J Plant Growth Regul (2002) 21:17–23
DOI: 10.1007/s003440010043

J P G R
Journal of Plant Growth Regulation

Identification of Cytokinin Biosynthesis Genes in *Arabidopsis*: A Breakthrough for Understanding the Metabolic Pathway and the Regulation in Higher Plants

Hitoshi Sakakibara* and Kentaro Takei

The Institute of Physical and Chemical Research (RIKEN), Plant Science Center, Wako, Saitama, Japan

Abstract

The primary biosynthetic reaction of cytokinin is thought to be the isopentenylation of an adenine nucleotide such as AMP with dimethylallylpyrophosphate. For many years, the nature of the enzyme catalyzing this reaction in higher plants had not been identified despite the physiological importance of these compounds. However, the completion of the genomic sequence of *Arabidopsis thaliana*, a model plant for genetic research, has provided us with new opportunities to solve these problems. Recent studies have revealed the cytokinin biosynthesis enzyme is encoded by a small multigene family that is structurally related to both bacterial adenylate isopentenyltransferase and tRNA isopentenyltransferase. Interestingly, biochemical studies of some of the gene products indicate that ADP and ATP, rather than AMP, are preferentially used as substrates for this biosynthetic reaction. These findings require reconsideration of the currently accepted cytokinin biosynthetic pathway. In addition, there is an increasing body of evidence suggesting that the expression of these cytokinin synthesis genes is affected by the availability of nutrients.

Key words: Adenylate isopentenyltransferase; *Arabidopsis thaliana*; AtIPT; Cytokinin; Nitrogen availability; tRNA-isopentenyltransferase

Introduction

Cytokinins are a group of phytohormones involved in various processes of growth and development of plants, such as cell division, photosynthesis, senescence, and nutrient metabolism (Mok 1994). Since a naturally occurring cytokinin, *trans*-zeatin (*t*-Z), was first isolated from maize in the early 1960s (Miller 1961; Letham 1963), a variety of cytokinin species have been identified in various plant species (Letham 1994). These studies demonstrated that naturally occurring cytokinins are N^6-substituted adenine derivatives that generally contain an isoprenoid or an aromatic derivative side chain. With the progress of chemical research, several cytokinin biosynthetic enzymes have been studied and characterized and some of the catalytic enzymes

Received: 3 October 2001; Accepted: 5 December 2001
Online publication: 30 April 2002
*Corresponding author. E-mail: sakaki@postman.riken.go.jp

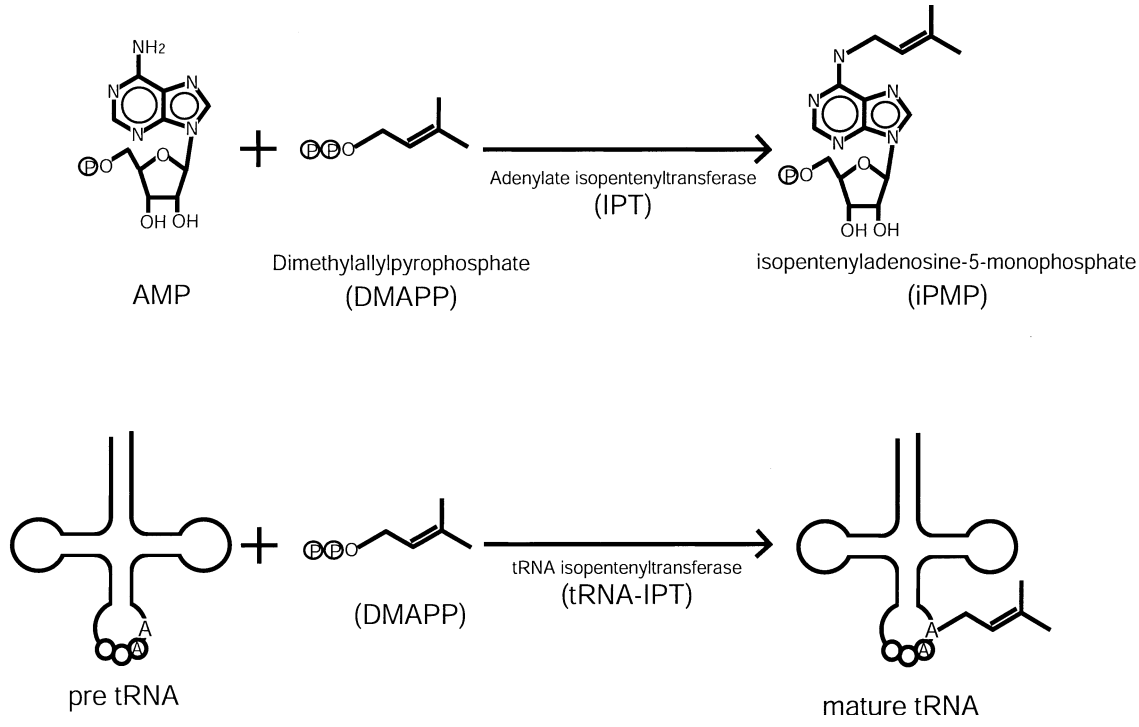

Figure 1. Schematic representation of the reaction of two types of isopentenyltransferases. Reactions that had been identified in bacterial enzymes are illustrated; pre tRNA = premature tRNA.

have been purified and the corresponding genes cloned (for reviews, see Mok and Mok 2001, and other articles in this issue). Our research has focused upon increasing the understanding of cytokinin biosynthetic enzymes in higher plants, for which relatively little is known.

The first identification of a gene encoding a cytokinin biosynthetic enzyme was carried out in *Agrobacterium tumefaciens*, a crown gall-forming bacterium (Akiyoshi and others 1984; Barry and others 1984). The gene, designated *tmr* (*ipt*), encoded an isopentenyltransferase associated with the synthesis of isopentenyladenosine 5′-monophosphate (iPMP) from dimethylallylpyrophosphate (DMAPP) and AMP *in vitro* (Blackwell and Horgan 1993). iPMP synthesis in higher plants has been assumed to be similar to that found in *A. tumefaciens.*Although many groups have attempted to purify and characterize plant isopentenyltransferase, only a few biochemical properties have been reported (Blackwell and Horgan 1994; Chen and Melitz 1979; Chen and Ertl 1994), and the protein has not been purified to homogeneity, perhaps because of low content or enzyme instability. Forward genetic approaches have also been employed, and, although information about the genes for metabolism and signal transduction is increasing (see other articles in this issue), little is known about the gene(s) related to cytokinin synthesis. The difficulty of this problem has resulted in the suggestion that the cytokinins may not be synthesized by higher plants at all, but instead are supplied by external symbiotic microorganisms (Holland 1997). Although this might be the case in some situations, it is difficult to accept this hypothesis for a phytohormone that is so central to plant growth and development. During 2000, the sequence of the *Arabidopsis* genome was completed; if cytokinins are endogenously produced in higher plants, then the gene(s) must be present in the genome, which has been estimated to encode about 24,000 proteins.

Isopentenyltransferases Involved in Cytokinin Production

Two types of isopentenyltransferases are known to produce cytokinins (Figure 1). One type of isopentenyltransferase modifies tRNA and is called tRNA-isopentenyltransferase (tRNA-IPT; EC 2.5.1.8). Mature tRNA molecules are decorated with many modified nucleosides. Some of the U-group tRNA molecules, such as Cys, Leu, Phe, Ser, Trp, and Tyr, contain an isopentenyladenosine (iPA) or a derivative residue at the site adjacent to the anticodon. tRNA-IPT catalyzes the first step of the modification

conjugating the isopentenyl moiety of DMAPP to the adenine residue. tRNA-IPT genes appear to exist ubiquitously in organisms: They have been experimentally identified in *Homo sapiens* (Golovko and others 2000), *Saccharomyces cerevisiae* (Dihanich and others l987), and *Escherichia coli* (Caillet and Droogmans 1988). In addition, a number of putative tRNA-IPT genes are registered in the GenBank database from other organisms.

Because degradation of tRNA could produce active cytokinins, it has been suggested as a possible source of the hormone. In fact, six different compounds, *cis*-zeatin riboside (*c*-ZR), *trans*-zeatin riboside (*t*-ZR), iPA, methylthio-iPA, *cis*-methylthio-ZR, and *trans*-methylthio-ZR, have been isolated from the hydrolytic products of plant tRNAs (Taller 1994). Among these, *c*-ZR is typically the most abundant cytokinin derived from plant tRNA. *Cis*-type cytokinins were therefore postulated to originate from tRNA degradation. However, based on calculations of the rates of tRNA turnover and cytokinin production, it is now accepted that the tRNA degradation pathway is not a major source of cytokinins (Klämbt 1992). Because comparable amounts of *cis*-zeatin(*c*-Z) and *trans*-zeatin (*t*-Z) are found in some plant species (Murofushi and others 1983; Parker and others 1989), we cannot yet exclude the hypothesis that tRNA degradation does not contribute at all to the pool of active cytokinins, but it is clearly not a major source. In such cases, Z *cis–trans* isomerase might be involved in the interconversion (Mok and Mok 2001).

Another type of isopentenyltransferase is an iPMP-forming enzyme, generally called adenylate isopentenyltransferase (IPT; EC 2.5.1.27). IPT has been identified in *Agrobacterium* (described above, Figure 1). IPT is structurally related to tRNA-IPT, suggesting that these transferases may have diverged from a common ancestral gene.

Identification of Cytokinin Synthesis Genes in *Arabidopsis*

An *in silico* search of the *Arabidopsis* genome for *tmr* homologs found the existence of nine putative genes, designated *AtIPT1* to *AtIPT9* (Takei and others 2001a; Kakimoto 2001). A phylogenetic analysis indicated that *AtIPT2* and *AtIPT9* are more similar in sequence to tRNA-IPT than to IPT (Takei and others 2001a; Kakimoto 2001; Figure 2); both gene products exhibited tRNA-IPT activity *in vitro* (Takei and others, unpublished data). Conversely, the other seven sequences (*AtIPT1* and *AtIPT3* to AtIPT8) are relatively closer to bacterial IPT than to tRNA-IPT (Takei and others 2001a; Kakimoto 2001; Figure 2). In the WS ecotype, *AtIPT6* appears to be a pseudogene as it contains a frameshift mutation (Kakimoto 2001).

E. coli transformants that express *AtIPT1* and *AtIPT3* to *AtIPT8* (henceforth called *AtIPT3–8*) excreted cytokinin isopentenyladenine (iP) and *t*-Z into the culture media. Although *t*-Z was detected in *E.coli* culture medium, it was not detected in the culture media of *S. cerevisiae* transformants expressing these genes (Takei and others, unpublished result). These results suggest two possibilities: (1) *E. coli* cells contain an unknown compound of terpenoid origin that functions as a side chain donor instead of DMAPP. If such a compound exists, the initial product of the reaction is not iPMP but *trans*-zeatin 5′-monophosphate (*t*-ZMP). (2) An unknown enzyme(s) having hydroxylase activity exists in *E.coli* cells. This enzyme converts isopentenyladenine-type cytokinins to zeatin-type cytokinins. Interestingly, Åstot and coworkers (2000) have suggested an alternative cytokinin biosynthetic pathway whose initial product was not iPMP but *t*-ZMP based upon *in vivo* deuterium-labeling experiments. Taken together, it is possible that a hydroxylated derivative of DMAPP, such as 4-pyrophosphate-2-methyl-*trans*-but-2-enol, exists as a substrate in plants and *E. coli* (Figure 3). Clearly, it will be essential to examine the catalytic activity of AtIPTs using the putative substrates *in vitro* and to identify the compounds in the plant tissues.

Extracts of *E. coli* transformants expressing *AtIPT1* and *AtIPT3–8* demonstrate IPT activity. In addition, overexpression of *AtIPT4* driven by a CaMV 35S promoter enabled calli to regenerate shoots even in the absence of externally applied cytokinins (Kakimoto 2001). These results strongly suggest that *AtIPT1* and *AtIPT3–8* encode cytokinin biosynthesis enzymes.

Recently, *AtIPT1* and *AtIPT4* were purified as recombinant enzymes and the cytokinin biosynthetic activity was identified (Takei and others 2001a; Kakimoto 2001). Takei and coworkers (2001a) found that purified AtIPT1 could synthesize iPMP from AMP and DMAPP *in vitro* and that the activity of *AtIPT1* was strongly inhibited in the copresence of ADP or ATP (using a rapid IPT assay that measures incorporation of radioisotope-labeled AMP into iPA; after Blackwell and Horgan 1993). The K_m value for AMP was found to be 185 ± 19 μM and that for DMAPP 50 ± 3 μM, values that are much higher than those for *A. tumefaciens* tmr (K_m for AMP, 85.7 nM; K_m for DMAPP, 8.3 μM; Blackwell and Horgan 1993). On the other hand, a biochemical analysis of

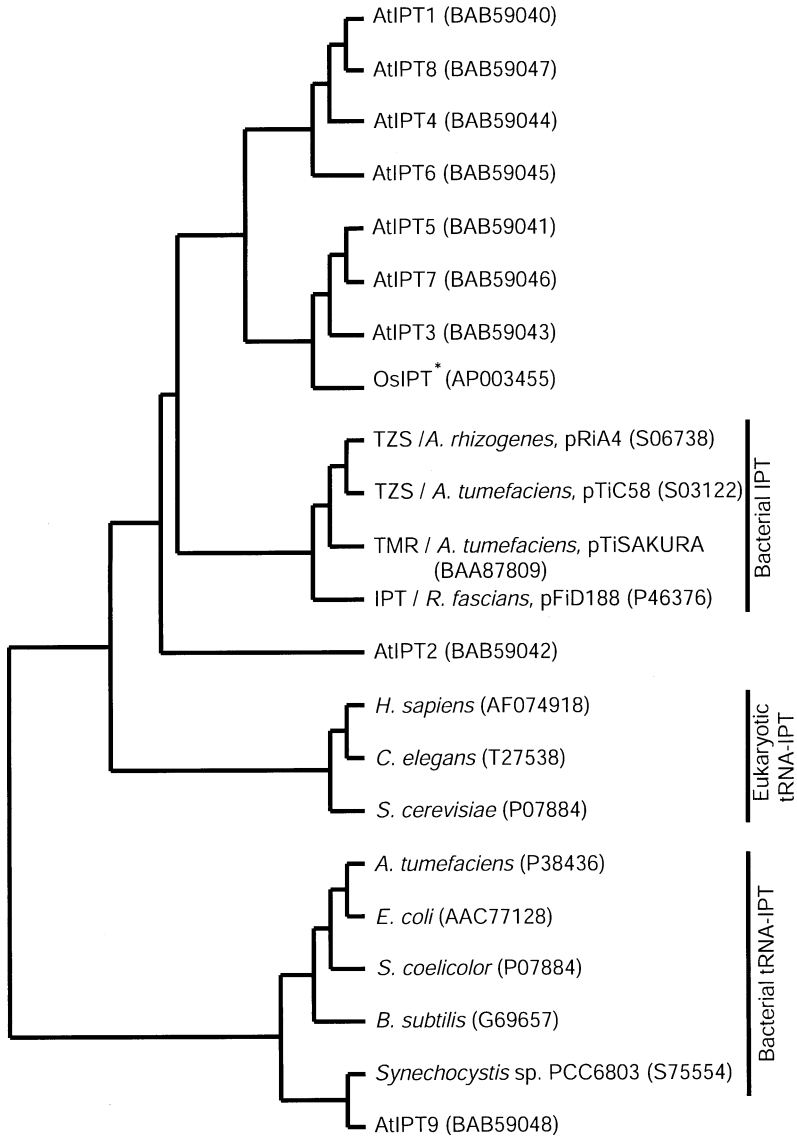

Figure 2. Phylogenetic tree of various isopentenyltransferases. Phylogenetic relationship was calculated using the CLUSTALW program at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp). Accession numbers of the amino acid sequence are shown in parentheses. *OsIPT sequence is deduced from the nucleotide sequence whose accession number is AP003455 (80946 to 79969). *O. sativa* = *Oryza sativa; A. rhizogenes* = *Agrobacterium rhizogenes; R.fascians* = *Rhodococcus fascians; C.elegans* = *Caenorhabditis elegans; S. coelicolor* = *Streptomyces coelicolor, B. subtilis* = *Bacillus subtilis; Synechocystis* = *Synechocystis sp.* PCC6803.

AtIPT4 revealed that the recombinant enzyme preferentially used ATP and ADP; AMP was not utilized as a substrate by the recombinant enzyme. The K_m value for ATP was 18 μM and that for DMAPP was 6.5 μM (Kakimoto 2001), which are much lower than the comparable values for AMP and DMAPP. *AtIPT1* also utilizes ATP and ADP preferentially, although the enzymatic parameters have not yet been determined (Takei and others, data not shown). The apparent inhibition by ATP and ADP in the IPT rapid assay is probably due to substrate competition between radioisotope-labeled AMP and cold ATP or ADP. These findings suggest that *AtIPT1* has cytokinin biosynthetic properties and that ADP and ATP are the preferential acceptors of the isopentenyl moiety during cytokinin synthesis in higher plants. Interestingly, while *AtIPT1* can utilize AMP, it is not used as a substrate by *AtIPT4*, suggesting that the substrate specificity of the AtIPTs differ. Although a complete biochemical characterization of the seven AtIPTs is not yet complete, the data strongly suggest that we should

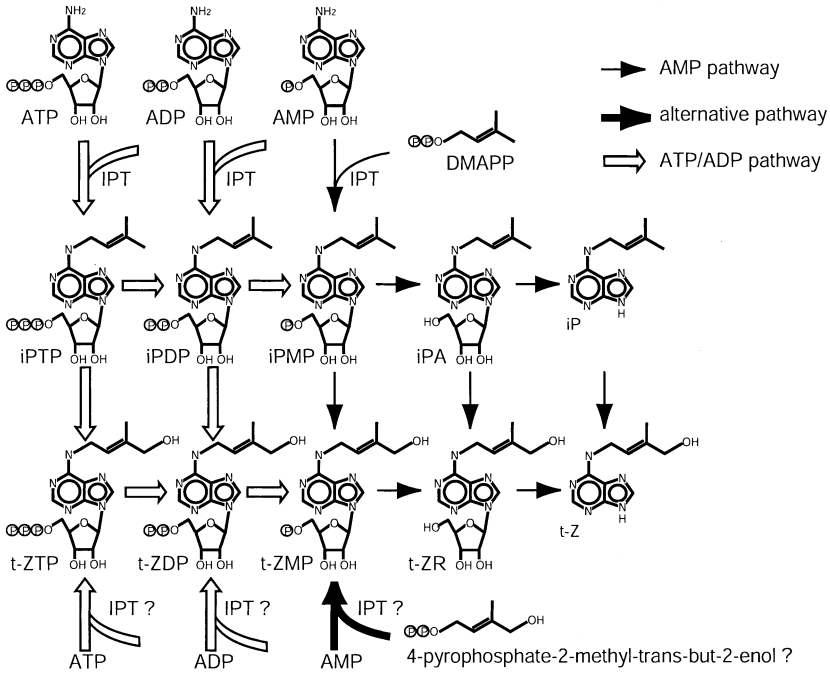

Figure 3. Current putative pathway of cytokinin biosynthesis. The pathway that had been accepted (AMP pathway) is shown by a thin arrow, an alternative pathway that was suggested by Åstot and coworkers (2000) is shown by a bold arrow, ATP/ADP pathway that was suggested by Kakimoto (2001) is shown by a open arrow. Details are given in the text.

reconsider the previous model for the cytokinin biosynthetic pathway (Figure 3).

Åstot and coworkers (2000) found that *t*-ZMP was preferentially synthesized independent of iPMP synthesis. If all AtIPTs prefer ATP and ADP as substrates, the reaction products may be *trans*-zeatin riboside 5′-diphosphate (*t*-ZDP) and *trans*-zeatin riboside 5′-triphosphate (*t*-ZTP), respectively. General procedures for purification and quantification of cytokinin contain a phosphatase treatment and, thus, cannot discriminate the various nucleotides. Therefore, we may have to reconsider quantification results that are focused on nucleotide derivatives of cytokinin.

Insight into Regulation of Cytokinin Biosynthesis

The existence of a family of genes that encode the cytokinin synthesis enzymes suggests that each gene may be specialized for a distinct physiological role. Although experimental data is not yet available, one can speculate that these genes may be expressed in distinct spatial and temporal patterns.

The accumulation of cytokinins is closely correlated with the nitrogen status of the plant in several species such as *Urtica dioica* (Wagner and Beck 1993), barley (Samuelson and Larsson 1993), cotton (Yong and others 2000), and maize (Takei and others 2001b). In maize, Takei and coworkers (2001b) reported that nitrogen stimulated the accumulation of iPMP first, and then Z-type cytokinins accumulated in the roots. In the xylem, both the exudation rate of xylem sap and the concentration of *t*-ZR were significantly increased, after which *t*-Z levels subsequently increased in the leaves (Takei and others 2001b). Cytokinins therefore would represent a long-distance root-to-shoot signal for nitrogen availability to regulate the expression of genes involved in photosynthesis (Sugiharto and others 1992) and His–Asp phosphorelay signal transduction (Sakakibara and others 1998, 1999; Taniguchi and others 1998). Although the accumulation of cytokinins can result from the conversion of glucosylated derivatives (Takei and others 2002), our study strongly suggests that *de novo* biosynthesis of cytokinin is activated by the nitrate replenishment in the roots. It should be noted that the methods used to measure iPMP in this study were not able to discriminate between isopentenyladenosine 5′-diphosphate (iPDP) and isopentenyladenosine 5′-triphosphate (iPTP) because the extracted cytokinins were dephosphorylated before their identifications (Takei and others 2001b). A similar accumulation of cytokinins in response to nitrate resupply was observed in *Arabidopsis* (Takei and others 2002), suggesting that

nitrogen-induced cytokinin biosynthesis is a common property of higher plants and that some *AtIPT* genes may be regulated by nitrogen availability in the root zone.

In addition to nitrogen, other macronutrients have been shown to affect cytokinin metabolism and action. For example, phosphate starvation has been associated with decreased concentrations of cytokinins in higher plant tissues (Horgan and Waering 1980; Salama and Waering 1979). Exogenously supplied cytokinins counteract the root growth stimulation induced under a low-nutrient condition (Kuiper 1988). Furthermore, during floral initiation in *Sinapis alba*, the length of the day is closely related to phloem sucrose content and cytokinin concentration in root tips (Bernier and others 1993). These findings imply that cytokinin synthesis may be regulated by changes in the availability of a number of macronutrients in addition to nitrogen.

Finally, the concentrations of biologically active cytokinins are also influenced by the rate of cytokinin degradation and interconversion pathways. In *Arabidopsis*, cytokinin oxidase, an enzyme that degrades a subset of cytokinins, is encoded by a small multigene family composed of seven genes (Bilyeu and others 2001). Other genes involved in the metabolic pathway have also been reported (Mok and Mok 2001). However, almost nothing is known about how these genes might respond to nutritional availability. The quantitative and qualitative balance of activities among the biosynthesis, degradation, and interconversion determines the level of biologically active cytokinins in tissues. There is little doubt that comprehensive studies, including biosynthesis, degradation, and interconversion, under various nutritional regimes are clearly needed to depict a whole view of the regulation for these important biosynthetic enzymes.

ACKNOWLEDGMENTS

This work was partly supported by Grants-in-aid for Scientific Research on Priority Areas (number 12142202 to HS) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc Natl Acad Sci USA 81:5994–5998.

Åstot C, Dolezal K, Nordström A, Wang Q, Kunkel T, Moritz T, Chua NH, Sandberg G. 2000. An alternative cytokinin biosynthesis pathway. Proc Natl Acad Sci USA 97:14778–14783.

Barry GF, Rogers SG, Fraley RT, Brand L. 1984. Identification of a cloned cytokinin biosynthetic gene. Proc Natl Acad Sci USA 81:4776–4780.

Bernier G, Havelange A, Houssa C, Petitjean A, Lejeune P. 1993. Physiological signals that induce flowering. Plant Cell 5:1147–1155.

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

Blackwell JR, Horgan R. 1993. Cloned *Agrobacterium tumefaciens ipt1* gene product, DMAPP:AMP isopentenyltransferase. Phytochemistry 34:1477–1481.

Blackwell JR, Horgan R. 1994. Cytokinin biosynthesis by extracts of *Zea mays.* Phytochemistry 35:339–342.

Caillet J, Droogmans L. 1988. Molecular cloning of the *Escherichia coli miaA* gene involved in the formation of Δ^2-isopentenyl adenosine in tRNA. J Bacteriol 170:4147–4152.

Chen CM, Ertl JR. 1994. Cytokinin biosynthetic enzymes in plants and slime mold. In: Mok DWS, Mok MC, editors. Cytokinins: Chemistry, Activity, and Function. Boca Raton, FL: CRC Press. p 81–85.

Chen CM, Melitz DK. 1979. Cytokinin biosynthesis in a cell-free system from cytokinin-autotrophic tobacco tissue cultures. FEBS Lett 107:15–20.

Dihanich ME, Najarian D, Clark R, Gillman EC, Martin NC, Hopper AK. 1987. Isolation and characterization of *MOD5,* a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of *Saccharomyces cerevisiae.* Mol Cell Biol 7:177–184.

Golovko A, Hjälm G, Sitbon F, Nicander B. 2000. Cloning of a human tRNA isopentenyltransferase. Gene 258:85–93.

Holland MA. 1997. Occam's razor applied to hormonology. Plant Physiol 115:865–868.

Horgan JM, Waering PF. 1980. Cytokinins and the growth response of seedlings of *Betula pendula* Roth and *Acer pseudoplatanus* L. to nitrogen and phosphorus deficiency. J Exp Bot 31:525–532.

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

Klämbt D. 1992. The biogenesis of cytokinins in higher plants: our present knowledge. In: Kaminek M, Mok DWS, Zazímalová E, editors. Physiology and Biochemistry of Cytokinins in Plants. The Hague: SPB Academic Publishing. p 25–27.

Kuiper D. 1988. Growth response of *Plantago major* L. ssp. *pleiosperma* (Pilger) to changes in mineral supply. Plant Physiol 87:555–557.

Letham DS. 1963. Zeatin, a factor inducing cell division from *Zea mays.* Life Sci 8:569–573.

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

Miller CO. 1961. A kinetin-like compound in maize. Proc Natl Acad Sci USA 47:170.

Mok DW, Mok MC. 2001. Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52:89–118.

Mok MC. 1994. Cytokinins and plant development—An overview. In: Mok DWS, Mok MC, editors. Cytokinins: Chemistry, Activity, and Function. Boca Raton, FL: CRC Press. p 155–166.

Murofushi N, Inoue A, Watanabe N, Ota Y, Takahashi N. 1983. Identification of cytokinins in root exudate of the rice plant. Plant Cell Physiol 24:87–92.

Parker CW, Badenoch–Jones J, Letham DS. 1989. Radioimmunoassay for quantifying the cytokinins *cis*-zeatin and *cis*-zeatin riboside and its application to xylem sap samples. J Plant Growth Regul 8:93–105.

Sakakibara H, Hayakawa A, Deji A, Gawronski S, Sugiyama T. 1999. His-Asp phosphotransfer possibly involved in a nitrogen signal transduction mediated by cytokinin in maize: Molecular cloning of cDNAs for two-component regulatory factors and demonstration of phosphotransfer activity *in vitro*. Plant Mol Biol 41:563–573.

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

Salama AMSEDA, Waering PF. 1979. Effects of mineral nutrition on endogenous cytokinins in plants of sunflower (*Halianthus annuus* L.). J Exp Bot 30:971–981.

Samuelson ME, Larsson CM. 1993. Nitrate regulation of zeatin riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. Plant Sci 93:77–84.

Sugiharto B, Burnell JN, Sugiyama T. 1992. Cytokinin is required to induce the nitrogen-dependent accumulation of mRNAs for phospho*enol*pyruvate carboxylase and carbonic anhydrase in detached maize leaves. Plant Physiol 100:153–156.

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

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

Takei K, Takahashi T, Sugiyama T, Yamaya T, Sakakibara H. 2002. Multiple routes communicating nitrogen availability from roots to shoots: a signal transduction pathway mediated by cytokinin. J Exp Bot. 53:971–977.

Taller BJ. 1994. Distribution, biosynthesis, and function of cytokinins in tRNA. In: Mok DWS, Mok MC, editors. Cytokinins: Chemistry, Activity, and Function. Boca Raton, FL: CRC Press, p 101–112.

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

Wagner BM, Beck E. 1993. Cytokinins in the perennial herb *Urtica dioica* L. as influenced by its nitrogen status. Planta 190:511–518.

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