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CYTOKININ METABOLISM AND ACTION

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■ **Abstract** Cytokinins are structurally diverse and biologically versatile. The chemistry and physiology of cytokinin have been studied extensively, but the regulation of cytokinin biosynthesis, metabolism, and signal transduction is still largely undefined. Recent advances in cloning metabolic genes and identifying putative receptors portend more rapid progress based on molecular techniques. This review centers on cytokinin metabolism with connecting discussions on biosynthesis and signal transduction. Important findings are summarized with emphasis on metabolic enzymes and genes. Based on the information generated to date, implications and future research directions are presented.

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DEDICATION

In memory of Professor Folke Skoog in whose laboratory cytokinin was discovered and a generation of scientists were inspired.

INTRODUCTION

Cytokinins are plant hormones promoting cell division and differentiation. Since the discovery of the first cytokinin, kinetin, by Skoog, Miller, and associates in 1955 (119), the number of chemicals fitting the definition of cytokinins has grown to include a large array of natural and synthetic compounds, adenine and phenylurea derivatives. The chemistry of these groups of cytokinins has been reviewed extensively by Shaw (160) and Shudo (162). The biological activities of cytokinins in whole plants and tissues cultures have also been summarized (126, 132). Moreover, endogenous cytokinins have been examined in many species (5, 84, 185). Despite the wealth of information concerning cytokinin chemistry and physiology, the transition from descriptive studies to molecular biology has been relatively slow compared with other hormones, particularly ethylene (86, 92) and gibberellin (71, 74, 169). It is still uncertain how cytokinins are synthesized in plants although it is generally assumed that the model adopted from *Agrobacterium* suffices (42a). Only recently have candidates for cytokinin receptors begun to emerge (89, 145). Although many metabolites have been identified, their significance and the regulation of the metabolic conversions are still largely unknown. In recent years, sophisticated methods have been employed to quantify a host of major and minor components. However, as the contribution of individual cytokinins to particular growth processes is uncertain, the relevance of cytokinin quantities is open to interpretation. In short, cytokinin research needs to capitalize on the power of molecular biology to resolve many of the critical issues.[1]

There are intrinsic challenges particular to cytokinins. In tissue culture bioassays, the effects of cytokinins are well defined. In contrast, the responses of whole plants to cytokinins are broad and unspecific, unlike the triple response, stem elongation, or abscission/dormancy induced by ethylene, gibberellins, and abscisic acid, respectively (117). The action of cytokinins is often masked by interaction with other hormones. For example, cytokinins can interact with auxins either synergistically or antagonistically and induce the production of ethylene; therefore,

[1]Abbreviations: AMP, adenosine-5′-monophosphate; BA, N^6-benzyladenine; CBP, cytokinin-binding protein; CPPU, *N*-phenyl-*N*′-[2-chloro-4-pyridyl]urea; DPU, *N*,*N*′-diphenylurea; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; i^{6}AMP, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; ipn^{6}Ade, N^6-isopentyladenine; ipn^{6}Ado, N^6-isopentyladenosine; ipt, isopentenyltransferase; TDPG, thymidine-5′-diphosphoglucose; UDPG, uridine-5′-diphosphoglucose; UDPX, uridine-5′-diphosphoxylose; ZMP, zeatin riboside-5′-monophosphate.

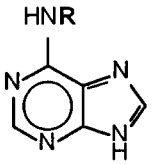
phenotypic changes are not easily identifiable as directly related to cytokinins (15, 46, 61, 179). Because mutant phenotypes may not be distinguishable from lesions affecting other hormones and severe impairment of cytokinin biosynthesis or action is likely to be lethal, isolating cytokinin mutants has not been very successful. Moreover, as naturally occurring cytokinins are adenine derivatives, it is necessary to distinguish metabolic changes unique to cytokinins from those associated with the biochemistry of purines. These constraints explain to some extent the slow progress. Nevertheless, recent advances in the identification of genes involved in cytokinin metabolism, perception, and response have added impetus to cytokinin research, signaling a fast transition to molecular approaches. Therefore, a summary of key findings with interpretations and identification of research areas deserving attention is timely. We take liberty in the latter with the comforting thought that scientific investigation is a self-correcting process and any predictions/models will serve as invitation for rigorous tests. The choice of metabolism as the connecting theme is based on our research interests and the premise that enzymes and genes involved in inter-conversions provide useful clues to both cytokinin biosynthesis and mode of action. The review consists of four parts: an overview of cytokinin metabolites, description of metabolic enzymes and genes, recent progress regarding cytokinin action, and implications related to recent findings.

CYTOKININ METABOLITES

The natural cytokinins are adenine derivatives and can be classified by the configuration of their N^6-side chain as isoprenoid or aromatic cytokinins (Figure 1). Cytokinins with an unsaturated isoprenoid side chain are by far the most prevalent, in particular those with a *trans*-hydroxylated N^6-side chain, *trans*-zeatin (101, 161) and its derivatives. Dihydrozeatin, the counterpart of zeatin with a saturated side chain, has been identified in many species, while *cis*-zeatin and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) are generally minor components although exceptions exist (51, 54). Kinetin and N^6-benzyladenine (BA) are the best known cytokinins with ring substitutions at the N^6-position. In the early years of cytokinin research, only cytokinins with an isoprenoid side chain were thought to be endogenous compounds; however, in the mid-1970s BA derivatives were identified as natural cytokinins (75, 76). The phenylureas constitute a group of synthetic cytokinins, some of which are highly active, e.g. CPPU (*N*-phenyl-*N′*-[2-chloro-4-pyridyl]urea) (170) and thidiazuron (130).

Adenine-Type Cytokinins

Modifications of the Adenine Ring The discovery of a new natural cytokinin is almost invariably followed by the report of its nucleoside and nucleotide (Figure 2). These conversions are primarily related to purine metabolism and only incidental to cytokinin metabolism. Other possible modifications of the adenine ring (Figure 2) include glucosylation of the adenine ring at the 3-, the 7-, or 9-position



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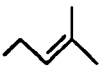
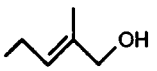
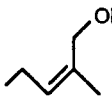
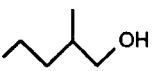
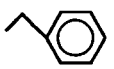
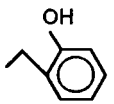
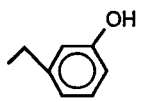
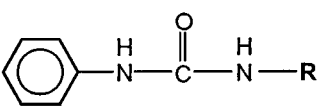
	N^6-(Δ^2-isopentenyl)adenine; i^{6}Ade
	*trans*-zeatin, zeatin
	*cis*-zeatin
	dihydrozeatin
	N^6-benzyladenine; BA
	N^6-(2-hydroxybenzyl)adenine, *ortho*-topolin
	N^6-(3-hydroxybenzyl)adenine, *meta*-topolin



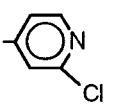
	*N*,*N*′-phenylurea; DPU
	*N*-phenyl-*N*′-(2-chloro-4-pyridyl)urea; CPPU
	*N*-phenyl-*N*′-(1,2,3-thidiazol-5-yl)urea, thidiazuron; TDZ

Figure 1 Structures of natural adenine and synthetic phenylurea cytokinins.

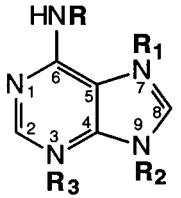


Figure 2 Modifications of the adenine ring.

R_1	β-D-glucopyranosyl
R_2	β–D-ribofuranosyl β–D-ribofuranosyl-5'-monophosphate β-D-glucopyranosyl alanyl
R_3	β-D-glucopyranosyl

and conjugation of alanine at the 9-position of zeatin, forming lupinic acid (50, 84, 166, 178). These conversions presumably can occur with most adenine-type cytokinins provided there is no steric hindrance. With the exception of the 3-glucosides, the *N*-glucosyl and alanyl conjugates generally exhibit no or low activity in bioassays (103). The ribose moiety of cytokinin nucleosides can also be further glycosylated (173, 178).

Modifications of the N^6-Isoprenoid Side Chain The most important changes are those affecting the N^6-side chain, since even small substitutions have pronounced effects on cytokinin activity, as demonstrated by extensive structure-activity relationship studies (100, 155, 156, 163). The modifications of the *trans*-zeatin side chain include reduction to dihydrozeatin, conjugation to *O*-glycosides (*O*-glucosides and *O*-xylosides), and side chain cleavage by cytokinin oxidases (Figure 3). *O*-Acetylation has been observed in a few species (84, 105). Modifications of the side chain of dihydrozeatin, such as *O*-glucosylation and *O*-xylosylation, are also known to occur (125, 182); however, an important difference between *trans*-zeatin and dihydrozeatin resides in the resistance of dihydrozeatin to cytokinin oxidases (3). The *cis*-isomer of zeatin is usually much less active than its *trans* counterpart in bioassays (156) and is somewhat susceptible to oxidases (3, 14). Evidence is emerging that the *O*-glucoside of *cis*-zeatin also occurs (111, 181) and isomerization of the *cis*- to the *trans*-configuration has been reported (11). Both i^6Ade and i^6Ado are highly susceptible to cytokinin oxidase attack (3) and can be converted to zeatin or zeatin riboside (38, 52), but reduction of the side chain from isopentenyl to isopentyl (to form ipn^6Ade or ipn^6Ado) has never been demonstrated.

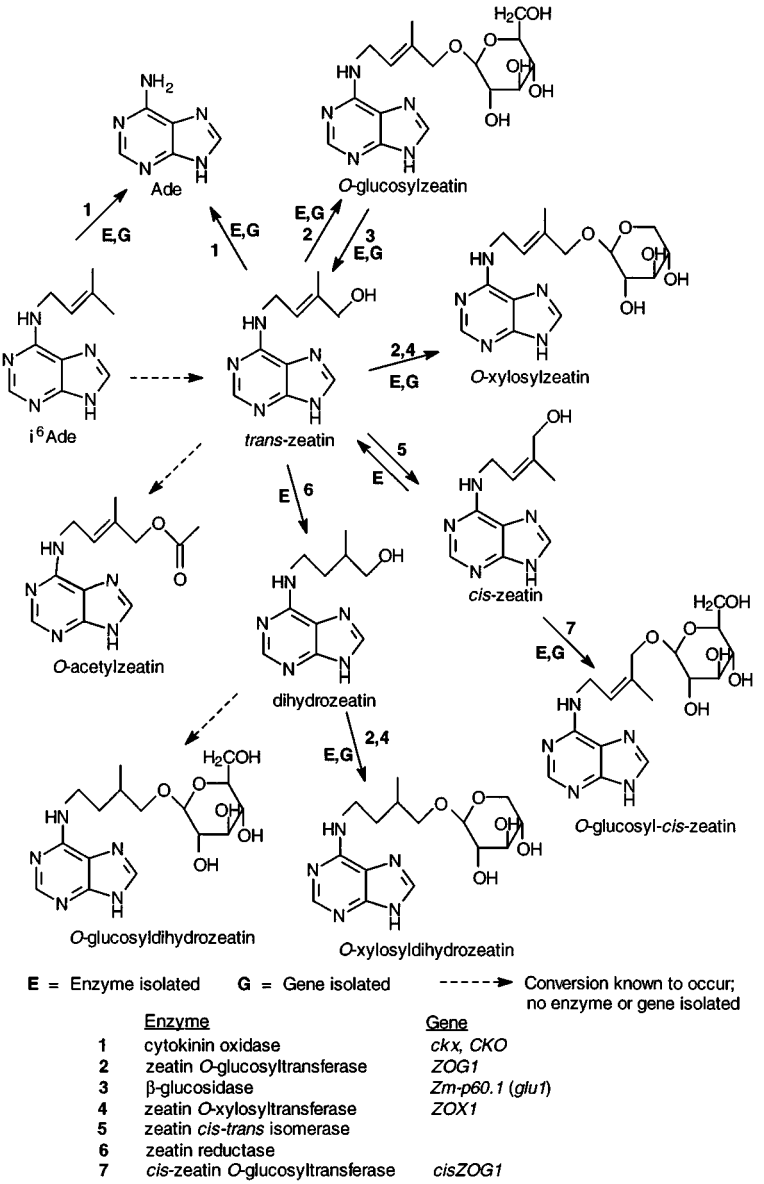


Figure 3 Enzymes (*E*) and genes (*G*) involved in the modifications of the N^6-isoprenoid side chain.

Aromatic Cytokinins BA-type compounds were recognized as naturally occurring based on their isolation from poplar (75, 76), *Zantedeschia* (33), anise (58), and tomato (136). Modifications of the adenine ring on aromatic cytokinins mostly parallel those of the isoprenoid-cytokinins (Figure 2). Hydroxylated forms of BA, *meta*- and *ortho*-topolin, occur naturally, with the accompanying nucleosides, nucleotides, and *O*-glucosides (166). *meta*-Topolin is much more active than the *para* and *ortho*-derivatives. Other metabolites include 3-, 7-, and 9-glucosides and 9-alanine on the adenine ring (166) and a glucoside linked to the ribosyl moiety (6). It is not known if the distribution of BA derivatives is limited to a few species or is more widespread since plants analyzed in early experiments have often not been re-examined for their occurrence nor have these compounds been searched for routinely in recent analyses.

Phenylurea-Type Cytokinins

Diphenylurea (DPU) (Figure 1) was the first cytokinin-active phenylurea identified (159). Although this discovery was linked to detection of the compound in liquid coconut endosperm, it was later found to be a contaminant from prior chemical analyses of DPU. This fortuitous discovery, however, led to the synthesis of a number of potent analogues such as CPPU and thidiazuron (Figure 1), with cytokinin activity exceeding that of zeatin (130, 132, 162, 170). In contrast to zeatin, these active phenylureas are highly stable. Major metabolites isolated from bean callus tissues grown on medium containing ^{14}C-thidiazuron are glucosyl derivatives (129). There is no evidence that any phenylurea cytokinins occur naturally in plant tissues.

Perspectives on the Discovery and Quantitation of Metabolites

In the early years of cytokinin research, metabolites were isolated primarily by analyses of chromatographic fractions showing cytokinin activity in bioassays. This approach led to the identification of metabolites formed in relatively large quantities. Subsequent protocols for the isolation of cytokinins were often based on the structures and properties of metabolites discovered earlier. With the advent of more refined analytical tools such as HPLC combined with ELISA and/or mass spectral analyses (4, 7, 47, 63, 70, 174), metabolites present at minute concentrations can be detected, but the tendency to focus on known structures continues. Obviously, it is difficult to devise protocols for the isolation of unknown compounds; moreover, less common and novel cytokinin derivatives can be overlooked. Such may be the case with lupinic acid, isolated from lupin (104), and *O*-xylosylzeatin, found in *Phaseolus vulgaris* (99, 177), which could also occur in other species. Additional routes to identify new metabolites are now available. For example, determining the substrate specificity of metabolic enzymes would help predict the possible occurrence of unknown/unexpected compounds. As an illustration, the zeatin *O*-xylosyltransferase of *P. vulgaris* was found to convert dihydrozeatin to its *O*-xylosyl derivative (177; see below), which led to the isolation

of *O*-xylosyldihydrozeatin from bean seeds (125). Prediction of metabolites could also be based on genomics. Newly cloned genes encoding cytokinin metabolic enzymes can be compared with GENBANK sequences to identify homologues. The recombinant proteins of candidate genes can then be tested in vitro to determine if (and which) cytokinins are substrates for the enzymes. Enzymatic products may represent cytokinin metabolites occurring in vivo even though they have not yet been revealed using conventional approaches. For instance, the cloning of a gene from maize encoding an *O*-glucosyltransferase specific to *cis*-zeatin (111; see below) suggests the presence of *cis*-zeatin-*O*-glucoside in this species. Eventually, metabolites may be predicted through the identification of sequences known to represent cytokinin binding sites.

Numerous studies have focused on measurements of cytokinin levels, but the significance of such quantitations is often unclear. Early on, levels of biologically active cytokinins were usually expressed as kinetin equivalents based on results of bioassays such as tobacco callus culture or chlorophyll retention tests. This approach, although seemingly undefined/unrefined, was operationally useful. As more cytokinin metabolites were discovered, results from cytokinin analyses gradually evolved into the display of arrays of metabolites and their quantities, the extent of which is limited mainly by the machinery and resources of the laboratories. The presentation of all measurable cytokinin components allows for a myriad of interpretations, but how such data sets relate to active cytokinin levels is not obvious. This is in part due to the uncertainty regarding the active form of cytokinin and the presumed reversibility of storage forms to active forms. The more cytokinin metabolites are discovered, the more difficult the task of interpreting the results of quantitative measurements becomes. As long as the function, or the qualitative importance, of each cytokinin remains unknown, the interpretation of prolific measurements will continue to be problematic. Moreover, current methods measure only extractable/free metabolites without including cytokinins bound to proteins or structural components. As the efficacy of each cytokinin is determined by a composite of factors such as cellular/tissue location, stability, and its metabolism, the quantity of that cytokinin extracted from mixed tissues or whole plants may or may not reflect its biological significance in vivo. Therefore, instead of relying on quantitation, it may be more informative to associate phenotypic variations or responses to environmental/chemical cues with changes in a specific metabolic step. In order for this approach to be successful, it is necessary to identify enzymes and genes regulating such metabolic conversions.

METABOLIC ENZYMES AND GENES

Conversions Between Cytokinin Bases, Nucleosides, and Nucleotides

Feeding experiments with cytokinin bases invariably result in the formation of corresponding nucleosides and nucleotides (102). Generally, such interconversions involve enzymes common to purine metabolism. These enzymes usually have

higher affinity for adenine, adenosine, and AMP than for the corresponding cytokinins (reviewed in 34). For example, a 5′-nucleotidase (EC 3.1.3.5) from wheat germ (36) and tomato (26) converts AMP to adenine and cytokinin nucleotides to cytokinin bases but has higher affinity to AMP. Similarly, adenosine nucleosidase (EC 3.2.2.7) from wheat germ (37) has a lower K_m for adenosine than i^6Ado. Formation of cytokinin nucleosides from their free bases is mediated by an adenosine phosphorylase (EC 2.4.2.1), which prefers adenine over i^6Ade (41). Nucleotides of cytokinins can be formed from nucleosides by adenosine kinase (EC 2.7.1.20) or directly from free bases via the action of adenine phosphoribosyltransferase (APRTase; EC 2.4.2.7) (40). Mutants deficient in APRTase were isolated from *Arabidopsis* (123) and two genes encoding this enzyme were cloned (122, 158). Although one of the two recombinant enzymes (APT1) has much higher affinity for adenine than BA, the other (APT2) has threefold higher affinity for BA. The affinities for isoprenoid cytokinins were not reported. It is not known whether the male sterility of the *apt1* mutant is due to changes in cytokinin composition or purine metabolism (66). Another gene, encoding an adenosine kinase, was isolated from the moss *Physcomitrella* by complementation of a purine auxotrophic *E. coli* mutant, but its substrate specificity has not yet been determined (180).

Other Modifications of the Adenine Moiety

A glucosyltransferase (EC 2.4.1.118) catalyzing the formation of cytokinin glucosides at the 7- and 9-positions was isolated from radish cotyledons (55, 57). Although the enzyme recognizes a large number of adenine derivatives as substrates, the rate of conversion is highest for compounds with N^6-side chains of at least three alkyl carbons and roughly correlates with cytokinin activity (57). The formation of 7-glucosides is favored, but the relative amounts of 7- and 9-glucosides formed differ between substrates, with ratios of about 2:1 for BA and 10:1 for zeatin. The K_m for zeatin is 150 μM. The enzyme has a mass of about 46.5 kD and a broad pH range with an optimum of 7.3. Both UDPG and TDPG can serve as glucosyl donors.

A transferase (EC 4.2.99.13) converting zeatin to its 9-alanyl derivative, lupinic acid, was partially purified from lupin seeds (56). This enzyme has low substrate specificity but shows a preference for cytokinins since the rate of product formation is 14 times higher with zeatin than adenine and the K_m is 0.88 mM for zeatin versus 26 mM for adenine. The donor substrate is *O*-acetyl-L-serine (K_m 47 μM). The mass of the enzyme is estimated as 64.5 kD.

Modification of the Isoprenoid Side Chain

Cytokinin Oxidases Cytokinin oxidases selectively degrade unsaturated N^6-isoprenoid side chains, converting active cytokinins such as zeatin and i^6Ade to adenine (Figure 3). Cytokinin bases and nucleosides but not the nucleotides are susceptible (3, 69, 87). The oxidation product of the i^6Ade-side chain has been identified as 3-methyl-2-butenal (23). Since the isolation of the first cytokinin oxidase from tobacco callus tissues 30 years ago (140), this type of enzyme has

been obtained from maize, beans, poplar, wheat, and *Vinca rosea* crown gall tissues (reviewed in 3, 69, 87). The wide occurrence of oxidases is matched by their diversity. They vary in mass, from 25 kD for the enzyme from *Vinca rosea* to 94 kD for maize (3, 118). Most but not all of these enzymes are glycoproteins, with pH optima ranging from 6.0 to 9.0 (3, 90). Based on the degradation products, the use of oxygen, and the enhancement by copper imidazole complexes (32), oxidases were classified as copper-dependent amine oxidases (EC 1.4.3.6). This classification has recently been questioned (147) because copper-dependent amine oxidase seems to oxidize primary amines specifically (98), whereas cytokinins are secondary amines. The finding that the recombinant cytokinin oxidases of maize are flavoproteins (14, 77, 133; see below) also suggests that reclassification may be in order. However, the diverse properties of cytokinin oxidases indicate that there may be more than one class of cytokinin degradation enzyme.

Maize cytokinin oxidase genes were recently cloned in two laboratories (77, 133). Both groups based their strategies on determining partial internal amino acid sequences of enzymes purified from immature maize kernels. A partial gene sequence was then obtained using PCR with degenerate primers. Morris et al (133) isolated the full-length gene by screening a genomic library, while Houba-Hérin et al (77) cloned the cDNA via RACE PCR from a maize kernel cDNA library. The ORF was expressed in *Pichia* or moss protoplasts and an active oxidase was secreted into the culture medium. The two groups may have isolated alleles of the same gene family since the two clones differ only in seven nucleotides (three amino acids). The gene, *ckx* or *CKO*, consists of three exons separated by two small introns. The ORF of 1602 bp encodes a 57-kD enzyme with a flavin-binding domain and eight possible glycosylation sites. BLAST searches revealed homology to seven *Arabidopsis* accessions as well as the *fas5* gene of *Rhodococcus fascians* (14, 77). The *Arabidopsis* homologs are 39% to 47% identical to the maize gene at the amino acid level. Of the four homologs tested, three encode proteins displaying cytokinin oxidase activity (14). Western analyses detected high levels of the enzyme in maize kernels but low or no enzyme in other plant parts. The K_m values for the recombinant enzyme (i^6Ade, 1.5 μM; i^6Ado, 11 μM; zeatin, 14 μM; *cis*-zeatin, 46 μM) are in good agreement with those of the native enzyme, but differ somewhat from earlier estimates reported for maize cytokinin oxidase by other laboratories (3, 118). The molecular weight and the pH optimum also differ from earlier findings, again suggesting the likely occurrence of additional types of cytokinin oxidases.

Many studies have shown that phenylurea-type cytokinins such as thidiazuron and CPPU are strong inhibitors of cytokinin oxidase activity (3, 25, 31, 96). Although characterizations of maize and bean oxidases suggested such inhibition is noncompetitive (3, 25), the latest kinetic analyses of ckx1 recombinant protein indicate that CPPU is a competitive inhibitor, at least to this maize oxidase (14). It should be noted that adenine-type cytokinins such as BA and kinetin, which do not serve as substrates to the enzyme, did not inhibit oxidase activity in bean callus

cultures (31). Whether these cytokinins show competitive inhibition with the ckx1 enzyme has not been determined.

Zeatin O-Glycosyltransferases *O*-Glucosylzeatin seems to be a ubiquitous metabolite in plants (178), although *O*-xylosylzeatin has been detected thus far only in *Phaseolus* (99, 125). As *O*-glucosylzeatin is resistant to cytokinin oxidase attack (3) and can be converted to the active aglycone by β-glycosidases, *O*-glycosylation may serve an important function in regulating the level of active cytokinins. Two zeatin *O*-glycosyltransferases have been isolated thus far, an *O*-xylosyltransferase (EC 2.4.1.204) from *P. vulgaris* (177) and an *O*-glucosyltransferase (EC 2.4.1.203) from *P. lunatus* (49). The two enzymes are similar in mass (about 50 kD) but differ in charge. The *O*-glucosyltransferase uses UDPG and UDPX as donor substrates but has much higher affinity to UDPG, whereas the *O*-xylosyltransferase uses only UDPX. The cytokinin substrate recognition of the enzymes is also highly specific; besides zeatin, the only other substrate is dihydrozeatin, which is used by both transferases to form *O*-xylosyldihydrozeatin in the presence of UDPX (49, 109, 124). Interestingly, the *O*-glucosyltransferase does not catalyze the formation of *O*-glucosyldihydrozeatin in the presence of UDPG. This is rather unexpected since *O*-glucosyldihydrozeatin is known to occur in *P. vulgaris* (182); therefore, it is likely that other *O*-glucosyltransferases exist capable of mediating conversion of dihydrozeatin. Neither *cis*-zeatin nor any of the ribosides serve as substrates for the enzymes. The stringent substrate specificity for cytokinins and sugar donors suggests that *O*-glycosylation is precisely regulated, tailored to individual cytokinins.

Genes encoding the zeatin *O*-glucosyl- and *O*-xylosyltransferase have been cloned from *P. lunatus* and *P. vulgaris*, respectively (112, 113). The first gene, *ZOG1* (for zeatin *O*-glucosyltransferase), was identified by screening a cDNA library from immature *P. lunatus* seeds with monoclonal antibodies (110). The second gene, *ZOX1* (for zeatin *O*-xylosyltransferase), was obtained by inverse PCR with primers based on the *ZOG1* sequence. Neither gene contains any introns. The ORFs of *ZOG1* and *ZOX1* encode polypeptides of 459 and 454 amino acids, respectively, with a mass of 51 kD. The recombinant proteins (112, 113) exhibit properties identical to those of the native enzymes (49, 177). The two genes exhibit 93% and 87% identity at the DNA and amino acid level, respectively. Northern analyses showed high expression of the genes in immature seeds but low in vegetative tissues. Recently, additional homologues have been isolated from *Phaseolus* as well as *Glycine max* (X Shan, RC Martin, MC Mok & DWS Mok, unpublished results).

The regions of the *ZOG1* gene relevant to UDPG affinity were determined using hybrid enzymes derived from domain exchanges of parental *ZOG1* and *ZOX1* genes (109). The N-terminal half of the enzyme is of critical importance in this aspect since hybrid enzyme having the N-terminal half of ZOG1 and C-terminal half of ZOX1 had exactly the same patterns of UDPG and UDPX utilization as ZOG1. Substituting the region between bp 401 and 637 of the *ZOG1* ORF with

the corresponding segment of *ZOX1* abolished utilization of UDPG as substrate, but UDPG still competed with UDPX when present in the reaction mix. Additional substitution of the segment from bp 74 to 400 eliminated binding of UDPG to the enzyme, as evidenced by the loss of competitive inhibition by UDPG in UDPG+UDPX incubation. Amino acids critical for UDPG binding to *ZOG1* may be pinpointed by site-directed mutagenesis.

Transgenic tobacco plants were generated harboring the *ZOG1* gene under the control of a constitutive (*35S*) and an inducible (*Tet*) promoter (114). Expression of the transgene resulted in elevated enzyme production and increased conversion of exogenous zeatin to its *O*-glucoside. In zeatin/NAA interaction experiments, Tet-*ZOG1* leaf discs cultured in the presence of tetracycline required tenfold higher zeatin concentrations for the formation of shoots and callus than the controls (Tet-no insert), which fits the hypothesis of *O*-glucosylzeatin being a storage product. On the other hand, 35S-*ZOG1* plants showed developmental variations including adventitious root formation on the lower stems and axillary shoot growth, the latter indicative of decreased apical dominance. Thus, increased zeatin *O*-glucosylation in detached, cytokinin-dependent tissues leads to cytokinin sequestering whereas in whole plants the effect can simulate a reduction or a rise in cytokinin activity, depending on the tissue and stage of development. The use of tissue- and stage-specific promoters will further clarify the role of cytokinin *O*-glycosylation and perhaps even allow targeted modifications of growth patterns.

Recently, a novel gene, *cisZOG1*, encoding a *cis*-zeatin *O*-glucosyltransferase, was isolated from maize (RC Martin, MC Mok, JE Habben & DWS Mok, unpublished results). This gene was cloned utilizing the sequence of *ZOG1* from *Phaseolus* to identify candidate genes from maize EST (expressed sequence tag) data banks of Pioneer Hi-Bred International (PHB). This gene also does not have any introns and the ORF of 1401 bp encodes a polypeptide of 50 kD. The enzyme recognizes *cis*-zeatin and UDPG as substrates, but not *trans*-zeatin, dihydrozeatin, *cis*-zeatin riboside, or UDPX. The *cisZOG1* and *ZOG1* genes are 60% identical at the DNA level and 40% at the deduced amino acid level. The occurrence of *trans*- and *cis*-specific enzymes again reinforces the precision of metabolic regulation. The finding suggests that *cis*-zeatin derivatives may play a more important role in cytokinin homeostasis than currently recognized and illustrates the possible use of genomic analyses to seek new cytokinin metabolites.

***β*-Glucosidases** In order to restore cytokinin activity, conversion of glucosides to the corresponding aglycones is necessary. Although β-glucosidases (EC 3.2.1.21) and genes encoding these enzymes have been identified, none showed specificity to cytokinins. A maize β-glucosidase of 60 kD (p60) was partially purified (27) and the gene cloned (24). The recombinant enzyme (64 kD) cleaves *O*-glucosylzeatin and kinetin-*N*3-glucoside (24) as well as a number of other artificial and natural substrates (42). The gene (*Zm-p60.1, glu1*) is about 5 kb in length and consists of 12 exons separated by 11 introns. A similar gene was isolated from *Brassica*

napus (60). The recombinant enzyme converts zeatin *O*-glucoside to zeatin, but related substrates, such as cytokinin *N*-glucosides, were not tested. Considering the low specificity of β-glucosidases, hydrolysis of glucosides may not be highly regulated, in contrast to *O*-glucosylation. Separation of cytokinin glycosides from β-glucosidases in different subcellular locations may provide a measure of control, as suggested by the finding that the *O*-glucoside of dihydrozeatin is localized in vacuoles (65).

Zeatin Reductase Reduction of the *trans*-zeatin side chain to form dihydrozeatin is mediated by a zeatin reductase isolated from immature seeds of *P. vulgaris* (115). The enzyme is highly specific for zeatin; it does not reduce *cis*-zeatin, *trans*-zeatin riboside, i^6Ade, or zeatin *O*-glycosides. The enzyme requires NADPH as a cofactor. Two isoforms of the enzyme, about 25 kD and 55 kD in size, were found in *Phaseolus* species. As dihydrozeatin is resistant to cytokinin oxidases, reduction of the zeatin side chain may preserve cytokinin activity, especially in tissues with high levels of oxidases.

Zeatin Isomerase A *cis-trans* zeatin isomerase was partially purified from *Phaseolus* (11). The enzyme favors conversion from the *cis*- to the *trans*- isomer. As the indirect pathway of cytokinin biosynthesis involves the breakdown of cytokinin-containing tRNAs (146, 171), the isomerase provides a possible route to convert the less active *cis*-zeatin to the highly active *trans*-isomer.

Hydroxylase Hydroxylation of the isopentenyl side chain was detected in a microsomal fraction of cauliflower (38). The enzyme catalyzed conversion of i^6Ade to zeatin and i^6Ado to zeatin riboside in the presence of NADPH. However, further purification and characterization of the enzyme has not been reported.

CYTOKININ ACTION

For close to 30 years, scientists have been searching for cytokinin-binding proteins (CBPs) that could serve as receptors (reviewed in 19–21; 18, 22, 64, 93–95, 120, 138). Many CBPs exhibit some of the properties expected of receptors, but clear proof for a true receptor is lacking. Based on the cytokinin concentrations in plant tissues, few of these proteins have K_d values low enough to qualify as receptors. Moreover, the function of these proteins is often unknown (reviewed in 15, 19, 20, 21).

The advent of molecular biology has brought about rapid progress in the field of plant hormone perception/signaling (61, 117). The patterns emerging for plant signal transduction resemble those established for other organisms (30, 117, 143). The first step involves binding of the hormone to a high-affinity receptor protein, resulting in a conformational change. This then sets into motion a signal transduction cascade, either through a his-asp phosphorylation/dephosphorylation chain or by guanine nucleotide binding and hydrolysis, eventually leading to changes in

transcription of a specific set of genes. Although information on the components of cytokinin perception and signal transduction is still very limited, recent findings suggest that this model may be applicable to cytokinin signaling as well.

Phosphorelay Signal Transduction

A gene encoding a putative cytokinin receptor, CKI1, was isolated from *Arabidopsis* through *Agrobacterium*-mediated activational tagging using a 35S promoter (89, 135). Tissues of the transformant harboring the promoter upstream of the *CKI1* gene were cytokinin-independent in vitro, indicative of either increased cytokinin production or sensitivity. The CKI1 protein contains a putative input domain with two membrane-spanning regions, a sensor histidine kinase, and a receiver domain. The phenotype of the mutant together with the similarity of the protein to bacterial two-component systems and ETR1, an ethylene receptor (29, 154), render a cytokinin receptor function plausible. However, binding of CKI1 to cytokinins has yet to be demonstrated.

The existence of a his-asp phosphorelay chain is further suggested by the identification of other possible components of such a pathway (44, 45). *Arabidopsis* genes rapidly induced by cytokinin, *IBC6/ARR5* and *IBC7/ARR4*, were isolated by two groups (17, 80, 91, 172). The induction of gene expression does not require protein synthesis, indicative of a primary response function. Moreover, highly active cytokinins, including thidiazuron, were able to induce these genes, whereas adenine was only weakly active (17). Maize genes homologous to *IBC/ARR* have also been identified (152, 153). The gene products are similar to the receiver domain of bacterial two-component systems (30) and contain two invariant aspartate residues and an invariant lysine residue. Although 14 members of this family were reported for *Arabidopsis* (45, 79), only those containing a receiver domain and a short C-terminal region (A-type) were rapidly induced by cytokinins. Those having a receiver domain and a longer C-terminal region (B-type) were not induced by cytokinin. Divergence between the two types is indicated by the low homology of amino acid sequences of receiver domains between members of the two groups (24% to 30%), in contrast to the high homology between members of the same group (60% to 96%). B-type ARR proteins have properties suggestive of transcription factors, including the presence of a Myb-related motif, nuclear localization, and the ability to activate transcription when fused to the GAL4 DNA-binding domain (79, 107).

A gene family (*AHP*) with potential action in the same signal transduction pathway as A-type ARR members was obtained by scanning the *Arabidopsis* EST database for genes containing histidine phospho-transfer domains (121, 168). Purified AHP1 and AHP2 protein, when previously phosphorylated by crude bacterial membranes, could phosphorylate ARR3 and ARR4 in vitro, indicating a possible role upstream of ARR in the pathway. It should be noted that genes similar to *AHP1* also occur in maize (151). A model was proposed by D'Agostino & Kieber (45), with CKI1 as the receptor, AHP downstream from CKI1, and ARR downstream

from AHP. However, a complication is the finding that AHP1 can phosphorylate the receiver domain of CKI1 as well (135). To provide an explanation, the authors speculated that the CKI1 receiver domain may negatively modulate its own signaling pathway through the removal of a phosphate from a previously phosphorylated AHP. The same set of AHP proteins can form stable complexes with B-type ARRs, but does not phosphorylate them (79). Another gene possibly interacting with ARR4 was obtained by a yeast two-hybrid screen (184). This gene (*AtDBP*) had been cloned earlier and encodes a DNA-binding protein (2).

CycD3, encoding a protein involved in the G1—S transition of the cell cycle, may be one of the primary genes induced by cytokinin (148, 165). Transcripts began to accumulate within one hour of cytokinin treatment of cell suspensions and intact seedlings (148). Moreover, tissues overexpressing CycD3 were cytokinin-independent in culture. The inability of cycloheximide to inhibit induction indicates that this is an early response gene, not requiring protein synthesis. Although there is no established connection with the phosphorelay genes described above, regulation through phosphorylation is implicated by the effects of phosphatase and kinase inhibitors (148). Another candidate gene is *cdc2*, a histone H1 kinase involved in G2—M transition with expression induced by cytokinin and auxin (73, 83, 186). As expected, many additional genes are induced or repressed by cytokinins, and in some cases expression was shown to be affected by phosphorylation (for review, see 43, 157). However, for most of these genes either the function is unknown or the lag time before changes in transcription is too long to be considered as early response genes. (See Note Added in Proof, p. 118.)

G-Protein Coupled Receptor

A different type of putative cytokinin receptor, belonging to the class of G-protein coupled receptors (59), was identified by Plakidou-Dymock et al (145) through screening of an *Arabidopsis* EST database for genes with homology to known bacterial G-protein receptors. The deduced amino acid sequence of the gene, *GCR1* (for G-protein cytokinin receptor), contains seven membrane-spanning domains and homology to 7TM G-protein coupled receptors. This protein was assigned a role in cytokinin perception due to the lower sensitivity to cytokinins of *Arabidopsis* transformants containing an antisense *GCR1* construct (145). Binding of this putative receptor to cytokinins and interaction with G-proteins has yet to be shown.

IMPLICATIONS

Significance of Metabolites and Enzymes

The metabolic complexity of natural cytokinins can be reduced substantially if inter-conversions between bases, nucleosides, and nucleotides are considered separately. This seems reasonable since these interchanges are likely adjunct to purine metabolism, as supported by the observation that none of the enzymes involved in the modification of the adenine ring has specificity for cytokinins (34). Mutation of

the *APT1* gene of *Arabidopsis* encoding an adenine phosphoribosyltransferase results in male sterility and recalcitrance in tissue culture (66, 123), and the *schizoid* mutant of *Arabidopsis*, having a lesion in a gene with high homology to adenosine kinases, has characteristics indicative of cytokinin overproduction (144). However, it is not known whether the phenotypic alterations are related to a change in cytokinin metabolism or general purine metabolism. Yet, the interconversions between the three forms can have a bearing on cytokinin activity and transport. The free base is usually the most active in bioassays (100, 116, 155, 156, 163), which may be related to rapid uptake and high intrinsic activity (97). Evidence has also been presented that the three forms are transported differentially via the xylem, with the nucleosides being predominant (102). Recently, a family of adenine transporters was discovered in *Arabidopsis* (67). Although they are not specific for cytokinins, their activity is likely to influence the cell-to-cell transport of cytokinin bases as well. Thus, despite the nonspecific nature of the interchanges, the relative proportions of the three forms may directly or indirectly influence cytokinin activity.

Side chain modifications appear to be highly specific. Not only can the enzymes distinguish between cytokinin bases, nucleosides, and nucleotides, but also between saturation, hydroxylation, and steric conformation. The former is illustrated by the fact that zeatin *O*-glycosyltransferases act only on cytokinin bases (49, 112, 113, 177). The latter is demonstrated by the characteristics of zeatin *O*-glycosyltransferases and reductase, with *O*-glycosyltransferases differentiating between *cis*-, *trans*-, and dihydro-zeatin (124) and the reductase recognizing *trans*-zeatin but not *cis*-zeatin or i^6Ade (115). Even though cytokinin oxidases are not as specific, cleaving all cytokinins with unsaturated isoprenoid side chains, the rate of degradation is also dependent on side chain configuration (14, 31). The properties of these enzymes imply that the metabolism of individual cytokinins is precisely regulated by a host of highly specific proteins, many of which yet to be discovered.

Metabolic enzymes and genes are useful tools in dissecting the function of cytokinin metabolites. The levels of enzymes and reaction products can be modulated by manipulating the expression of genes encoding the enzymes. For example, cloning of the *ZOG/ZOX* genes allows more precise determination of the effects of *O*-glycosylation on plant development. The fact that leaf discs of transgenic tobacco (with increased *ZOG1* expression) required significantly higher zeatin concentrations for shoot regeneration (114) confirmed a storage role of *O*-glucosylzeatin. However, in intact plants, increased *O*-glucosylation can also lead to characteristics indicative of higher cytokinin activity, which could not have been predicted from the theoretical consequences of cytokinin sequestering through *O*-glucosylation. Although there are a number of possible explanations for the seemingly opposite effects on detached and intact plants tissues (114), the observations suggest that local cytokinin composition in organs at particular stages of development may be more important than total cytokinins in whole plants.

External factors may influence the expression of metabolic genes and composition of metabolites. In a recent study, zeatin *O*-glycosyltransferase levels were determined in roots of maize and beans (106) using antibodies specific to the bean enzymes (110). At low temperatures, 10° and 4°C, the amount of antigenic protein was substantially higher than at 25°C. Thus, low temperatures may induce sequestering of active compound by triggering zeatin *O*-glycoside formation. If confirmed, this would establish the relationship between a specific metabolic step and an environmental cue. In general, identification of physical and chemical factors influencing the expression of metabolic genes will provide valuable information on the regulation of metabolite levels and plant development.

The function of *cis*-zeatin is unclear. Although it is only weakly active in bioassays (156), *cis*-zeatin and its derivatives can be the predominant cytokinins in some species, such as chickpea (54), or in specific organs, such as male flower buds of *Mercurialis* (51). *cis*-Zeatin and derivatives were also detected in rice roots (82), potato tubers (137, 167), hops (183), wheat (142), and oats (142). It is conceivable that *cis*-zeatin has either a highly specialized function or can serve as a precursor for *trans*-zeatin. For instance, *cis*-isomers produced in roots may be transported to shoots, where light can enhance isomerization to *trans*-isomers (11). Manipulations involving the *cisZOG1* gene of maize (RC Martin, MC Mok, JE Habben & DWS Mok, unpublished results) may be useful to further elucidate the function of *cis*-derivatives, particularly in plant species with substantial amounts of *cis*-zeatin.

The fact that cytokinin oxidase level in whole plants and callus cultures is stimulated by both adenine- and phenylurea-cytokinins (3, 31, 134, 141, 175) indicates that these enzymes are important in the catabolism of excessive cytokinins. Another possible function of oxidases may be the prevention of cytokinin movement into or out of specific tissues or organs. An example may be found in developing seeds where high cytokinin oxidase activity occurs in the seed coat (176), limiting movement of cytokinins to and from developing embryos (102). Now that an oxidase gene from maize has been cloned, determining the distribution of oxidases among endosperm, embryo, aleurone, and pericarp of maize may be an initial step in examining the concept of oxidases as regulators of cytokinin trafficking between tissues.

Cytokinin and cytokinin oxidase activity during grain development have been studied rather extensively, with the ultimate goal of manipulating yield (8, 48, 85, 88, 150). At early embryo development of cereals, there is a narrow period (days) when cytokinin content rises, after which it declines rapidly. This led to the hypothesis that seed fill and the eventual size/yield of the seeds may be related to, and can be manipulated by, maintaining a higher level of cytokinins. However, external application of adenine- or urea-type cytokinins had sporadic effects on yield (8, 78), while transformation with the *ipt* (isopentenyltransferase) gene is beneficial only if expression of the gene could be regulated rather precisely. Since the decline in cytokinin level is accompanied by an increase in cytokinin oxidase, lowering the level of cytokinin oxidases may be a viable alternative. In general, cytokinin genes can conceivably be used to optimize agronomic characteristics.

Biosynthesis

Our knowledge of cytokinin biosynthesis in plants is still very limited. It is generally assumed that i^6Ade and zeatin have a common origin and that the first product of biosynthesis is i^6AMP (16, 35). Although this pathway seems logical, particularly in light of the action of the *ipt* gene of *Agrobacterium tumefaciens* (1, 10), evidence for this pathway in plants is still inconclusive. Only rather crude tobacco extracts were found to contain ipt activity (30). As zeatin-type compounds are predominant in plants, hydroxylation has to be a crucial step in the direct pathway but the general occurrence of this conversion is still uncertain. Incorporation of radiolabeled i^6Ade and i^6Ado into zeatin and its riboside by a microsomal fraction of cauliflower was reported (38). Conversion of exogenous, unlabeled i^6Ade to a product coeluting with zeatin was also observed in *Actinidia* (52), although the product was not further authenticated. On the other hand, callus tissues of *Phaseolus* did not incorporate any significant amount of label from ^{14}C-i^6Ade or ^{14}C-i^6Ado into zeatin or its riboside (28, 131). It is possible that hydroxylation is limited to certain plant species, as supported by a survey (53), accounting for the inconsistent occurrence.

It is also possible that there are other substrates for the direct biosynthesis of zeatin in plants. For example, the formation of ZMP could occur through transfer of a hydroxylated side chain. In fact, this function could also be mediated by the enzyme encoded by the *tzs* gene of *A. tumefaciens*. Expression of this *tzs* in bacteria resulted in the recovery of zeatin in the culture medium (13, 72). The explanation given was that side chain hydroxylation occurred after the formation of i^6AMP/i^6Ado/i^6Ade, through the action of bacterial enzymes. That explanation, however, cannot be reconciled with the observation that *Agrobacterium* without the Ti plasmid secreted only i^6Ade, derived from tRNA (68). Further support in favor of direct transfer of a hydroxylated side chain was recently provided by the dynamics of deuterium in vivo labeling of cytokinins in *ipt*-transformed and wild-type *Arabidopsis* (4). Moreover, transferases could conceivably differ in their relative affinities to isopentenyl and hydroxylated side chains, with plant enzymes preferring the hydroxylated side chains. However, plant transferases are unlikely to have high homology to their bacterial counterparts since extensive probing of plant genomes did not reveal complementary sequences to *ipt/tzs* (12). Nevertheless, the *Arabidopsis* data bank contains a number of candidate genes with low homology to these genes.

For many years, tRNA as a source of cytokinins has been the subject of debate. Measurements of tRNA breakdown indicated that this process can contribute up to 50% of the free cytokinins (9, 108). An often encountered argument against tRNA being a considerable source of cytokinin is the fact that *cis*-zeatin is the major cytokinin in tRNA. However, this problem can potentially be accommodated by the isomerization of *cis*-zeatin to *trans*-zeatin by *cis-trans* isomerases (11). Another objection is the general nature of tRNA breakdown, occurring in all tissues, while cytokinin production is localized in root tips, shoot meristems, and immature seeds (102), and must be highly regulated. Therefore, if tRNAs were a major source of

cytokinins, regulatory mechanisms must operate at the level of the metabolism of the released *cis*-zeatin.

The occurrence of *cis*-zeatin as a minor cytokinin constituent could be explained by its origin from tRNA, but its presence as the major component in some plants or plant organs (51, 54, 82) may signify a direct pathway of biosynthesis. The isopentenyl side chain can possibly be hydroxylated to the *cis* configuration or, as proposed above for *trans*-zeatin, a *cis*-hydroxylated side chain is directly transferred to the adenine moiety. The biosynthetic pathway leading to aromatic cytokinins is entirely unknown. To date, no enzymes mediating the conjugation of a benzyl ring to adenine have been identified. It is apparent that the pathway for the biosynthesis of these cytokinins should be substantially distinct from that proposed for the isoprenoid cytokinins, perhaps closer to the metabolism of phenolics (166). A precursor suggested for topolin is the amino acid phenylalanine (185). In the same vein, isoprenoid cytokinins could be derived from compounds related to the amino acid leucine. However, these are theoretical pathways, the confirmation of which will have to come from the identification of the enzymes and genes involved. Nevertheless, the possibility that cytokinins with specific side chain configurations have separate origins, rather than having a common intermediate, must be considered.

Perception

The action of cytokinins at the molecular and the whole plant level is still largely unknown. In addition to putative receptor/signal transduction genes such as *CKI1*, *ARR*, and *GCR1*, proposed cytokinin targets include cell cycle genes and genes affecting shoot meristem formation. Candidate genes related to the cell cycle are *CycD3* and *cdc2* (83). The *knotted* homeobox gene of maize and *Arabidopsis* was also implicated in cytokinin action since transgenic plants overexpressing the *ipt* gene or *kn1* have similar phenotypes (139) and *ipt* transformants have increased mRNA levels of homeobox genes (149). As cytokinins stimulate cell division and formation of meristematic tissues, a link between these events and cytokinin action is expected. However, with the exception of *CycD3* (148), there is no evidence for primary action of these genes and they likely function further downstream from the signal transduction chain.

The study of action/perception will invariably encounter the paradox unique to cytokinins, the apparently indistinguishable biological effects of adenine and phenylurea cytokinins. It has been debated whether the phenylurea derivatives exert direct effect or indirect effect by modifying the biosynthesis and metabolism of endogenous cytokinins. The direct-effect hypothesis is supported by steric modeling studies suggesting that both adenine and urea cytokinins have a conformation capable of binding to the same receptor protein (62, 81). If that is the case, a true cytokinin receptor should display affinity to both types of cytokinins. The report of CBPs with affinity to both types of compounds (138) seems to suggest the occurrence of proteins with epitopes fitting both classes of cytokinins. The induction of the *ARR4* and *ARR5* genes by both adenine and phenylurea cytokinins (17) is also

compatible with the direct-action hypothesis. Signal-receiving molecules capable of binding to both types of compounds, however, will also have to conform to the rules for structure-activity relationship of cytokinins, especially regarding the configuration of the N^6-side chain (163). The notion of ligands (cytokinins) with such different chemical structures recognizing the same signal-receiving proteins is challenging. The alternative, i.e. phenylureas acting on endogenous cytokinin metabolism or biosynthesis, is supported by the observations that they are inhibitors of cytokinin oxidases (14, 32) and are able to induce cytokinin-autonomous growth of cultured cells (127). In addition, conversion of cytokinin nucleotides to nucleosides is stimulated by phenylurea cytokinins (28). These properties, coupled with the stability of the phenylureas, are expected to alter the level/action of endogenous cytokinins. Unfortunately, the relative contributions of the direct and indirect effects on cytokinin action cannot easily be determined. Nevertheless, the studies of phenylurea cytokinins indicate quite clearly that in general, the apparent cytokinin activities of chemicals may not necessarily correlate with their affinities to cytokinin receptors since indirect effects and differential stability will influence their efficacy.

FUTURE RESEARCH DIRECTIONS

Cytokinin research based on traditional concepts and approaches will continue to be necessary in updating and confirming earlier findings. Molecular biology coupled with innovative and testable hypotheses will be needed if significant progress is to be made. For example, it may be useful to consider the possibility of independent pathways for the direct biosynthesis of cytokinins with distinct N^6-side chains, rather than continue to pursue a single pathway based on the *Agrobacterium* model. The contribution of the indirect pathway involving tRNA should also be reinvestigated. Parallel pathways may operate in cytokinin biosynthesis, just as for auxin biosynthesis (164), and different pathways may operate in different species. The metabolism of diverse classes of adenine-type cytokinins is likely to be regulated by different sets of genes encoding enzymes with precise recognition based on the configuration of the N^6-side chain. The various metabolites may have unique functions via differential subcellular localization or accumulation in tissues. Therefore, determining the location of enzymes as well as metabolites in tissues and cells will be important. Quantifying cytokinins may be more meaningful if selective measurements are taken of particular tissues at specific stages of development. Determination of changes in cytokinin components associated with defined phenotypic variations should be more useful than data taken on whole plants. Many areas of cytokinin research require attention but the greatest need is the cloning and characterization of additional genes, be they involved in cytokinin biosynthesis, metabolism, or perception/signal transduction. Employing genomics, transgenics, and reverse genetics as routine tools to study cytokinins will be crucial to gene discovery and characterization. Concentrating efforts on

model plants extensively used for cytokinin analyses in the past, such as tobacco, beans, and maize, or those with sequenced genomes, such as *Arabidopsis*, should accelerate the pace of progress.

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NOTE ADDED IN PROOF

A gene, *CRE1* (cytokinin response 1), encoding a histidine kinase was identified in *Arabidopsis*. When expressed in yeast lacking the endogenous enzyme SLN1, *CRE1* confers a cytokinin dependent growth phenotype, indicative of receptor properties (80a).