cytokinin level, together with auxin, is important for determining the relative growth rate of roots and shoots (Skoog and Miller 1957). Regulation of the cell cycle and the number of cycles that cells undergo in the meristems and organ primordia are the primary regulatory targets of cytokinins. The hormone is a limiting factor for cell division activity in the shoot and has a negative regulatory function in the root. The observation that transgenic plants with reduced cytokinin content display reduced apical dominance and wild-type leaf senescence is surprising but is consistent with recent interpretations of the role of cytokinins in these processes (see below). However, as a note of caution, it should be kept in mind that the changes observed in the *AtCKX* transgenic plants depend upon the promoter driving gene expression. For example, it is possible that effects on early embryogenesis are missed because the 35S promoters are not very active during this developmental stage (Custers and others 1999). Likewise, an overall reduction of cytokinins in leaves might generally interfere with the normal sink-source partition and, in turn, cause side effects that are not directly due to

an altered cytokinin content. Therefore, studies including cell- or tissue-specific expression of the cytokinin-degrading enzymes are required.

Analyses of Conditional Transgenic Cytokinin Overproducers and Novel Mutants Argue Against a Role for Cytokinins as a Long-Range Root-Borne Signal in the Regulation of Shoot Branching

Roots, and in particular root-tips, are a rich source of cytokinins, which are transported from the root to the shoot (Letham 1994). Numerous regulatory functions have been proposed for root-borne cytokinin. One is the functional counterpart of auxin in the regulation of shoot branching, auxin being inhibitory to lateral bud growth and cytokinin stimulatory to bud growth. The antagonistic actions of auxin and cytokinin on bud growth was reported soon after the discovery of cytokinins (Wickson and Thimann 1958). Two models have been proposed to account for the involvement of root-borne cytokinin in the regulation of shoot branching. One model proposes that gradients of auxin and cytokinin along the shoot axis control shoot branching, with the ratio of the two hormones being the relevant factor (Stafstrom 1993). A second model proposes that auxin negatively regulates the export of cytokinin from the root. In fact, following removal of the shoot apex, cytokinin export from roots increases, which can be blocked by application of auxin to the decapitated stump (Bangerth 1994). Although there is ample evidence that cytokinins are exported from the root and that treatment with cytokinins causes growth of lateral buds (reviewed by Cline 1991), it has never been demonstrated unequivocally that it is root-derived cytokinin that triggers the release of lateral buds from dormancy after reducing apical dominance. The following section reviews experiments with transgenic plants in which systemic and local cytokinin syntheses are controllable and the site of cytokinin synthesis and the proposed site of action can be separated experimentally. These plants have been used to examine the relevance of cytokinin synthesis in the root, or in the bud itself, to bud growth. The outcome of these experiments argues against a role for root-derived cytokinins in the control of shoot branching.

Faiss and coworkers (1997) used a tetracycline (tc)-dependent gene expression system to conditionally control *IPT* gene expression and cytokinin enhancement in transgenic tobacco plants. Exogenous addition of tc to single buds of these plants caused the treated buds to grow, indicating that local synthesis of cytokinins is sufficient to trigger this effect (Figure 3A). Feeding of tc through roots of hydroponically grown transgenic plants led to release of all lateral buds from dormancy (Figure 3A). This could be due to either locally enhanced cytokinin production because of transport of tc to the aerial plant part or to enhanced cytokinin export from the root. To answer this question, reciprocal grafts between wild-type plants and *IPT* transgenics were performed, thereby restricting the response to tc and enhanced cytokinin synthesis to either the rootstock or the scion. The ability of the plant to transport tc to aerial parts and to cause bud breakage was clearly demonstrated when *IPT* transgenics were grafted on wild-type root stocks. As a consequence of tc feeding to roots, all lateral buds of the transgenic graft part were released from dormancy. Also, when wild-type scions were grafted onto transgenic root stocks, the phenotypic consequences of tc feeding through roots were localized to the transgenic tissue, although an increased amount of cytokinins reached the transpiration stream (Faiss and others 1997). Even with simultaneous tc treatment, removal of the apex reduced the apical dominance in the wild-type scion, and only the two apical buds of the scion started to grow just as it happens in ungrafted wild-type plants. This indicated that no activity derived from the dominant apical bud suppressed the cytokinin effect on the lateral buds. The authors concluded that these results question the classical view of the role of cytokinins as a root-borne signal in the control of shoot apical dominance and proposed a function in paracrine signaling, that is, restricted to the vicinity of the site of production (Faiss and others 1997).

In an extension of the above experiments, Böhner and Gatz (2001) constructed transgenic tobacco plants that harbor the *IPT* gene under control of a dexamethasone (dx)-inducible and tc-repressible promoter (dx-on/tc-off system). Systemic induction of enhanced cytokinin synthesis by dx caused outgrowth of all lateral buds. Simultaneous treatment of selected buds with the anti-inducer tc suppressed growth in these buds (Figure 3B). Continued tc-mediated suppression of lateral bud growth was even effective when the rest of the plants showed pleiotropic consequences of the enhanced cytokinin content (for example, ectopic shoots, leaf greening). Control experiments showed that tc did not inhibit the outgrowth of buds in general, proving that the effect was due to the specific down-regulation of the

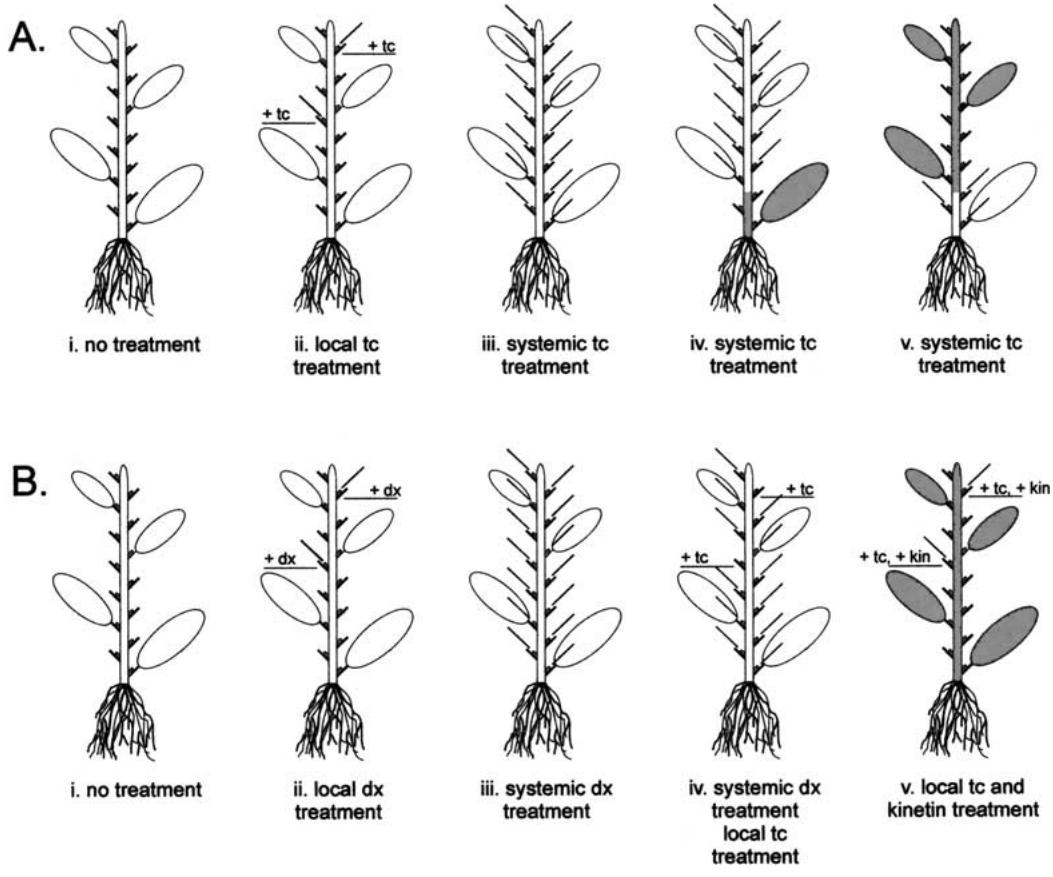

Figure 3. Experiments with conditionally cytokinin-overproducing transgenic tobacco plants that investigate the question of whether cytokinins have a function as long-range root-to-shoot signal in the regulation of shoot branching. **(A)** Experiments carried out with plants harboring a tc-inducible *IPT* gene *(35So::IPT;* Faiss and others 1997). **(i)** Non treated transgenic plants look similar to wild type and are not branched. **(ii)** Local induction of the *IPT* gene by tc only induces growth of the induced bud. **(iii)** Systemic *IPT* gene induction by feeding tc through the root system leads to release of all lateral buds from dormancy. **(iv)** Transgenic scion grafted on a wild-type root stock. Tc feeding through roots induces growth of all buds in the transgenic scion. **(v)** Wild-type scion grafted on a transgenic root stock. Tc feeding through roots induces growth of all buds in the transgenic root stock. Buds of the wild-type scion remain inhibited. **(B)** Experiments carried out with plants harboring the *IPT* gene under control of a dx-on/tc-off promoter (Böhner and Gatz 2001). **(i)** Nontreated transgenic plants look like wild type and are not branched. **(ii)** Local induction of the *IPT* gene by dx induces only growth of the induced bud. **(iii)** Systemic *IPT* gene induction by feeding dx through the root system leads to release of all lateral buds from dormancy. **(iv)** Like in **(iii)** but selected buds were treated with tc. Only tc-treated buds do not grow. **(v)** Control experiment for iv. Simultaneous treatment of single buds with kinetin and tc induces growth of the bud, demonstrating that tc has no inhibitory effect on bud growth. Arrows indicate growths of lateral buds following the indicated treatment. Shaded plants and graft parts are wild type. Abbreviations: dx-dexamethason; kin-kinetin; tc-tetracycline.

transgene. This suggests that buds that are compromised for enhanced local cytokinin synthesis do not perceive a mobile cytokinin signal from elsewhere and that local cytokinin synthesis is needed to release buds from dormancy.

These data are in conflict with those of McKenzie and colleagues. (1998) who used a root-specific copper-inducible gene expression system to regulate *IPT* gene transcription. After application of copper, lateral bud growth was detected in the whole plant, which would support the classical concept that apical dominance is influenced by root-borne cytokinin. However, McKenzie and coworkers (1998) could not exclude the possibility that the Cu-inducible branching phenotype is due to residual activity of the chimeric transgene in the shoot. Root specificity of the promoter was determined using *GUS* as a reporter (Mett and others 1996). However, that reporter might not be sensitive enough to detect low-level transcriptional enhancement that may be sufficient to cause morphological changes as a result of *IPT* gene expression.

In conclusion, the data obtained from *IPT* transgenic tobacco plants indicate that cytokinins are not

the mobile signal that informs the lateral bud about the loss of the shoot apex. This function must be executed by other signals, which in turn could trigger local cytokinin synthesis. The differentially and locally restricted expression of members of the *Arabidopsis* cytokinin-synthesizing *AtIPT* gene family in shoot tissues is in accordance with this proposal (Kakimoto 2001b). In fact, an expression analysis of *AtIPT* genes after decapitiation indicated cytokinin synthesis at the node (Shimizu–Sato and Mori 2001).

The conclusion that signals other than root-derived cytokinins play a role in branching control is supported by the analysis of branching mutants. Investigation of the *ramosus* (*rms*)-increased-branching mutants of pea has provided evidence that factors other than the auxin/cytokinin ratio are involved in regulating branching. Four of the five *rms* mutants (*rms1, rms3, rms4, rms5*) with increased shoot branching have reduced cytokinin concentrations in the root xylem sap (Beveridge and others 1997a, 1997b; Morris and others 2001). This showed that branching in pea is associated with the down-regulation of root cytokinin export, probably under the control of the shoot apices. Grafting different shoot and rootstock combinations indicated that two of the studied loci, Rms1 and Rms5, cause increased branching through alteration of a long-distance, acropetally moving signal that is not cytokinin (Morris and others 2001; Foo and others 2001). It is noteworthy that the strongly reduced cytokinin export from roots in branched mutants led to neither a reduced cytokinin content of leaves nor an earlier onset of leaf senescence (C. Beveridge personal communication) suggesting that the amount of cytokinin export from roots might be less relevant for the regulation of the onset of leaf senescence than was thought previously.

Cytokinins Carry Nutritional Information Over Long Distances and Coordinate Root and Shoot Development

What functions could root-derived cytokinins have in the shoot if not the regulation of branching? Earlier investigations have yielded indications that cytokinins could carry information about the nutritional status of organs. For example, in barley roots cytokinin accumulates in response to an increased nitrogen supply (Samuelson and others 1992). In *Urtica dioica,* the amount of total cytokinins exported by the root is higher in nitrogen-sufficient plants than in nitrogen-depleted plants (Beck and Wagner 1993). Cytokinin

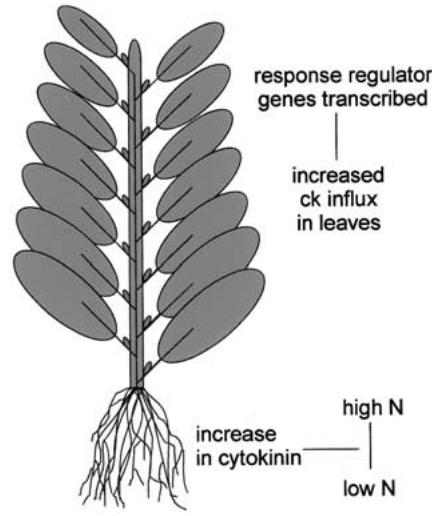

Figure 4. Model for a role of cytokinin in long-distance root-to-shoot signaling. An increase in nitrogen supply to nitrogen-depleted roots causes an increase in cytokinin content in roots, then in the xylem sap and finally in leaves, where cytokinin signals via the two-component system. In turn, transcription of response regulator genes is switched on.

accumulates in maize roots in response to an improved N supply (Sakakibara and others 1998). Yong and coworkers (2001) showed that increased N supply increases the actual delivery rate per unit leaf area of cytokinin to the leaves of cotton. The enhancement of delivery rate was dependent on the CO_2 concentration in the air (Yong and others 2001). Taken together, these data indicated a role for cytokinins in root–shoot communication for N nutrition (Figure 4).

Recent data provide the first clues to understanding the molecular mechanism of N-dependent regulation. Sakakibara and colleagues (1998) showed that *ZmRR* (formerly *ZmCIP1*), a response regulator gene of *Z. mays,* is rapidly induced in leaves either by the addition of cytokinin or by supplying nitrate or ammonium ions to the roots. Similarly, transcripts of different type-A response regulator genes of *Arabidopsis* accumulated after addition of nitrate into the culture medium of hydroponically grown plants, indicating that ARRs are nitrate-responsive in the leaves of *Arabidopsis* (Taniguchi and others 1998). Takei and coworkers (2001b) characterized the spatial and temporal accumulation pattern of cytokinins in nitrogen-starved maize plants in response to nitrate supply and the accumulation of mRNAs encoding response regulators in more detail. Cytokinins started to increase in roots within 1 h after nitrate addition to nitrogen-starved maize plants. An increased level of cytokinins in leaves was measured 4 h after nitrate

resupply, about the same time as the *ZmRR1* transcripts started to accumulate (Takei and others 2001; Sakakibara 1998). The cytokinin level remained elevated for at least 24 h, while *ZmRR1* transcript accumulation was transient. Additional cytokinin during the 24-h period did not reinduce *ZmRR1* transcript, implying a feedback regulation of cytokinin signaling (Takei and others 2001b). In conclusion, cytokinins might act as a long-distance signal to coordinate root and shoot development dependent on nitrogen availability, and the molecular link may be the two-component signaling system (Figure 4). Nitrogen supply also has a root-specific effect, namely regulation of root growth and architecture. High nitrate preferentially inhibits root growth and decreases the formation of lateral roots. On the other hand, local application of nitrate to roots of nitrogen-limited plants leads to local proliferation of lateral roots (Zhang and Forde 1998; reviewed by Stitt 1999). It is well possible that cytokinins and the two-component signaling system are also involved in mediating nitrogen-dependent root development.

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REFERENCES

Bangerth F. 1994. Response of cytokinin concentration in the xylem exudate of bean (*Phaseolus vulgaris* L.) plants to decapitation and auxin treatment, and relationship to apical dominance. Planta 194:439–442.

Baskin TI, Cork A, Williamson RE, Gorst JR. 1995 *STUNTED PLANT 1*, a gene required for expansion in rapidly elongating but not in dividing cells and mediating root growth responses to applied cytokinin. Plant Physiol 107:233–243.

Beemster GT, Baskin TI.2000. Stunted plant 1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of *Arabidopsis.* Plant Physiol 124:1718–1727.

Beck E, Wagner BM. 1994. Quantification of the daily cytokinin transport from the root to the shoot of *Urtica dioica* L. Bot Acta 107:342–348.

Beveridge CA, Murfet IC, Kerhoas L, Sotta B, Miginiac E, Rameau C. 1997a. The shoot controls zeatin riboside export from pea roots: evidence from the branching mutant *rms4.* Plant J 11:339–345.

Beveridge CA, Symons GM, Murfet IC, Ross JJ, Rameau C. 1997b. The *rms1* mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by graft-tansmissible signals. Plant Physiol 115:1251–l258.

Bilyeu KD, Cole JL, Laskey JL, Riekhof WR, Esparza TJ, Kramer MD, Morris RO. 2001. Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol 125:378–386.

Böhner S, Gatz C. 2001. Characterization of novel target promoters for the dexamethasone-inducible/tetracycline-repressible regulator TGV using luciferase and isopentenyl transferase as sensitive reporter genes. Mol Gen Genet 264:860–870.

Brandstatter I, Kieber JJ. 1998. Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell 10:1009–1020.

Cary AJ, Liu W, Howell SH. 1995. Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thatiana* seedlings. Plant Physiol 107:1075–1082.

Cline MG. 1991. Apical dominance. Bot Rev 57:318–358

Custers JBM, Snepvangers SCHJ, Jansen HJ, Zhang L, van Lookeren Campagne MM. 1999. The 35S-CaMV promoter is silent during early embryogenesis but activated during non-embryogenic sporophytic development in microspore culture. Protoplasma 208:257–264.

D'Agostino IB, Deruère J, Kieber JJ. 2000. Characterization of the response of the *Arabidopsis* response regulator gene family to cytokinin. Plant Physiol 124:1706–1717.

D'Agostino IB, Kieber JJ. 1999. Phosphorelay signal transduction: the emerging family of plant response regulators. Trends Biochem Sci 24:452–456.

Doerner P, Jorgensen JE, You R, Steppuhn J, Lamb C. 1996. Control of root growth and development by cyclin expression. Nature 380:520–523.

Faiss M, Zalubilova J, Strnad M, Schmülling T. 1997. Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. Plant J 12:401–415.

Foo E, Turnbull CGN, Beveridge CA. 2001. Long-distance signaling and the control of branching in the *rms1* mutant of pea. Plant Physiol 126:203–209.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270:1986–1988.

Galuszka P, Frébort I, Šebela M, Sauer P, Jacobsen S, Peè P. 2001. Cytokinin oxidase dehydrogenease? Mechanism of cytokinin degradation in cereals. Eur J Biochem 268:450–461.

Haberer G, Kieber J. 2002 Cytokinins: new insights into a classic phytohormone. Plant Physiol 128:345–362.

Houba–Hérin N, Pethe C, d'Alayer J, Laloue M. 1999. Cytokinin oxidase from *Zea mays*: purification, cDNA cloning and expression in moss protoplasts. Plant J 17:615–626.

Hwang I, Sheen J. 2001. Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413:383–389.

Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Kiba T, Ueguchi C, Sugiyama T, Mizuno T. 1999. Compilation and characterization of *Arabidopsis thaliana* response regulators implicated in His-Asp phosphorelay signal transduction. Plant Cell Physiol 40:733–742.

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T. 2001. Identification of CRE1 as a cytokinin receptor from *Arabidopsis.* Nature 409:1060–1063.

Kakimoto T. 2001a. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyl transferases. Plant Cell Physiol 42:677–685.

Kakimoto T 2001b. Biosynthesis and perception of cytokinins. 17th International Conference on Plant Growth Substances, p 56 [abstract]. Bruo, Czech Republic, 1–6 July 2001.

Kiba T, Taniguchi M, Imamura A, Ueguchi C, Mizuno T, Sugiyama T. 1999. Differential expression of genes for response regulators in response to cytokinins and nitrate in *Arabidopsis thaliana.* Plant Cell Physiol 40:767–771.

Letham DS. 1994. Cytokinins as phytohormones — sites of biosynthesis, translocation and function of translocated cytokinin. In Mok DWS, Mok MC, editors. Cytokinins: Chemistry, Activity and Function. Boca Raton, FL:CRC Press, p 57–80.

Lohrmann J, Buchholz G, Keitel C, Sweere C, Kircher S, Bäuerle I, Kudla J, Harter K. 1999. Differentially-expressed and nuclear-localized response regulator-like proteins from *Arabidopsis thaliana* with transcription factor properties. Plant Biol 1:495–506.

Lohrmann J, Sweere U, Zabaleta E, Bäuerle I, Keitel C, Kozma–Bognar L, Brennicke A, Kudla J, Schafer E, Harter (2001) The response regulator ARR2: A pollen-specific transcription factor involved in the expression of nuclear-encoded mitochondrial complex I genes. Mol Gen Genomics 265:2–13.

Mähönen AP, Bonke M, Kaupinnen L, Riikonen M, Benfey PN, Helariutta Y (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev 14:2938–2943.

Martin RC, Mok C, Mok DWS. 1999a. A gene encoding the cytokinin enzyme zeatin *O*-xylosyltransferase of *Phaseolus vulgaris.* Plant Physiol 120:553–570

Martin RC, Mok C, Mok DWS. 1999b. Isolation of a cytokinin gene, *ZOG1*, encoding zeatin O-glucosyltransferase of *P.lunatis.* Proc Natl Acad Sci USA 96:284–289.

Martin RC, Mok MC, Habben JE, Mok DWS. 2001. A maize cytokinin gene encoding an O-glucosyltransferase specific to *cis*-zeatin. Proc Natl Acad Sci USA 98:5922–5926.

McKenzie MJ, Mett V, Reynolds PHS, Jameson PE. 1998. Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. Plant Phys 116:969–977.

Mett VL, Podivinsky E, Tennant AM, Lochhead LP, Jones WT, Reynolds PH. 1996. A system for tissue-specific copper-controllable gene expression in transgenic plants: nodule-specific antisense of aspartate aminotransferase-P2. Transgenic Res 5:105–113

Mizukami Y, Fischer RL. 2000. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. Proc Natl Acad Sci USA 97:942–947.

Morris RO, Bilyeu KD, Laskey JG, Cheikh NN. 1999. Isolation of a gene encoding a glycosylated cytokinin oxidase from maize. Biochem Biophys Res Commun 255:328–333.

Morris SE, Turnbull CGN, Murfet IC, Beveridge CA. 2001. Mutational analysis of branching in pea. Evidence that *Rms1* and *Rms5* regulate the same novel signal. Plant Physiol 126:1205–1213.

Osakabe Y, Urao, T, Shoinozaki K, Yamaguchi–Shinozaki K. 2001. Functional analysis of *Arabidopsis* response regulators, ATRR1/ARR4/IBC7 and ATRR8/ARR8 in transgenic plants. 12th Arabidopsis Conference, Poster 224, Madison, Wisconsin, 23–27 June 2001.

Riechmann JL, Heard J, Martin G, Reber L, Jiang L, Keddie J, Adam L, Pineda O, Ratcliffe J, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Gandehari D, Sherman BK, Yu G. 2000. *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. Science 290:2105–2110.

Sakakibara H, Suzuki M, Takei K, Deji A, Taniguchi M, Sugiyama T. 1998. A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. Plant J 14:337–344.

Sakai H, Aoyama T, Oka A. 2000. *Arabidopsis* ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J 24:703–711.

Sakai H, Honma T, Aoyama T, Sato S, Kato T, Tabata S, Oka A. 2001. ARR1, a transcription factor for genes immediately responsive to cytokinins. Science 294:1519–1521.

Samuelson ME, Eliasson L, Larsson C. 1992. Nitrate-regulated growth and cytokinin responses in seminal roots of barley. Plant Physiol 98:309–315.

Scheres B, DiLaurenzio L, Willemsen V, Hauser M-T, Janmaat K, Weisbeek P, Benfey PN. 1995. Mutations affecting the radial organization of the *Arabidopsis* root display specific defects throughout the embryonic axis. Development 121: 53–62.

Schmülling T. 2001. CREam of cytokinin signalling: receptor identified. Trends Plant Sci 6:281–284.

Schmülling T, Schäfer S, Romanov G. 1997. Cytokinins as regulators of gene expression. Physiol Plant 100:505–519.

Shimizu–Sato S, Mori H. 2001. Control of outgrowth and dormancy in axillary buds. Plant Physiol 127:1405–1413.

Skoog F, Miller CO. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro.* Symp Soc Exp Biol 11:118–131.

Stafstrom J. 1993. Axillary bud development in pea: apical dominance, growth cycles, homonal regulation and plant architecture. In: Amasino R, editor. Cellular communication in plants. New York: Plenum Publishing. pp 75–86.

Stitt M. 1999. Nitrate regulation of metabolism and growth. Curr Opin Plant Biol 2:178–186.

Suzuki T, Miwa K, Ishikawa K, Yamada H, Aiba H, Mizuno T. 2001. The *Arabidopsis* sensor kinase, AHK4, can respond to cytokinin. Plant Cell Physiol 42:107–113.

Sweere U, Eichenberg K, Lohrmann J, Mira–Rodado V, Bäurle I, Kudla J, Nagy F, Schäfer E, Harter K. 2001. Interaction of the response regulator ARR4 with phytochrome B in modulating red light signaling. Science 294:1108–1111.

Takei K, Sakakibara H, Sugiyama T. 2001a. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana.* Biol Chem 276: 26405–26410.

Takei K, Sakakibara H, Taniguchi M, Sugiyama T. 2001. Nitrogen-dependent accumulation of cytokinins in root and the translocation to leaf: Implication of cytokinin species that induces gene expression of maize response regulator. Plant Cell Physiol 42:85–93.

Taniguchi M, Kiba T, Sakakibara H, Ueguchi C, Mizuno T, Sugiyama T. 1998. Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate. FEBS Lett 429: 259–262.

Ueguchi C, Koizumi H, Suzuki T, Mizuno T. 2001. ovel family of sensor histidine kinase genes *in Arabidopsis thaliana.* Plant Cell Physiol 42:231–235.

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

Wickson M, Thimann KV. 1958. The antagonism of auxin and cytokinin in apical dominance. Plant Physiol 11:62–74.

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Mizuno T. 2001. The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42:1017–1023.

Yong JWH, Wong SC, Letham DS, Hocart CH, Farquhar GD. 2000. Effects of elevated [CO_2] and nitrogen nutrition on cytokinins in the xylem sap and leaves of cotton. Plant Physiol 124:767–779.

Zhang H, Forde BG. 1998. An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. Science 279:407–409.