Cytokinins: Activity, Biosynthesis, and Translocation

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Annu. Rev. Plant Biol.
2006. 57:431–49

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

doi: 10.1146/annurev.arplant.57.032905.105231

First published online as a Review in Advance on February 1, 2006

1543-5008/06/0602-0431$20.00

Key Words

adenosine phosphate-isopentenyltransferase, *Agrobacterium tumefaciens*, cytochrome P450 monooxygenase, plant hormones, plastid, root/shoot signaling, *trans*-zeatin

Abstract

Cytokinins (CKs) play a crucial role in various phases of plant growth and development, but the basic molecular mechanisms of their biosynthesis and signal transduction only recently became clear. The progress was achieved by identifying a series of key genes encoding enzymes and proteins controlling critical steps in biosynthesis, translocation, and signaling. Basic schemes for CK homeostasis and root/shoot communication at the whole-plant level can now be devised. This review summarizes recent findings on the relationship between CK structural variation and activity, distinct features in CK biosynthesis between higher plants and *Agrobacterium* infected plants, CK translocation at whole-plant and cellular levels, and CKs as signaling molecules for nutrient status via root-shoot communication.

Contents

INTRODUCTION

CK: cytokinin
tZ: *trans*-zeatin

Half a century has passed since the discovery of cytokinin (CK). Triggered by the isolation of kinetin in autoclaved products of herring sperm DNA as a cell division promoting factor in 1955 (4, 62, 63), a number of compounds with CK activity have been identified, including *trans*-zeatin (tZ) as a naturally occurring CK (54), diphenylurea as a synthetic compound (66, 84), and several natural CKs with aromatic side chains (36, 93). Subsequent studies have clarified structural requirements for CK activity. Our current understanding is that naturally occurring CKs are adenine derivatives carrying either an isoprene-derived or an aromatic side chain at the N^6 terminus (66, 93); conventionally, these families are called isoprenoid CKs and aromatic CKs, respectively. In both groups, there are small variations in side-chain structure such as the absence or presence of hydroxyl groups and their stereoisomeric position; the physiological significance of these variations has not yet been fully elucidated.

CK plays a crucial role in regulating the proliferation and differentiation of plant cells, and also controls various processes in plant growth and development, such as delay of senescence (30, 67), control of shoot/root balance (112, 113), transduction of nutritional signals (77, 80, 100), and increased crop productivity (6). In spite of its biological and agricultural importance, only in the past few years have the basic molecular mechanisms of biosynthesis and signal transduction been elucidated. This progress was facilitated by the identification of genes encoding enzymes and proteins controlling key steps in CK biosynthesis and signaling, by in-depth analysis of the biochemical properties of the enzymes and proteins, and by thorough determination of the whole-plant and subcellular compartmentation of CKs. Generally, CK biosynthesis and homeostasis are finely controlled by internal and external factors such as other phytohormones and inorganic nitrogen sources (77). Recent findings demonstrated that the CK biosynthesis pathway in *Agrobacterium* infected plants is distinct from that in higher plants in terms of substrate choice, and that this difference is part of the surviving strategy of *Agrobacterium* in the host plant cells (78).

The previously widely accepted idea that CK and auxin are synthesized only in root tips and shoot apices, respectively, is now overturned. Both hormones have coordinated functions as long-distance messengers as well as local paracrine signals; they are synthesized and act at various sites in a plant body, although the physiological differentiation and the mechanisms of the dual signaling system have not been fully elucidated. In basipetal polar auxin transport, the AUX1/PIN-PGP system takes a major role, and the molecular basis of auxin translocation has been characterized (16, 70). The nature of CK translocation systems is less clear, but several lines of evidence point to a common translocation mechanism for purines, nucleosides, and CKs.

In this review, I focus on activity, biosynthesis, translocation, and root-shoot communication, which complements recent excellent

reviews on CK signaling (44) and metabolism (66).

STRUCTURAL VARIATION AND BIOLOGICAL ACTIVITY

Structural Diversity of Natural Cytokinins

Both isoprenoid and aromatic CKs are naturally occurring, with the former more frequently found in plants and in greater abundance than the latter. Common natural isoprenoid CKs are N^6-(Δ^2-isopentenyl)-adenine (iP), tZ, *cis*-zeatin (cZ), and dihydrozeatin (DZ) (**Figure 1**). Among them, the major derivatives generally are tZ and iP as well as their sugar conjugates, but there is a lot of variation depending on plant species, tissue, and developmental stage. For instance, tZ- and iP-type CKs are the major forms in *Arabidopsis*, whereas substantial amounts of cZ-type CKs are found in maize (109), rice (41), and chickpea (24). As for aromatic CKs, *ortho*-topolin (oT), *meta*-topolin (mT), their methoxy-derivatives (meoT and memT, respectively), and benzyladenine (BA) are only found in some plant species (93) (**Figure 1**). Several synthetic derivatives possess CK activity (40, 69, 86, 89) but have not been found in nature so far. Human urine contains kinetin (8), but there is no evidence for its occurrence in plants. Usually, all natural CK nucleobases have the corresponding nucleosides, nucleotides, and glycosides (**Figure 2**).

Biological Activity

Results from classical bioassays such as those using tobacco pith (81) and moss (*Funaria hygrometrica*) (92) suggested that CK nucleobases are the active forms. In these assays, tZ and iP generally exhibited higher activities than cZ. tZ and iP generally exhibited higher activity, but cZ had lower or no activity. However, the results between different bioassays were not always consistent (55). It is likely that exogenous-applied CKs could be further

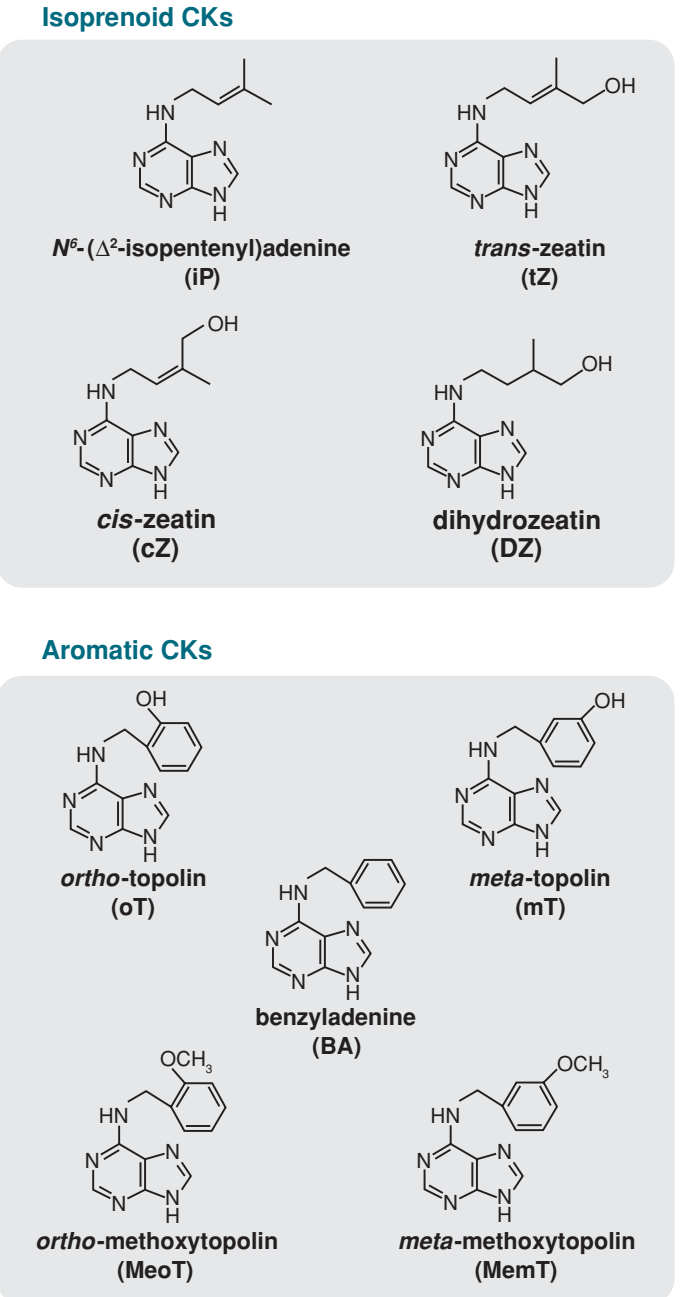

Figure 1

Structures of representative active cytokinin (CK) species occurring naturally. Only trivial names are given, with commonly used abbreviations in parentheses.

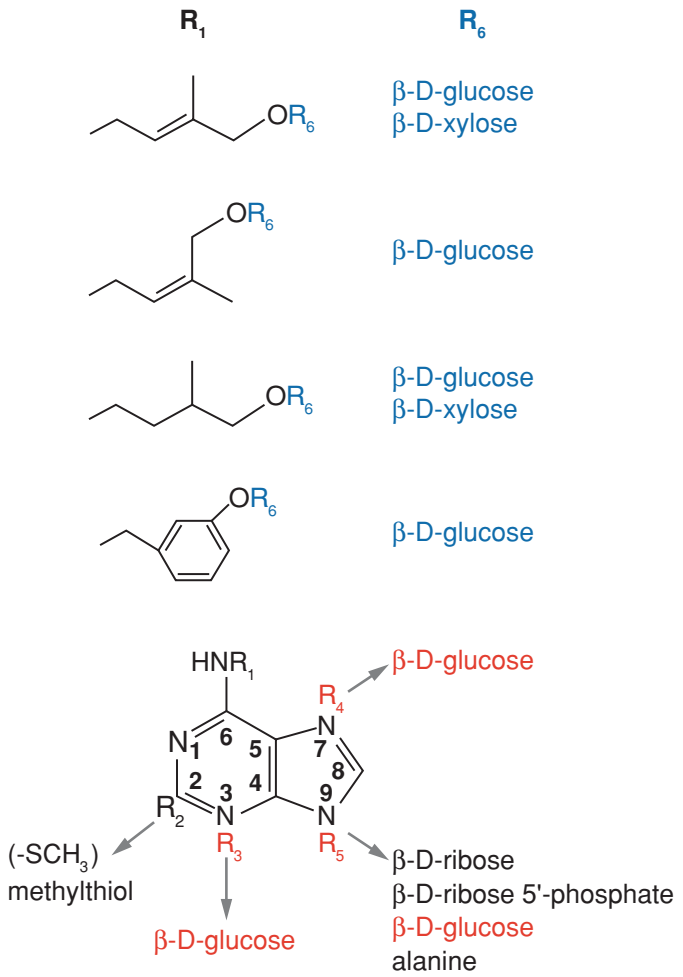

Figure 2

Cytokinin (CK) conjugates with sugars, sugar phosphates, and others. *O*-Glycosylation of side chain (colored in *blue*) is catalyzed by zeatin *O*-glucosyltransferase or *O*-xylosyltransferase. *N*-glucosylation of adenine moiety (colored in *red*) is catalyzed by cytokinin *N*-glucosyltransferase.

converted into other metabolites during the experiments. The identification of CK receptors and the detailed analysis of their ligand specificity helped us to better understand the relative activity of various CKs. CK receptors in higher plants are encoded by a small gene family (39, 97, 108, 115). Bioassays based on heterologous expression of CK receptors in budding or fission yeast (39, 97) and in *Escherichia coli* (91, 97, 114, 115) confirmed that CK nucleobases are the primary ligands for the receptors whereas the sugar conjugates are less active or inactive. In a heterologous assay system, a CK receptor from maize (ZmHK1) responded to cZ with a similar sensitivity to tZ, and another one (ZmHK2) responded to tZ riboside (tZR) as well as to tZ (115). The *Arabidopsis* CK receptor, AHK3, was as sensitive to tZR and tZR 5′-monophosphate (tZRMP) as to tZ (91), suggesting that each receptor has a specific spectrum of ligand preference (91, 114, 115). Thus, structural variations of CK side chains and modifications of the adenine moiety confer specificity of the CK-receptor interaction.

iP: N6-(Δ2-isopentenyl)adenine

cZ: *cis*-zeatin

CYTOKININ BIOSYNTHESIS AND METABOLISM

Basic Schemes of Cytokinin Metabolism

Cytokinin metabolism and homeostasis. CK metabolic pathways can be broadly classified into two types: the modification of the adenine moiety and that of the side chain. The concomitant occurrence of CK nucleobases with the corresponding nucleosides and nucleotides in plant tissues suggests that important metabolic steps are shared with the purine metabolic pathway [i.e., salvage pathway (22, 66, 76)]. Thus, the metabolic flow from CK nucleotides to the active nucleobases is probably not unidirectional but circular (**Figure 3**). In fact, exogenously applied CK nucleobases are rapidly metabolized into the corresponding nucleotides and nucleosides in plant tissues (56, 65, 88, 115). Enzymes of the purine salvage pathway in plants are encoded by small multigene families. It is now supposed that some of the isoenzymes have broad substrate specificities, enabling them to act on CKs as well as on authentic adenine. In this regard, two isoenzymes of adenine phosphoribosyltransferase from *Arabidopsis*, APT2 and APT3, have a particularly strong preference for CK nucleobases as compared with other isoenzymes (3, 83). Such preference is also

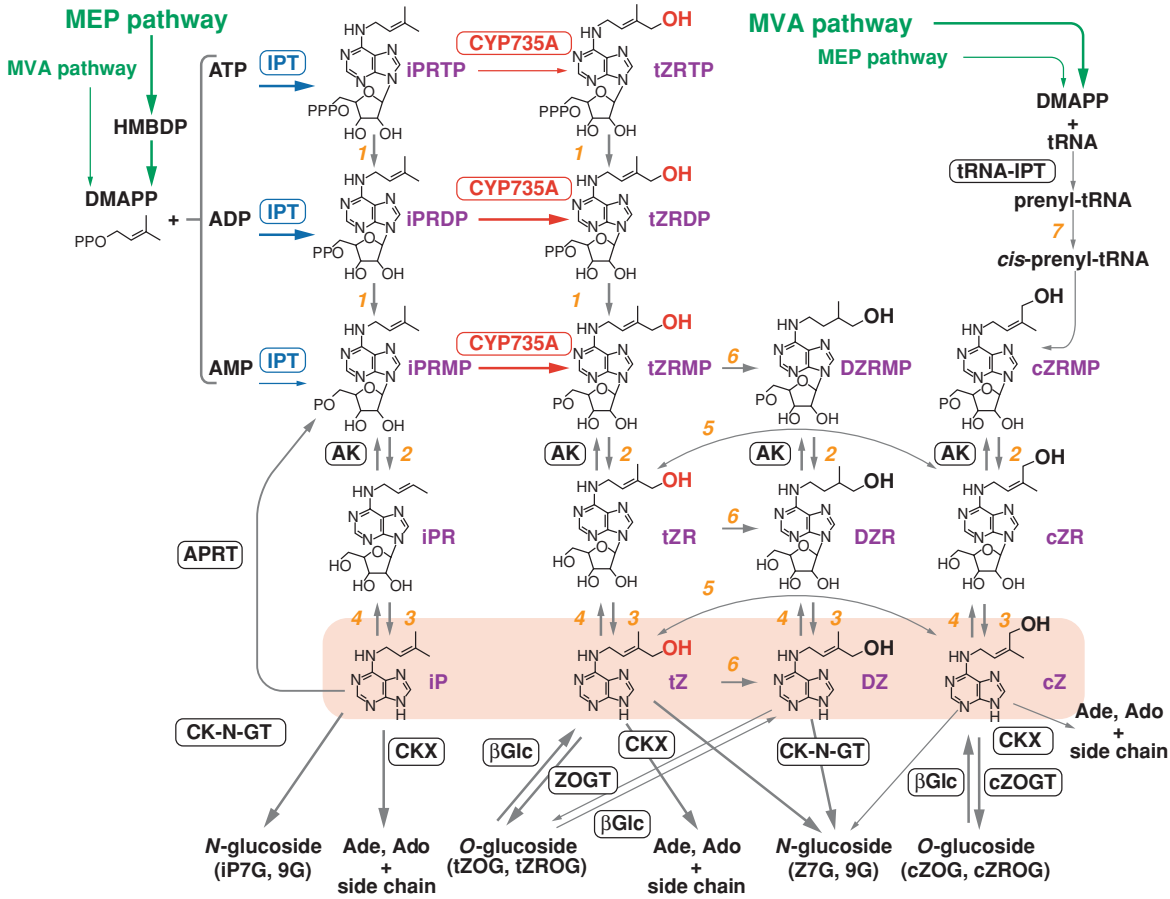

Figure 3

Current model of isoprenoid cytokinin (CK) biosynthesis pathways in *Arabidopsis*. Isoprenoid side chains of N^6-(Δ^2-isopentenyl)adenine (iP) and *trans*-zeatin (tZ) predominantly originate from the methylerythritol phosphate (MEP) pathway, whereas a large fraction of the *cis*-zeatin (cZ) side chain is derived from the mevalonate (MVA) pathway (*green arrows*) (46). Plant adenosine phosphate-isopentenyltransferases (IPTs) preferably utilize ATP or ADP as isoprenoid acceptors to form iPRTP and iPRDP, respectively (*blue arrows*) (43, 76). Dephosphorylation of iPRTP and iPRDP by phosphatase (*1*), phosphorylation of iPR by adenosine kinase (AK), and conjugation of phosphoribosyl moieties to iP by adenine phosphoribosyltransferase (APRT) create the metabolic pool of iPRMP and iPRDP. APRT utilizes not only iP but also other CK nucleobases. The CK nucleotides are converted into the corresponding tZ-nucleotides by CYP735A (*red arrows*). iP, tZ, and the nucleosides can be catabolized by CKX to adenine (Ade) or adenosine (Ado). cZ and tZ can be enzymatically interconverted by zeatin *cis-trans* isomerase (*5*). tZ can be reversibly converted to the *O*-glucoside by zeatin *O*-glucosyltransferase (ZOGT) and β-glucosidase (βGlc). CK nucleobases also can be converted to the *N*-glucoside by CK *N*-glucosyltransferase (CK-N-GT). The width of the arrowheads and lines in the green, blue, and red arrows indicates the strength of metabolic flow. Flows indicated by black arrows are not well characterized to date. tZRDP, tZR 5′-diphosphate; tZRTP, tZR 5′-triphosphate; *2*, 5′-ribonucleotide phosphohydrolase; *3*, adenosine nucleosidase; *4*, purine nucleoside phosphorylase; *6*, zeatin reductase; *7*, CK *cis*-hydroxylase. Modified from Reference 104.

reported from an isoform of tobacco adenosine kinase, ADK2S (50).

On the other hand, the enzymes for the *N*-glucosylation of the adenine moiety (37) and those for the hydroxylation (104), *O*-glucosylation, and *O*-xylosylation of the side chain (59–61) are specific for particular CK substrates.

Glycosylation of Cytokinins. Glucosylation of CK has been observed at the *N3*, *N7*, and *N9* position of the purine moiety as *N*-glucosides, and at the hydroxyl group of the side chains of tZ, DZ, and cZ as *O*-glucosides or *O*-xylosides (**Figure 2**). *O*-glucosylation is reversible; the deglycosylation is catalyzed by β-glucosidase (18). On the other hand, *N*-glucoconjugates are not efficiently cleaved by β-glucosidase (18); as a result, *N*-glucosylation is practically irreversible. The physiological consequences of the differences in stability of *N*-glucosides and *O*-glucosides are not fully understood to date. However, it has been suggested that the readily cleaved *O*-glucosides represent inactive, stable storage forms of CKs.

Although genes involved in the *O*-glycosylation of CKs have been well characterized (59–61, 68, 109), information on the molecular biology of *N*-glucosylation is scarce. In *Arabidopsis*, two enzymes (UGT76C1 and UGT76C2) glucosylate a series of CK nucleobases at the *N7*- or *N9*-positions, and also tZ-*O*-glucoside (tZOG) at *N7* (37). Both enzymes prefer glucosylation at *N7* to that at *N9*, which corresponds well with the concentrations of various CK *N*-glucosylates in *Arabidopsis* tissues (78).

Determinants of the levels of active Cytokinins. Steady-state levels of active CK in planta are determined by the rate of release of CK nucleobase from the conjugates and that of CK degradation and inactivating conjugation. CK oxidase/dehydrogenase (CKX) catalyzes the irreversible degradation by cleavage of the side chain (5, 28, 82), and glycosyltransferases, described above, inactivate CKs by glycosylation (**Figure 3**). Phosphoribosylation of CK nucleobase by adenine phosphoribosyltransferase also reduces the biological activity (**Figure 3**). The deribosylation of CK nucleoside to release the nucleobase might be an important step to regulate the level of active CKs, but the enzymes catalyzing it and their genes have not been identified yet.

Stability among CKs also has an effect on biological activity and is determined by its affinity to metabolic enzymes. For instance, tZ and iP are readily catalyzed by CKXs from various plant species (13, 29), whereas cZ is generally less amenable (13). Because CKXs recognize the double bond of the isoprenoid side chain, DZ and aromatic CKs are resistant to CKX (5).

CKX: cytokinin oxidase/dehydrogenase

DMAPP: dimethylallyl diphosphate

HMBDP: hydroxymethylbutenyl diphosphate

IPT: adenosine phosphate-isopentenyltransferase

Cytokinin Biosynthesis

Primary reaction of de novo cytokinin biosynthesis. The first step in the isoprenoid CK biosynthesis is *N*-prenylation of adenosine 5′-phosphates (AMP, ADP, or ATP) at the N^6-terminus with dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP); this reaction is catalyzed by adenosine phosphate-isopentenyltransferase (IPT; EC 2.5.1.27) (**Figure 3**). It had long been thought that DMAPP and AMP were the only substrates for CK biosynthesis, but now it seems clear that substrate specificities of IPTs vary depending on the origin and the species.

The first identification of substrates for the isoprenoid CK biosynthesis was achieved in the slime mold, *Dictyostelium discoideum*, which produces discadenine [3-(3-amino-3-carboxypropyl)-N^6-(Δ^2-isopentenyl)adenine], an inhibitor of spore germination (1). The first step of discadenine biosynthesis is the IPT-catalyzed prenylation of AMP. *D. discoideum* IPT utilizes AMP or ADP as a prenyl side-chain acceptor, but not ATP or cyclic AMP (38, 107). The K_m value for AMP is 100 nM and for DMAPP it is 2.2 μM (38).

The first characterization of an *IPT* gene was carried out in *Agrobacterium tumefaciens*, a crown gall-forming bacterium (2, 9). *A. tumefaciens* has two IPT genes, *Tmr* and *Tzs*. *Tmr* is encoded on the T-DNA region of the Ti-plasmid and *Tzs* is located on the virulence region of nopaline-type Ti-plasmids, whose role is to promote T-DNA transfer efficiency (42, 73). After infection, *Tmr* is integrated into the host nuclear genome and functions in the host cell, whereas *Tzs* acts within the bacterial cells. Both recombinant enzymes have been purified and their kinetic parameters characterized: The K_m value of Tmr for AMP is 85 nM (14) and those for DMAPP and HMBDP are 10.1 μM and 13.6 μM, respectively (78). Tzs also utilizes HMBDP as an isoprene donor (48), and the affinities for HMBDP and DMAPP are similar (H. Sakakibara, unpublished results). Both Tmr and Tzs do not utilize adenosine phosphates other than AMP.

The reaction pathway of iP riboside 5′-moophosphate (iPRMP) synthesis in higher plants has been assumed to resemble that found in *D. discoideum* and *A. tumefaciens*. Although many attempts have been made to purify and characterize plant IPT, only a few biochemical properties have been reported (15, 21), probably due to low contents or enzyme instability. Higher plant *IPT* genes have been identified in *Arabidopsis* (43, 99), petunia (116), and hop (79). In *Arabidopsis*, seven *IPT* genes (*AtIPT1* and *AtIPT3* to *AtIPT8*) are involved in CK biosynthesis (43, 95, 99). Biochemical studies strongly suggest that plant IPTs predominantly use ADP or ATP rather than AMP as prenyl acceptors, resulting in the production of iP riboside 5′-diphosphate (iPRDP) or iP riboside 5′-triphosphate (iPRTP) (43, 76, 79, 103). The K_m values of AtIPT1 for AMP, ADP, and ATP were 185 μM, 14.6 μM, and 11.4 μM, respectively, and for DMAPP it was 8.3 μM (99, 103). AtIPT4 had K_m values of 9.1 μM for ADP, 3.4 μM for ATP, and 11.6 μM for DMAPP (43, 76). The predominant accumulation of iP-type CKs in transgenic lines of *Arabidopsis* that overexpress any of the AtIPTs suggested that all AtIPTs have similar substrate preferences in terms of the isoprenoid donor (78).

Although some *Arabidopsis* IPTs could utilize HMBDP as a prenyl donor in vitro, the affinities were low and there is little evidence that tZ-type species are formed via this reaction in vivo (78, 98).

MEP: pathway: methylerythritol phosphate pathway

MVA: pathway: mevalonate pathway

Metabolic origin of the isoprenoid side chain. HMBDP is a metabolic intermediate of the methylerythritol phosphate (MEP) pathway, which occurs in bacteria and plastids (33). Thus, in higher plants, it is believed that HMBDP is formed only in plastids. DMAPP is synthesized via the MEP pathway and the mevalonate (MVA) pathway, which is commonly found in the cytosol of eukaryotes (57, 75). When DMAPP is used as a substrate for CK biosynthesis, the primary product is iP nucleotide (**Figure 3**). tZ nucleotide can be formed directly when IPT acts on HMBDP. Before the characterization of the MEP pathway (57, 75), the MVA pathway was the only reaction sequence known to produce the isoprenoid precursors DMAPP and isopentenyl diphosphate in higher plants. In cultured tobacco BY-2 cells, lovastatin, an inhibitor of the MVA pathway, significantly decreases CK accumulation (51, 52), corroborating the idea that the MVA pathway is the metabolic origin of isoprenoid CK side chains. However, in *Arabidopsis*, a large number of the AtIPTs (AtIPT1, AtIPT3, AtIPT5, and AtIPT8) are located in the plastids (46), and AtIPT3 and AtIPT5 are the dominant isoforms in young and mature plants under normal conditions (64, 102). Thus, it seems that plastids are the major subcellular compartment for iP-type CK biosynthesis in higher plants. Selective labeling experiments using ^{13}C-labeled precursors specific for either the MEP or MVA pathway demonstrate that the isoprenoid side chain of iP- and tZ-type CKs predominantly originates from the MEP pathway (46), which is consistent with the IPT subcellular location. On the other hand, AtIPT4 and AtIPT7 are localized in the cytosol and mitochondria,

P450: Cytochrome P450 monooxygenase is a heme-containing enzyme that catalyzes the oxidative reaction of a wide variety of organic compounds by utilizing atmospheric O_2

respectively (46). Therefore, a major role of the MEP pathway in the biosynthesis of tZ- and iP-type CK does not rule out a greater contribution of the MVA pathway to the synthesis of these CKs under different growth or environmental conditions, if the relative abundance of IPT isoenzymes is modulated.

The hydroxylation step in tZ biosynthesis. In higher plants, there are two possible pathways for tZ biosynthesis, the iP nucleotide-dependent and the iP nucleotide-independent one (7, 72, 104) (**Figure 3**). In the iP nucleotide-dependent pathway, tZ synthesis is catalyzed by a cytochrome P450 monooxygenase (P450); two such enzymes, CYP735A1 and CYP735A2, were recently identified in *Arabidopsis* (104). Although previous studies on microsomal fractions isolated from cauliflower showed *trans*-hydroxylation of iP and iPR only (20), CYP735A1 and CYP735A2, which lack *cis*-hydroxylation activity (104), utilize iP nucleotides but not the nucleoside and free-base forms (104). Comparison of the specificity constants (k_{cat}/K_m) for iP-nucleotides suggests that CYP735As predominantly acts on iPRMP or iPRDP rather than on iPRTP (104). Although the physiological role of the CK nucleotides has not been fully clarified, the nucleotide-specific hydroxylation indicates that they form a metabolic pool for side-chain modifications. If the CK nucleobases iP and tZ serve distinct physiological functions that are determined by side-chain structure, the metabolic compartmentalization of the corresponding nucleotides would be important to maintain the physiological division of tasks at the nucleobase level.

In the iP nucleotide-independent pathway, tZ nucleotides are assumed to be produced directly by IPT using an unknown hydroxylated side-chain precursor (7). This precursor probably is derived from the MVA pathway, because mevastatin, an inhibitor of that pathway, reduces the rate of tZ biosynthesis (7). Although the biochemical nature of the iP nucleotide-independent pathway remains obscure, one possible explanation is that tZ biosynthesis via the iP nucleotide-independent pathway might be mediated by *cis-trans* isomerization of cZ derivatives, in which the prenyl side chain is primarily derived from the MVA pathway (46) (**Figure 3**). It will be necessary to identify the postulated isomerase (10) to understand the fabric of the pathway.

The strategy of Agrobacterium to modify cytokinin biosynthesis. In vitro studies on the substrate preference of Tmr (78) and measurements of CK contents in *Tmr*-overexpressing transgenic plants (7, 25, 78) and crown galls (71, 94) suggested that Tmr utilizes HMBDP as the primary substrate and produces tZRMP. However, because Tmr lacks any apparent sequences for subcellular localization, it was believed that Tmr functions in the cytosol. However, recent work demonstrated that Tmr is targeted to and functions in the plastids of infected host plant cells (78) (**Figure 4**). In the stroma of the

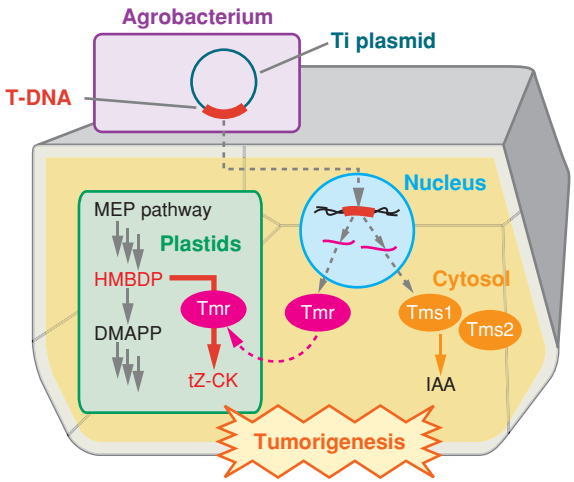

Figure 4

Proposed mechanism for the modification of cytokinin (CK) biosynthesis by Tmr upon *Agrobacterium* infection. On infection, the T-DNA region is transferred to the host plant cells and integrated into the nuclear genome. A series of genes including biosynthesis of CK (Tmr) and auxin (Tms1 and Tms2) are expressed in the host plants. Among them, Tmr is targeted into the plastids and directly produces *trans*-zeatin (tZ)-type CK by using 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate (HMBDP).

plastids, Tmr creates a CK biosynthesis bypass using HMBDP, an intermediate of the MEP pathway, without the requirement for CYP735A-mediated hydroxylation (78). This bypass enables *A. tumefaciens* to produce high amounts of tZ in order to induce tumorigenesis while the host's CYP735A-mediated CK hydroxylation activity could be repressed by auxin (104). Moreover, HMBDP reductase produces isopentenyl diphosphate and DMAPP at a ratio of 5:1 (74), and these compounds are used as common building blocks for all isoprenoids. Therefore, the pool size of HMBDP available for Tmr may be larger than that of DMAPP in the plastids.

Aromatic cytokinins. Aromatic CKs, BA, and topolins (**Figure 1**) were identified in several plant species including poplar (93) and *Arabidopsis* (106), but it is not yet clear whether they are ubiquitous in plants. Although they exhibit strong CK activity, their biosynthesis and degradation pathways remain to be elucidated. The mechanisms of glycosylation of aromatic CKs and of their interaction with the cellular signaling system appear to be shared with isoprenoid CKs because the enzymes and receptors involved recognize members of both groups (39, 66, 68, 114). In tomato pericarp tissue, the modification of BA is greatly inhibited by metyrapone, a potent inhibitor of P450 enzymes (58). Although details are unknown, CYP735A or some other P450s may be involved in the biosynthesis of topolins.

A neglected pathway: cytokinins derived from tRNA degradation. Shortly after the discovery of CKs, it was assumed that tRNA is a major source of CKs because isoprenoid CKs were identified in the hydrolysates of tRNAs (45, 90, 110, 111). Several of the tRNA species with anticodons complementary to codons beginning with uridine, such as $tRNA^{Leu}$ and $tRNA^{Ser}$, carry a prenylated adenosine adjacent to the anticodon. Thus, tRNA prenylation could contribute, at least to some extent, to CK production. The first step of the pathway leading to CKs is catalyzed by tRNA-isopentenyltransferase (tRNA-IPT; EC 2.5.1.8) (32) (**Figure 3**). Because the prenyl-moiety of the tRNA contains a *cis*-hydroxylated group (111), tRNA-degradation is a source of cZ-type CKs; the biochemical nature of the *cis*-hydroxylating enzyme has not been characterized yet. A large fraction of the cZ side chain in *Arabidopsis* is derived from the MVA pathway, suggesting that plants are able to independently modulate the levels of tZ and cZ (46). Early calculations of turnover rates of tRNA led to the conclusion that tRNA degradation was not a major pathway of CK synthesis (47). However, tRNA-derived CKs should not be neglected as some plant species such as maize and rice contain substantial amounts of cZ-type CKs.

Regulation of Cytokinin Biosynthesis

Spatial expression of AtIPTs. Analyses of spatial expression patterns of *AtIPTs* using their promoter::reporter genes revealed tissue- and organ-specific patterns of CK synthesis by IPT (64, 102). *AtIPT1* is expressed in xylem precursor cell files in root tips, leaf axils, ovules, and immature seeds; *AtIPT3* is expressed in phloem companion cells; *AtIPT4* and *AtIPT8* are expressed in immature seeds with highest expression in the chalazal endosperm; *AtIPT5* is expressed in lateral root primordia, columella root caps, upper parts of young inflorescences, and fruit abscission zones; *AtIPT6* is expressed in siliques; *AtIPT7* is expressed in phloem companion cells, the endodermis of the root elongation zones, trichomes on young leaves, and occasionally in pollen tubes. CK biosynthesis in aerial organs was also confirmed by in vivo deuterium labeling methods (72).

Regulation by plant hormones. The expression of key genes for CK biosynthesis and homeostasis such as *IPT*, *CKX*, and *CYP735A* is regulated by phytohormones including CKs, auxin, and abscisic acid (ABA). In *Arabidopsis*, the accumulation of the transcripts of *AtIPT5* and *AtIPT7* is promoted by

auxin in roots, whereas the transcript levels of *AtIPT1*, *AtIPT3*, *AtIPT5*, and *AtIPT7* are negatively regulated by CK (64). On the other hand, the expression of both *CYP735A1* and *CYP735A2* is upregulated by CKs in roots but downregulated by auxin or ABA (104). Genes for CKX in maize are upregulated by CK and ABA (17). These regulation patterns suggest that the enzymes antagonistically regulate cellular CK levels and the balance between iP and tZ, which interact with auxin and/or ABA. The interdependent regulation of phytohormones might provide a basis for the variable morphogenetic responses of plants to environmental factors.

Cytokinins as a local signal. In the control of outgrowth and dormancy of axillary buds, the mutual regulation of auxin, ABA, and CKs has been proposed to play a central role (85). In pea, the expression of two IPT genes, *PsIPT1* and *PsIPT2*, which are expressed in nodes, is negatively regulated by auxin (105). Although an antagonistic role of auxin and CKs in the regulation of axillary bud outgrowth has been postulated for a considerable time, little is known about the underlying molecular mechanisms. Recent studies revealed that one role of apex-derived auxin in apical dominance is to repress CK biosynthesis in the nodes and that after decapitation CKs are locally synthesized in the stem rather than being transported to the stem from the roots (105).

Regulation by nitrogen supply. Inorganic nitrogen strongly affects plant growth and development. Plants constantly sense the nutrient status and modulate their metabolic activities and developmental program to adapt efficiently to the nutritional environment. CK is a pivotal signaling substance communicating the nitrogen nutrient status from root to shoot via the xylem vessels (87, 100, 101). Recent studies reveal a molecular mechanism of nitrogen-dependent CK biosynthesis and regulation (**Figure 5**); *AtIPT3* and *AtIPT5* are regulated differentially depending on the nitrogen sources available (64, 102). *AtIPT3* rapidly and specifically responds to NO_3^- under nitrogen-limited conditions whereas *AtIPT5* responds to both NO_3^- and NH_4^+ under long-term treatment (102). This dual-response system might be important for plants coping with unpredictably changing nitrogen availability. AtIPT3 and AtIPT5 are the dominant IPTs in *Arabidopsis* under normal conditions (64, 102); they are localized in plastids (46), strongly suggesting that CK biosynthesis is affected by nitrogen sources. In a *Ds* transposon-insertion mutant of *AtIPT3*, NO_3^--dependent CK accumulation was greatly reduced (102), indicating that *AtIPT3* is a key determinant of CK biosynthesis in response to rapid changes in NO_3^- in the soil.

Cytokinins as a long-range signal. Expression of *CYP735A2* in roots is strongly upregulated by CK application (104). In this context the fact that *AtIPT3* is upregulated by NO_3^- seems to explain the observation that applying NO_3^- induces tZ-nucleotide accumulation in roots (100) (**Figure 5**). tZR is the major species of CKs in the xylem (53, 80, 100). Interestingly, its translocation rate in xylem vessels is controlled by NO_3^- in the root medium (100). Thus, xylem-mediated signaling by tZ-type CKs in the transpiration stream appears important for the communication of nutrient signals on the whole-plant level. However, leaf exudates contain mainly iP-type CKs (23). Note that NO_3^--dependent expression of *AtIPT3* occurs in phloem tissue (64, 102). One possible explanation is that *trans*-hydroxylation controls the differential compartmentalization of CK species and thus also controls the direction of CK translocation, although the detailed mechanisms remain to be elucidated.

TRANSLOCATION

Cytokinin Nucleobase Transport

Because de novo CK biosynthesis catalyzed by IPTs is tissue- and cell-specific, the CKs must

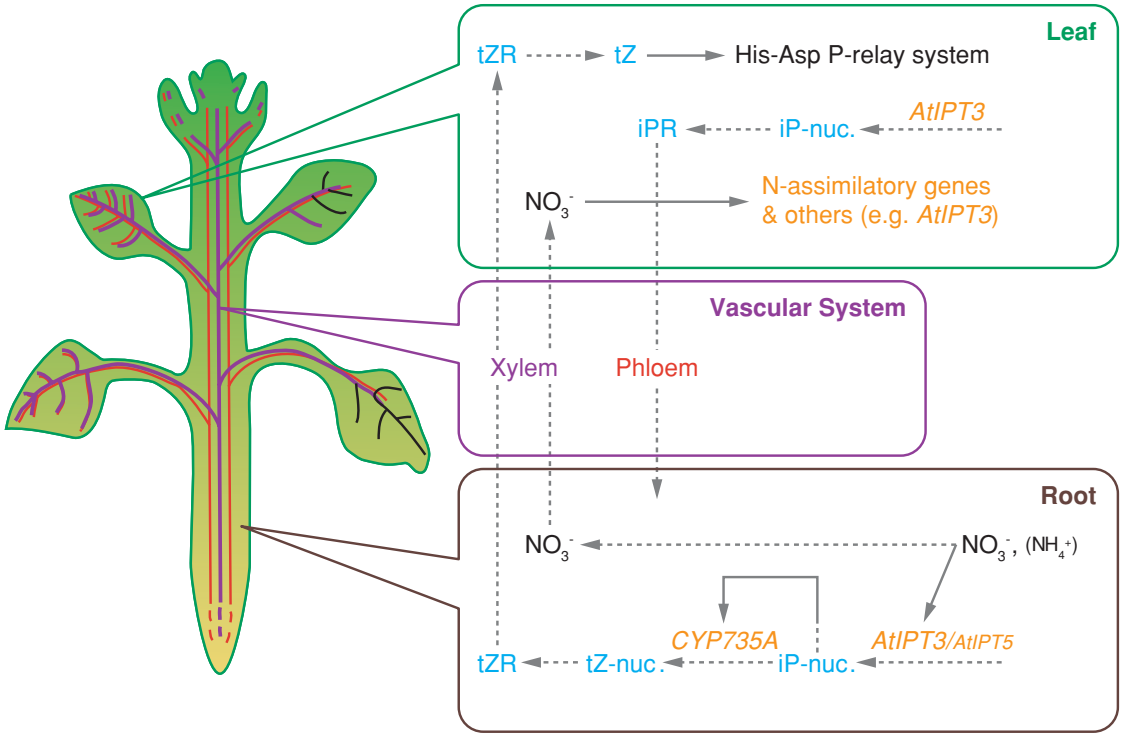

Figure 5

Nitrogen-dependent regulation of cytokinin (CK) biosynthesis and root/shoot communication via xylem and phloem. Solid arrows indicate positive regulation of gene expression. Broken lines with arrowheads show metabolic flow or translocation of CKs. Xylem stream and phloem stream are indicated in violet and red lines, respectively. Although CK functions as both a local signal and a long-range signal, only the long-range signal is illustrated in this figure. iP-nuc., iP nucleotides; tZ-nuc., tZ nucleotides. Other abbreviations as defined in the text. Modified from Reference 77.

be moved to target cells by diffusion and/or by selective transport systems. The idea of selective transport is supported by reports that plant cells are capable of absorbing CK nucleobases (19, 27) and nucleosides (88), and that tZ-type and iP-type CKs accumulate in xylem and phloem, respectively (23, 53, 100).

In cultured *Arabidopsis* cells, adenine and CK nucleobase cross membranes using the same proton-coupled high-affinity transport system (19). Two genes for *Arabidopsis* purine permeases, *AtPUP1* and *AtPUP2*, encode transporters that mediate CK nucleobase uptake (19, 31). When expressed in budding yeast cells, AtPUP1 and AtPUP2 mediate tZ uptake, and the affinity of AtPUP1 for tZ (K_i = 20–35 μM) is comparable to that of other CK metabolizing enzymes for their substrates (19, 31). PUPs have a broad substrate specificity and mediate uptake of several adenine derivatives, such as adenine itself and caffeine. The expression of *AtPUP1* observed in the epithem of hydathodes and the stigma surface suggests a role for this transporter in the retrieval of CK from xylem sap to prevent loss during guttation; similarly, *AtPUP2* expression in the phloem implies a function in the long-distance transport of CKs (19).

PUP: purine permease

Cytokinin Nucleoside Transport

Although CK nucleobases are present in xylem as well as phloem, the major CK forms in the xylem are nucleosides such as tZR (11,

ENT: The equilibrated nucleoside transporter (ENT) catalyzes the transport of various kinds of nucleosides down their concentration

12, 100). In addition, the mobility of kinetin riboside in the xylem exceeds that of BA and kinetin (34). Therefore, nucleosides are considered the major translocation form of CKs. In higher plants, some members of the equilibrative nucleoside transporter (ENT) family appear to mediate the selective translocation of CK nucleosides. The rice genome contains four *ENT* genes (*OsENT1* - *OsENT4*); one of the gene products, OsENT2, mediates the uptake of CK nucleosides as well as that of adenosine (35). OsENT2 prefers iPR (K_m = 32 μM) over tZR (K_m = 660 μM), suggesting that it may be responsible in part for the selective transport of CK nucleosides in the vascular tissues (35). *OsENT2* expression was detected in the scutellum during germination and in the vascular tissues in germinated seedlings, suggesting that OsENT2 participates in the retrieval of endosperm-derived nucleosides in the germinating embryo and in the long-distance transport of nucleosides in growing plants (35). In *Arabidopsis*, an ENT gene, *SOI33/AtENT8*, was also suggested to function in CK nucleoside transport (96). In summary, our current understanding of CK translocation in planta is that CK transport is achieved by the translocation systems that also mediate the transport of purine derivatives and nucleosides.

Compared with the highly elaborated polar transportation system for auxin, CK transport appears somewhat unspecific. However, differential loading into xylem or phloem might be sufficient for acropetal and systemic transport of CKs, respectively. It is interesting, though, that CK transport seems to occur in parallel with that of fundamentally important building blocks of the genetic apparatus and the energy transduction machinery, such as purine and nucleosides, which may be related to the CKs' function as a signal in the coordination of growth and development.

Intracellular Traffic of Cytokinin

Plastids are the major subcellular compartments of de novo CK biosynthesis through IPT (46, 102). This leads us to speculate on a transport system of CKs from plastids to the cytosol. At present, we have no evidence as to which form of CKs is transported across the plastid membranes. There is a report that AtIPT7 is localized in mitochondria (46). CK glycosides accumulate in the vacuole (26), and the deglucosylating enzyme, β-glucosidase, is localized in the plastids (49). Such complexity in the subcellular location of CK biosynthesis and metabolism points to an intricate intracellular CK transport network. It is still unknown whether the intracellular traffic of CK is also based on a transporting system for structurally related compounds.

SUMMARY POINTS

1. Structural variations at the side chain of isoprenoid and aromatic CKs affect the interaction with CK receptors, perhaps signifying functional specificity.
2. The initial step of CK biosynthesis (iP nucleotide synthesis) is catalyzed by adenosine phosphate-isopentenyltransferase; plastids are the major subcellular compartment for this initial step.
3. *trans*-Hydroxylation in tZ biosynthesis is catalyzed by a P450 enzyme, CYP735A. The hydroxylation predominantly occurs at the nucleotide step.
4. Tmr of *Agrobacterium* is targeted to the plastids of infected cells despite lacking a typical plastid-targeting sequence, and creates a CK biosynthesis bypass using an intermediate in the plastid-specific MEP pathway to synthesize tZ.

5. CK translocation shares the same transport systems moving purines and nucleosides at the whole-plant and perhaps at the cellular level.
6. Root/shoot signaling may be related to movement of tZ and iP derivatives, respectively, in the xylem and phloem streams.
7. CK biosynthesis and homeostasis are fine-tuned by internal and external factors such as phytohormones and inorganic nitrogen sources, which appears important in linking nutrient signals and morphogenetic responses.

FUTURE ISSUES TO BE RESOLVED

1. Identification of genes involved in cZ biosynthesis and cZ-tZ isomerization: It is essential to understand the physiological function of cZ and the physiological significance of tRNA-derived CK production.
2. Identification and characterization of genes encoding CK nucleosidases: Because CK nucleobase is the active form, their release from the nucleobases is one of the critical steps to control active CK level.
3. Analysis of knockout mutants of genes involved in side-chain modification: It is important to understand the possible unique properties and physiological roles of different isoprenoid and aromatic CKs.
4. Determine the molecular mechanism of Tmr import into the plastid: It would be of interest to know if the Toc-Tic system is involved in the translocation.

ACKNOWLEDGMENTS

The author acknowledges Dr. David W. S. Mok for his critical reading of the manuscript and for giving helpful comments. The writing of this review and research conducted in the author's laboratory are supported by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Agriculture, Forestry, and Fisheries, Japan.

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Volume 57, 2006

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