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On the Biogenesis of Cytokinins in Roots of *Phaseolus vulgaris*

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Abstract. Roots of intact bean plants were supplied with [^{14}C]adenine by pulse-chase experiments. The rate of incorporation of radioactivity into tRNA and oligonucleotides of roots as well as the content of radioactive labeled cytokinin nucleotides in these RNA fractions were determined. On the average, 1/70 of the radioactivity incorporated into tRNA was localized in $N^6(\Delta^2$isopentenyl)adenosine. The half life of tRNA was estimated to be 65–70 h. Shortly after the pulse period, oligonucleotides contained zeatin riboside at a ratio of 1:800, on the basis of radioactivity. The half life of these oligonucleotides was determined to be about 8 h. The main free radioactive cytokinin of roots and leaves was zeatin. Comparing the rate of degradation of ^{14}C-labeled tRNA and the oligonucleotides of roots and the rate of appearance of radioactive cytokinins in roots and leaves, we found strong indications for their dependency. The results contradict the hypothesis of de novo synthesis of cytokinins in roots of intact bean plants.

Key words: Cytokinin (biosynthesis) – *Phaseolus* – Root

Introduction

The possibility of there being one of two different pathways for the biosynthesis of cytokinins in higher plants is under discussion. The first pathway indicates a de novo synthesis deriving from adenine monomers (Chen and Petschow 1978; Chen et al. 1976; Chen and Melitz 1979; Burrows 1978; Stuchbury et al. 1979; Taya et al. 1978), while the second pathway assumes cytokinin-containing tRNA and oligonucleotides to be precursors for the free cytokinins (Holtz and Klämbt 1975, 1978; Klemen and Klämbt 1974; Helbach and Klämbt 1981).

We now present results about the half lives of tRNA and oligonucleotides in bean roots as well as the yield of radioactive labeled cytokinins in bean roots and leaves. The data given here favor the occurrence of the second pathway.

Abbreviations: AMP = adenosine monophosphate; IPA = $N^6(\Delta^2$isopentenyl)adenosine; IPAde = $N^6(\Delta^2$isopentenyl)adenine; Z = zeatin; ZR = zeatinriboside; TLC = thin-layer chromatography; HPLC = high performance liquid chromatography

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Material and Methods

Cytokinins were products of Sigma, St. Louis. [^{14}C]adenine (10.582 GBq/mmol) was purchased from Amersham-Buchler, Braunschweig, FRG. [^{3}H]IPAde (10.852 GBq/mmol) was a special synthesis by NEN, Dreieich. [8-^{14}C]zeatin (0.4329 GBq/mmol) was a generous gift from Dr. E. Knegt, Wageningen, Netherlands. Alkaline phosphatase (EC 3.1.3.1., 400 u/mg) was purchased from Boehringer, Mannheim, FRG; Dowex 50 WX 4 cation exchange resin was purchased from Serva, Heidelberg, FRG, Sephadex LH-20 from Pharmacia, Freiburg, FRG, DEAE-Cellulose DE 52 from Whatman, Maidstone. Silica gel TLC-plates F-254 were products of Merck, Darmstadt, FRG. The HPLC system was purchased from Latek, Heidelberg, FRG with a steel column (0.3·25 cm, silica gel RP ODS/C 18101 S). Molecular ultrafiltration cells with filters PSAC 025 10, NMWL 10^3 were products of Millipore, Neu-isenburg; X-ray films NS-2 T no screen were products of Eastman-Kodak.

Beans (*Phaseolus vulgaris* var. Saxa) were grown in aerated liquid medium at 22° C with 12 h light-dark change. For the experiments, 4-week-old plants were used whose buds had been excised a week before harvesting. After washing the roots with sterile medium and surface drying them, 3 plants per test were put into 50 ml-glasses, each containing 5 ml sterile culture liquid plus 1.48 MBq (= 28 μM) [^{14}C]adenine. Six hours of pulse labeling was followed by a chase with a solution of 28 mM unlabeled adenine for another 6 h. Plants were harvested after 6, 12, 24, 48, 72, and 96 h, respectively, after the beginning of the pulse. Leaves and roots were weighed separately and immediately frozen in liquid nitrogen, after being exhaustively washed.

Isolation of Cytokinins. The frozen samples were ground in 80% ethanol and stirred overnight. The filtrate of the extracts was evaporated to dryness, in vacuo, at 40° C, dissolved in a small volume of 80% ethanol, acidified to pH 3.5, and applied to a watercooled Dowex column (2.2·20 cm). Elution was carried out by 5 M ammo-

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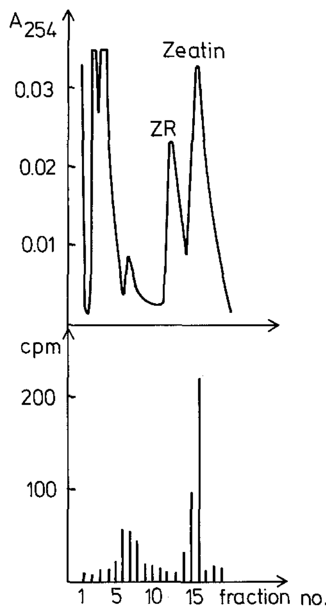

Fig. 1. HPLC chromatography of the predominant, free radioactive cytokinin in leaves, found after pulse application of 1.48 MBq [^{14}C]adenine to the roots of intact plants. Zeatin and zeatinriboside were added as markers. *Upper*: UV-absorbance, *lower*: corresponding radioactivity of the fractions

nia in 80% ethanol. The eluate was evaporated and dissolved in a small volume of 35% ethanol, which was applied to a Sephadex LH-20 column (2·47 cm, 2·35 cm) and equilibrated with 35% ethanol. The eluate from 0.8 and 2.3 column volumes were collected and evaporated to dryness. According to Armstrong et al. (1969) the most common cytokinins eluate at these volumes. The residue solved in 0.5 ml 80% methanol was applied to silica gel TLC and developed in chloroform:methanol (7:3, v/v). The zones containing cytokinins were scraped off, extracted with 80% methanol, evaporated to a small volume, and applied again to a silica gel TLC (1st solvent: n-butanol:acetic acid:water (12:3:5, v/v/v), 2nd solvent: n-butanol:25% ammonia:water (6:1:2, v/v/v, upper phase). The dried chromatograms were exposed to X-ray films. For cytokinin identification the radioactive spots were scraped out and extracted with 80% methanol. These extracts were vacuum dried, after which the residues were dissolved in 50 µl 80% methanol and chromatographed in HPLC together with authentic samples of cytokinins (Fig. 1). By adding [^{3}H]IPAde to the original extract, the loss of cytokinins during the whole procedure was estimated to be about 50%.

Isolation of tRNA. Roots already used for cytokinin extraction were once again ground in buffer 1 (10 mM Tris-HCl pH 7.5, 100 mM NaCl). After the deproteinization with equal volumes of phenol (1 kg phenol, 220 ml buffer 1, 124 ml m-cresol, 1.2 g 8-hydroxyquinoline), the aqueous phases were treated overnight with 2.5 vol. ethanol containing 2% K-acetate at −18° C. The precipitate was collected by centrifugation, suspended in 3 M Na-acetate (pH 6.0), and stirred for 8 h at 4° C. The insoluble highmolecular RNA was collected by centrifugation and discarded, while the supernatant was diluted 1:1 with water and the tRNA precipitated with 2 vol. of ethanol. The pellet which was resuspended in a small volume of buffer 1 was applied to a DEAE cellulose column (1.5·15 cm) equilibrated with buffer 2 (10 mM Tris-HCl, pH 7.5, 400 mM NaCl). After elution with buffer 3 (10 mM Tris-HCl, pH 7.5, 1 M NaCl), the UV-absorbing tRNA in the peak fractions was precipitated with 2 vol. of ethanol and redissolved in 1 ml buffer 1. Aliquots were measured for their radioactivity and UV-absorbance. The yield of tRNA was about 50%.

Isolation of Oligonucleotides. The isolation of oligonucleotides from roots was carried out by treatment with phenol, according to the tRNA preparation. The aqueous phases were extracted with ether, to remove residual phenol, and lyophilized. The dissolved residue was applied to a DEAE cellulose column, which was eluted with 10 mM Tris-HCl pH 7.5, 400 mM NaCl. The oligonucleotides were collected by membrane filtration with an excluding molecular weight of 1,000 and finally dissolved in a small volume of buffer 1 for measurements of radioactivity and UV-absorbance.

Alkaline Hydrolysis. Aliquots of tRNA and oligonucleotide preparations, respectively, were hydrolyzed by treatment with 0.5 to 1 M KOH at 37° C for 8 h, neutralized with $HClO_4$, and adjusted to pH 8.3 with Tris-HCl. The hydrolysate was incubated with 10 units alkaline phosphatase at 35° C for 14 h. The solution was evaporated to dryness and the residue dissolved in a small volume of 80% methanol. The steps for cytokinin identification were described above.

Results

[^{14}C]Adenine was applied to the bean roots in pulse-chase experiments. The half life of the tRNA which was labeled in that way was determined to be about 65 to 70 h (Fig. 2). This is in accordance with an earlier report on primary corn roots (Klemen and Klämbt 1974). After alkaline hydrolysis and phosphatase digestion the nucleosides were separated by silica gel TLC. In chloroform/methanol (7:3, v/v), adenosine was separated from the more hydrophobic cytokinins (adenosine $R_f = 0.25$, ZR and IPA $R_f > 0.5$). The zone above adenosine, i.e., R_f 0.3–0.9, was scraped, eluted with 80% methanol, and used for the TLC in two dimensions. Using co-chromatography in HPLC, IPA could be determined as the predominant cytokinin. The ratio of labeled cytokinin to total labeled nucleosides for two experiments are given below:

842,778 cpm totally – 10,083 cpm IPA = 1:84
503,220 cpm, totally – 8,031 cpm IPA = 1:62

More than 98% of the radioactivity corresponding to cytokinin activity belonged to IPA.

The oligonucleotides were also investigated for their half life and cytokinin content. The ratio of the absorbances 260 nm/280 nm of the isolated oligonucleotides was about 2. As Fig. 3 shows, the specific activity of the oligonucleotides decreases rather quickly after the pulse period. Later on the radioactivity decreases more moderately. This probably reflects the different sources of oligonucleotides from the different RNA-pools, which all reveal various half life times. Therefore, there is no uniform half life for the oligonucleotides. From the first part of the curve, a half life of the quickly metabolized oligonucleotides is estimated to be about 8 h.

The identification of cytokinins after KOH hydrolysis and phosphatase digestion of oligonucleotide fractions showed more than 90% ZR. The ratio of [^{14}C]cytokinin to total [^{14}C]nucleosides within the

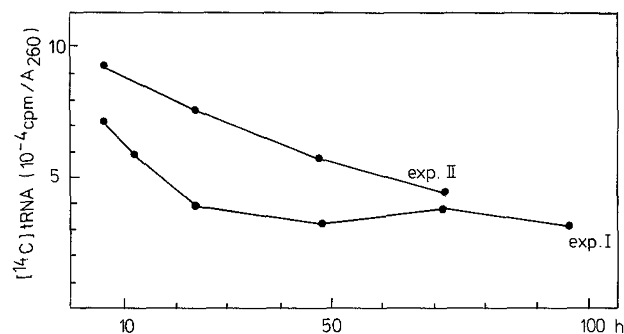

Fig. 2. Decrease of ^{14}C-labeled tRNA from roots after pulse labeling with 1.48 MBq [^{14}C]adenine during 6 h to the roots of intact plants and following chase for another 6 h with the 1,000 fold concentration of unlabeled adenine. Two characteristic experiments out of four

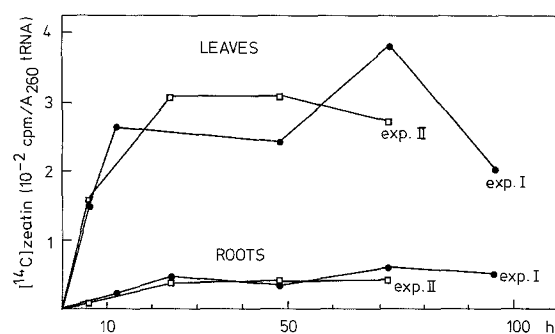

Fig. 4. Amount of [^{14}C]zeatin in roots and leaves after pulse labeling with 1.48 MBq [^{14}C]adenine during 6 h to the roots of intact plants and following chase for another 6 h with the 1,000 fold concentration of adenine

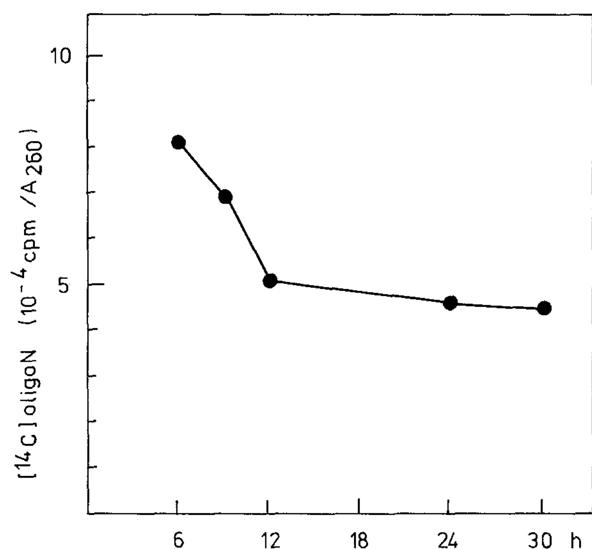

Fig. 3. Decrease of ^{14}C-labeled oligo N from roots after pulse labeling with 1.48 MBq [^{14}C]adenine during 6 h to the roots of intact plants and following chase for another 6 h with the 1,000 fold concentration of adenine

oligonucleotides was different, according to the quickly or moderately metabolized samples. While the first ones gave a ratio of 1:810, the second had only 1:2,600. The corresponding figures were:

1,687,370 cpm totally – 2,081 cpm ZR = 1:810
478,408 cpm totally – 184 cpm ZR = 1:26,000.

Free radioactive cytokinins were isolated from the leaves. On the average, 80% zeatin predominated all other radioactive spots found on the chromatograms. The minor spots were not identified. In order to compare the data of the different samples at a different time, it was useful to relate all of the data to the absorbance units (A_{260}) of root tRNA (Fig. 4). This eliminated the different physiological activities of the three-plant samples.

Table 1. Metabolism of 0.37 MBq [^{3}H]IPAde fed in pulse-chase-experiments (6 h pulse; 6 h chase with 50 fold concentration) to the roots of intact plants. Aliquots were measured

Material	Incubation time	Activity IPAde/IPA fraction cpm	Activity Z/ZR fraction cpm	Activity Ade/Ado fraction cpm
Roots	6 h	55,250	33.071	238,680
	12 h	66,926	29,142	564,857
	24 h	13,640	29,089	219,742
Leaves	6 h	10,332	29,898	76,383
	12 h	7,749	23,150	100,123
	24 h	14,328	37,791	123,608

0.37 MBq [^{3}H]IPAde in 3 ml medium (10 μM) were fed to the roots of intact plants to elucidate the turnover rates. After 6 h a chase was carried out with the 50-fold concentration of cold IPAde. As Table 1 shows IPAde was quickly metabolized. Besides the main product, adenine/adenosine zeatin/ZR was also found.

Discussion

Besides the root tips, there are other meristematic tissues which are known sites of cytokinin production (Torrey 1976). In our work, the buds had been excised to exclude an influence from these sources. The tRNA of pea shoots and wheat germ contains mainly IPA and ZR as cytokinins (Vreman et al. 1974, Burrows et al. 1970). In bean root tRNA we found about 98% IPA of the cytokinin radioactivity. The labeling of cytokinins by [^{14}C]adenine amounted to 1.1–1.6% of the total radioactivity within the tRNA. Holtz and Klämbt (1975, 1978) found that their isolated isopentenyltransferase also accepted oligonucleotides, besides tRNA, as a substrate. Therefore the oligonucleo-

Cytokinin biogenesis in bean roots

The proposed pathway

The de novo synthesis

[^{14}C] - adenine
(6 h pulse)
(6 h chase)
[^{14}C] - adenosine
[^{14}C] - AMP
[^{14}C] - ADP
[^{14}C] - ATP
transcription
precursor of
[^{14}C] - rRNA, [^{14}C] - tRNA, [^{14}C] - mRNA
processing
Δ^2 IPP, IPT ?
[^{14}C] - rRNA, [^{14}C]-tRNA ^{IPA}, [^{14}C] - mRNA $^{(IPA\ ?)}$
RNase
Δ^2 IPP, IPT
[^{14}C] - oligonucleotides ^{IPA}
hydroxylation
IPA → ZR
[^{14}C] - oligonucleotides ^{ZR}
RNase
phosphatase
[^{14}C] - zeatin derivatives
[^{14}C] - adenine derivatives
etc.

Δ^2 IPP, IPT
[^{14}C] N^6 -(Δ^2 - isopentenyl) - adenosine derivatives
hydroxylation
[^{14}C] - zeatin derivatives
(synthesized not longer than 6 - 8 h after pulse begin)

Fig. 5. Flow diagram of two possible pathways of cytokinin biogenesis in bean roots after feeding [^{14}C]adenine for a 6 h pulse to the roots of intact plants. *Δ^2IPP*, Δ^2-isopentenyldiphosphate; *IPT*, isopentenyltransferase

tides were also investigated. The main cytokinin bound in the oligonucleotides was ZR. By applying [14C]adenine to the bean roots a maximal content of $1.25\,^0/_{00}$, related to the total radioactivity within the oligonucleotides, could be determined. In order to get reliable correlations between the turnover rates of tRNA and oligonucleotides within the roots and the appearance of free radioactive cytokinins within the leaves, the half lives had to be measured. The half life of tRNA of 65–70 h is in good correspondence with the measurements from corn roots (Klemen and Klämbt 1974). Oligonucleotides, being degradation products of the diverse RNA species, do not show an uniform half life. This is because the pool of radioactive oligonucleotides is in constant fluctuation due to the influx of the different oligonucleotides. Shortly after the pulse period, a half life of 8 h was calculated. This might be explained by the turnover of short-living RNA species, like mRNA, whose polyadenyl-sequences might have been isopentenylated. The half life of the ZR-poor and slowly degrading oligonucleotides was calculated to be greater than 80 h.

While IPA was the main cytokinin isolated from the tRNA of roots, zeatin was the predominant free cytokinin in the leaves and roots (more than 80%). This is in contrast to the publications of Wang and Horgan (1978) and Wang et al. (1977), who isolated mainly dihydrozeatin-O-glucoside and, as minor derivative, dihydrozeatinriboside from bean leaves. These cytokinins could not be detected. In bioassays, the cytokinin glucosides are associated with weak cell division activity. Therefore they are proposed to be the storage or transportation form (Gordon et al. 1974; Parker et al. 1975; Laloue 1977). The discrepancy may be interpreted in the following way. As the plants used in our tests were about to flower, and therefore in a state of high physiological activity, the newly produced cytokinins in roots might have been

detected in the leaves as the free base instead of as glucosyl- or ribosyl-derivatives.

Within the tRNA, IPA could mainly be found, while zeatin was the most abundant free cytokinin. Since the oligonucleotides mainly contain ZR, we conclude that IPA has to be transformed during the RNA-breakdown. A rapid hydroxylation of the isopentenyl side chain of IPAde to Z is reported by Letham (1978) and also shown by our results for bean roots (Table 1).

Figure 5 shows diagrams of the two extreme possible pathways of cytokinin biogenesis in bean roots. Assuming the possible de novo synthesis of cytokinins as the main pathway, the [^{14}C]cytokinins have to be related to the [^{14}C]adenine pulse regime by some delay, due to the half life of cytokinins. In fact, the [^{14}C]adenine pool is actively diluted by the chase incubation with 1,000 fold the concentration of adenine from the 6th to the 12th h, as recorded by the half life measurements of tRNA (Fig. 2). In contrast, the amount of [^{14}C]Z increases in roots and leaves within 2–3 days after the pulse incubation (Fig. 4). These data clearly support the hypothesis that cytokinins are produced via the turnover of poly- and oligonucleotides. A mathematical approach also supports this hypothesis (Maaß and Klämbt 1981). This is in contradiction to the publications of Burrows (1978), Taya et al. (1978), Chen and Petschow (1978), Chen and Melitz (1979), and Stuchbury et al. (1979). These authors mainly exclude the cytokinin biosynthesis via the turnover of tRNA and prefer the de novo synthesis.

The investigations of Taya and his group on the slime mold Dictyostelium revealed that the 5′-AMP is a precursor for IPAde transformed to discadenine. Burrows, Chen and Stuchbury, on the contrary, used higher plants like tobacco callus or crown gall tissue. With the application of labeled adenine they reported a de novo synthesis of cytokinins. The tRNA was not considered in these publications. Burrows (1978) treated 150 g cytokinin autonomous tobacco tissue with 370 kBq [^{3}H]adenine (925 kBq/nmol) in 100 ml for 1 h. The supernatant after homogenization and centrifugation were spiked with authentic samples of IPA, IPAde, ZR, and Z, which he finally found radioactive. He measured 1.49 kBq cytokinins all together, which means 0.4% of the applied [^{3}H]adenine. If we assume that within one hour the uptake of the given adenine might not be more than half or even less, the percentage of cytokinin synthesized rised to 1% and more in relation to the intracellular content of [^{3}H]adenine. The concentration of the adenine-adenosine-AMP-ADP-ATP pool within the cytoplasm is in the range of 1 mM. This means that within 1 h cytokinins are produced up to a concentration of 10^{-5} M. Such a concentration has never been reported, which, according to Burrows (1978) moreover, should be produced within 1 h. His results are questionable. Chen and Melitz (1979) reported an enzyme system, isolated from tobacco callus tissue, which synthesizes cytokinins from 5′-AMP, directly. The enzyme isolated by Holtz and Klämbt (1975, 1978) does not accept monomer adenine derivatives in physiological concentrations. Perhaps there are different independent enzyme systems. Very recent work by Barnes et al. (1980) shows that 40% of the biosynthesis of cytokinins in potato cells can be referred to the breakdown of RNA. They claimed that the rest of the free cytokinins will arise via a second route – perhaps oligonucleotides or de novo. In our experiments bean roots produced cytokinin only via RNA-hydrolysis.

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References

Armstrong, D.J., Burrows, W.J., Evans, P.K., Skoog, F. (1969) Isolation of cytokinins from tRNA. Biochem. Biophys. Res. Commun. **37**, 451–456

Barnes, M.F., Tien, C.L., Gray, J.S. (1980) Biosynthesis of cytokinins by potato cell cultures. Phytochemistry **19**, 409–412

Burrows, W.J. (1978) Incorporation of [^{3}H]adenine into free cytokinins by cytokinin-autonomous tobacco callus tissue. Biochem. Biophys. Res. Commun. **84**, 743–748

Burrows, W.J., Armstrong, D.J., Kaminek, M., Skoog, F., Bock, R.M., Hecht, S.M., Dammann, L.G., Leonard, N.J., Occolowitz, J. (1970) Isolation and identification of four cytokinins from wheat germ tRNA. Biochemistry **9**, 1867–1872

Chen, C.M., Eckert, R.L., McChesney, J.D. (1976) Evidence for the biosynthesis of tRNA-free cytokinin. FEBS Lett. **64**, 429–434

Chen, C.M., Petschow, B. (1978) Cytokinin biosynthesis in cultured rootless tobacco plants. Plant Physiol. **62**, 861–865

Chen, C.M., Melitz, D.K. (1979) Cytokinin biosynthesis in a cell-free system from cytokinin-autotrophic tobacco tissue cultures. FEBS Lett. **107**, 15–20

Gordon, M.E., Letham, D.S., Parker, C.W. (1974) The metabolism and translocation of zeatin in intact radish seedlings. Ann. Bot. **38**, 809–825

Helbach, M., Klämbt, D. (1981) On the biosynthesis of cytokinis in *Lactobacillus acidophilus* ATCC 4963. Physiol. Plant. (in press)

Holtz, J., Klämbt, D. (1975) tRNA-Isopentenyltransferase from *Lactobacillus acidophilus* ATCC 4963. Hoppe-Seyler's Z. Physiol. Chem. **356**, 1459–1464

Holtz, J., Klämbt, D. (1978) tRNA-Isopentenyltransferase from *Zea mays* L. Characterization of the isopentenylation reaction of tRNA, oligo (A) and other nucleic acids. Hoppe-Seyler's Z. Physiol. Chem. **359**, 89–101

Klemen, F., Klämbt, D. (1974) Half-life of sRNA from primary roots of *Zea mays*. A contribution to the cytokinin production. Physiol. Plant. **31**, 186–188

Laloue, M. (1977) Cytokinins: 7-glucosylation is not a prerequisite of the expression of their biological activity. Planta **134**, 273–275

Letham, D.S. (1978) Cytokinins In: Phytohormones and related

compounds: A comprehensive treatise, pp. 205 Vol. 1. Letham, D.S., Goodwin, P.B., Higgins, T.J.V., eds.

Maaß, Klämbt (1981) Cytokinin biosynthesis in higher plants. Report at "Le Metabolisme et les Activites Moleculaires des Cytokinines" Gif-sur-Yvette, France Sept 1980. Monography by Springer Berlin (in press)

Parker, C.W., Letham, D.S., Wilson, M.M., Jenkins, I.D., MacLeod, J.K., Summons, R.E. (1975) The identity of two new cytokinin metabolites. Ann. Bot. **39**, 375–376

Stuchbury, T., Palni, L.M., Horgan, R., Wareing, P.F. (1979) The biosynthesis of cytokinins in crown-gall tissue of *Vinca rosea*. Planta **147**, 97–102

Taya, Y., Tanaka, Y., Nishimura, S. (1978) 5′-AMP is a direct precursor of cytokinin in *Dictyostelium discoideum*. Nature (London) **271**, 545–547

Torrey, J.G. (1976) Root hormones and plant growth. Annu. Rev. Plant Physiol. **27**, 435–459

Vreman, H.J., Schmitz, R.Y., Skoog, F., Playtis, A.J., Frihart, C.R., Leonard, N.J. (1974) Synthesis of 2-methylthio-cis- and transribosylzeatin and their isolation from *Pisum* tRNA. Phytochemistry **13**, 31–37

Wang, T.L., Thompson, A.G., Horgan, R. (1977) A cytokinin glucoside from the leaves of *Phaseolus vulgaris*. Planta **135**, 285–288

Wang, T.L., Horgan, R. (1978) Dihydrozeatin riboside, a minor cytokinin from the leaves of *Phaseolus vulgaris*. Planta **140**, 151–153

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