AN ABSTRACT OF THE THESIS OF

Yun-Hwa Lee for the degree of Master of Science

in Horticulture presented on October 12, 1984

Title: [8-^{14}C] Zeatin Metabolism in Phaseolus Embryos

Abstract approved: _____
Dr. David W. S. Mok

The metabolism of [8-^{14}C]zeatin was examined in embryos of Phaseolus vulgaris cv. Great Northern (GN) and P. lunatus cv. Kingston (K) in an attempt to detect genetic variations in organized plant tissues. Metabolites were fractionated by HPLC, and identified by chemical and enzymatic tests and GC-MS analyses. Five major metabolites were recovered from P. vulgaris embryo extracts, ribosylzeatin, ribosylzeatin 5'-monophosphate, the O-glucoside of ribosylzeatin and two novel metabolites, designated as I and II. Based on results of degradation tests and GC-MS analyses, I and II were tentatively identified as O-ribosides of zeatin and ribosylzeatin. In embryos of P. lunatus, however, metabolites I and II were not present. The major metabolites were ribosylzeatin, ribosylzeatin 5'-monophosphate and the O-glucosides of zeatin and ribosylzeatin. The zeatin metabolites recovered were the same for embryos of different sizes but their quantities varied with embryo size and incubation time. The genetic differences appear to be embryo-specific and may

be useful in the studies of the possible relationship between abnormal interspecific hybrid embryo growth and hormonal derangement in Phaseolus. In addition, analyses of both organized (intact) and unorganized (callus) tissues of the same genotype may provide an opportunity to address the problem of differential expression of genes regulating cytokinin metabolism during plant development.

[8-^{14}C]Zeatin Metabolism in *Phaseolus* Embryos

by

Yun-Hwa Lee

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed October 12, 1984

Commencement June 1985

APPROVED:

Professor of Horticulture in charge of major

Head of Horticulture Department

Dean of Graduate School

Date thesis is presented October 12, 1984

Typed by Mary Ann (Sadie) Airth for Yun-Hwa Lee

ACKNOWLEDGEMENTS

With deep appreciation I wish to thank Dr. David W. S. Mok for his guidance, patience and all he has taught me throughout this research. The support, expertise and collaboration of Dr. Machteld C. Mok was a great aid and encouragement to me during my graduate studies. I gratefully acknowledge the assistance of Mr. Donald A. Griffin for the analysis of my samples with mass spectrometer. To Dr. Dallice Mills I express my sincere gratitude for serving on my committee and his valuable instruction.

In addition to the above, I give my thanks to all who have been very supportive of me in this effort: my husband, parents and friends. It was only through the assistance of many people that I was able to begin and complete this project.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ade:	adenine
Ado:	adenosine
AMP:	adenosine-5'-monophosphate
ATP:	adenosine-5'-triphosphate
CI:	chemical ionization
DMSO:	dimethylsulfoxide
DZMP:	trans-dihydrozeatin 5'-monophosphate
DZ-O-G:	O-glucosyldihydrozeatin
EI:	electron impact ionization
GC-MS:	gas chromatography-mass spectrometry
HPLC:	High-performance Liquid Chromatography
i^6Ado:	N^6-(Δ^2-isopentenyl) adenosine
i^6Ade:	N^6-(Δ^2-isopentenyl) adenine
i^6AMP:	N^6-(Δ^2-isopentenyl)adenosine 5'-monophosphate
ipn^{6}Ade:	N^6-(Δ^2-isopentyl)adenine
ipn^{6}Ado:	N^6-(Δ^2-isopentyl)adenine
RDZ:	dihydrozeatin riboside, 6-(3-methylbutylamino)-9-ribofuranosylpurine
RDZ-O-G:	O-glucosyl, 9-ribosyldihydrozeatin
RZ:	ribosylzeatin, 9-β-D-ribofuranosyl-trans-zeatin
cis-RZ:	cis-ribosylzeatin, 9-β-D-ribofuranosyl-cis-zeatin
RZ-O-G:	O-glucosyl, 9-ribosylzeatin
TBAP:	tetrabutylammonium phosphate
TEA:	triethylamine
TFA:	trifluoroacetic acid

[^{14}C]Zeatin, [8-^{14}C]Zeatin, Z: trans-zeatin, 6-(4-hydroxy-3-methyl-trans-2-butenyl-amino)purine

Z-7-G: 7-glucosylzeatin, 7-β-D-glucopyranosyl-trans-zeatin

Z-9-G: 9-glucosylzeatin, 9-β-D-glucopyranosyl-trans-zeatin

ZMP: trans-zeatin 5'-monophosphate

Z-O-G: O-glucosylzeatin, O-β-D-glucopyranosyl-trans-zeatin

[8-^{14}C]Zeatin Metabolism in *Phaseolus* Embryos

I. INTRODUCTION

As part of a program to study the genetic regulation of cytokinin metabolism in *Phaseolus*, callus culture bioassays have been used to identify genotypic variations of interest. Genetic differences in cytokinin structure-activity relationships, cytokinin requirements (cytokinin-autonomous vs. cytokinin-dependent growth) and responses to phenylurea-type cytokinins have been detected (Mok *et al.*, 1978; Mok *et al.*, 1979; Mok *et al.*, 1980; Mok *et al.*, 1982a and Mok *et al.*, 1982b). These studies have led to the characterization of interspecific differences in cytokinin destruction by nuclear genes and the identification of one major locus controlling cytokinin autonomy in *P. vulgaris*.

We are also interested in defining genetic variations in organized tissues of *Phaseolus* plants. Therefore, the metabolism of [^{14}C]zeatin was examined in tissues of a number of *Phaseolus* species and genotypes. In the present paper we report the metabolism of [^{14}C]-zeatin in immature embryos of *P. vulgaris* and *P. lunatus*.

II. LITERATURE REVIEW AND BACKGROUND INFORMATION

Cytokinins are compounds that promote cell division (Skoog et al., 1965). "Kinetin" was the first synthetic cytokinin (6-furfurylaminopurine) identified when Miller et al. (1955, 1956) isolated the substance upon dehydrolation of DNA from herring sperm. Zeatin, found in immature corn kernels, was the first naturally occurring cytokinin to be isolated. Its structure was identified as 6-(4-hydroxy-3-methyl-trans-2-butenyl-amino)purine (Letham, 1963; Letham et al., 1964; Miller, 1961 and Letham and Miller, 1965) and was confirmed later by Shaw and Wilson (1964) via direct synthesis.

There are two broad classes of compounds that possess cytokinin activities (as defined by bioassays). The first group of compounds are N^6-substituted amino purine analogues such as i^6Ade, benzyladenine and zeatin. The second group are substituted phenylureas such as diphenylurea, N-phenyl-N'-(4-pyridyl)urea derivatives and N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuran) (Shantz and Steward, 1955; Bruce and Zwar, 1966; Bruce et al., 1965; Isogai, 1981; Mok et al., 1976 and Mok et al., 1982a). Although the chemical structures of the two classes of compounds are distinct, the biological activities are similar. The mode of action of phenylurea type cytokinins in relation to that of adenine-type cytokinins has not been defined.

The structure-activity relationship of the compounds in the two classes have been studied, and were reviewed by Skoog and Armstrong

(1970), Strong (1958), Leonard (1974) and Matsubara (1980). Generally, an alkyl group as the N^6-substituent (with the optimum length of 4-6 atoms) gives high activity. A double bond and a hydroxyl group further increase the activity. In addition, side chains which enhance the planarity of the molecule promote biological activity (Hecht et al., 1970). Among phenylurea-type cytokinins, substitutions in the phenylring, increase activity generally particularly with electronegative substituents.

A. Cytokinin Biosynthesis

Cytokinins occur naturally as components of specific t-RNA species, and as free forms in plant cells. At present, little is known about the mechanism of free cytokinin biosynthesis in plants. There are two hypotheses: degradation of t-RNA (Palni and Horgan, 1983) and de novo synthesis presumably via adenine or adenosine as precursors (Nishinari and Syono, 1980). Mevalonic acid (MVA) is generally considered as a precursor of the isoprenoid side chain. Both hypotheses have been reported but no definite evidence supporting either has been presented. Burrows and Fuell (1981) have suggested that free cytokinin production in cytokinin-autonomous and crown-gall tissues of tobacco takes place by a route not involving t-RNA. Palni and Horgan (1983) have found, however, the high level of trans-RZ observed from Vinca rosea crown-gall tissue is associated exclusively with the crown-gall tRNA. (For review see Letham and Palni, 1983).

B. Cytokinin Metabolism

Cytokinins have been found endogenously in a wide range of plant tissues. Naturally occurring cytokinins and some examples of the plant sources of these compounds are listed below: Z, from corn kernels (Letham, 1963) and cones of hops (Watanabe et al., 1981); cis-Z, from cones of hops (Watanabe et al., 1981); RZ, from corn kernels (Letham, 1966), Pinus radiata (Taylor et al., 1984) and cones of hops (Watanabe et al., 1981); cis-RZ, Z-7-G, from radish seeds (Summons et al., 1977); Z-9-G, from Vinca rosea crown-gall tissue (Peterson and Miller, 1977; Morris, 1977; Scott et al., 1980); RDZ and DZOG, from Phaseolus vulgaris leaves (Wang et al., 1977 and Wang and Horgan, 1978); Z-O-G, from Phaseolus vulgaris roots (Scott and Horgan, 1984a) and V. rosea crown-gall tissue (Peterson and Miller, 1977); RZOG, from V. rosea crown-gall tissue (Peterson and Miller; 1977) and cones of hops (Watanabe et al., 1981); cis-RZ, from cones of hops (Watanabe et al., 1981) and a glucoside of RZ in which the glucose moiety is attached directly to the ribose from Pinus radiata (Taylor, et al., 1984).

The metabolism of cytokinins has also been studied using radioactively labelled cytokinins applied exogenously to various plant organs, tissues, and cells.

Formation of the 7-glucoside of benzyladenine in tobacco callus was first demonstrated by Fox et al. (1973, 1974). Exogenously applied zeatin in radish roots and to derooted radish seedling (Parker and Letham, 1973; Cowley et al., 1978 and Gordon et al.,

1974) yield Ade, Ado, AMP, RZ, ZMP and Z-7-G. When [^{3}H]Zeatin was supplied to Zea mays L. seedlings (Cowley et al., 1978; Parker and Letham, 1974 and Parker et al., 1973) with roots excised, the metabolites were identified as AMP, Ado, Ade, Z-7-G (a minor metabolite). The principal metabolites formed from zeatin by the roots of intact Z. mays seedlings were Ade, Ado, AMP, RZ, ZMP, Z-7-G (a minor metabolite) and Z-9-G (a major metabolite). When zeatin was supplied to excised leaves of Populus alba, the principal metabolites formed were Ado, Z-O-G, DZ-O-G and RDZ-O-G; minor metabolites were AMP, Z-7-G, Z-9-G, DZ and RZ (Duke et al., 1979 and Letham et al., 1976). Labelled zeatin was supplied through the transpiration stream to derooted lupin (Lupinus angustifolius L.) seedlings. The major compounds were β - [6-(4-hydroxy-3-methylbut-trans-2-enylamino)-purin-9-yl]alanine (lupinic acid) and Z-O-G. The rest of the metabolites were DZ, RZ, RDZ, ZMP, DZMP, Z-7-G, Z-9-G,RZ-O-G, RDZ-O-G, dihydrolupinic acid, Ade, Ado and AMP (Parker et al., 1978; Duke et al., 1978 and Parker et al., 1975). All the above metabolites have been identified unequivocally by comparison with compounds obtained via direct syntheses.

The biological significance of various metabolites is still unclear. The O-glucosides and N-glucosides may be storage forms or may facilitate cytokinin transport (Letham et al., 1982 and Summons et al., 1980). They are also reported to be more stable and have higher resistance to some of the degradative enzymes.

C. Enzymes Involved in the Biosynthesis and Metabolism of Cytokinins

A number of enzymes involved in cytokinin biosynthesis and metabolism have been purified. Chen and Eckert (1977) reported the formation of N^6-(Δ^2-isopentenyl)adenosine-5'-monophosphate (i^6AMP) from i^6Ado and ATP by an adenosine kinase isolation from wheatgerm. In addition, nucleotides could be formed directly from the free base by the enzyme adenine phosphoribosyltransferase (Chen et al., 1982). A 5'-nucleotidase (Chen and Kristopeit, 1981a) converts nucleotides to corresponding nucleosides. The enzyme adenosine nucleosidase (Chen and Kristopeit, 1981b) catalyzes the deribosylation of i^6Ado (to i^6Ade). Adenosine phosphorylase (Chen and Petschow, 1978) catalyzes the ribosylation of i^6Ade to i^6Ado.

Paces et al., (1971) identified enzyme activity in crude extracts of tobacco tissues that could cleave the side chain of i^6Ado and other adenine derivatives with unsaturated N^6-side chains. This enzyme is similar to the cytokinin oxidase purified from Zea mays kernels by Witty and Hall (1974). The reaction converting i^6Ade, i^6Ado, Z and RZ to adenine and/or adenosine (McGaw and Horgan, 1983a) requires oxygen. The aldehyde, 3-methylbut-2-enal, is one of the intermediate products derived from the reaction of side chain cleavage (Brownlee et al., 1975). Dihydrozeatin (Palmer et al., 1981) and N^6-benzyladenine (Parker et al., 1973) are not substrates for the enzyme. Cytokinin oxidase has also been purified from Vinca rosea tumor tissue, which is also similar to the maize enzyme referred to

above (Scott et al., 1982). Another cytokinin oxidase has been partially purified from callus tissues of Phaseolus vulgaris L. cv. Great Northern (Chatfield and Armstrong, 1984). This enzyme exhibits similar specificity for substrates, cytokinins with an unsaturated N^6-side chain. The presence of glucosyl or ribosyl groups in the 7- or 9-position or an alanyl group in the 9-position of purine moiety has little effect on their susceptibility to cytokin oxidase, but O-glucosyl derivatives are resistant to oxidation (McGaw and Horgan, 1983b). In detached P. vulgaris leaves (Palmer et al., 1981) and V. rosea crown gall tissues (Horgan et al., 1981), side chain cleavage is also the fate of the majority of exogenously applied zeatin.

Cytokinin can occur naturally as glucosides with β-D-glucose as the sugar substituent. An enzyme has been purified from radish (Raphanus satives) cotyledons, cytokinin-7-glucosyltransferase, which utilizes uridine diphosphate glucose as the glucose donor and converts zeatin into its 7-and 9-glucosides (Entsch et al., 1979).

β-(9-Cytokinin)alanine synthase, derived from immature Lupinus luteus seeds, required the unusual and unstable substrate O-acetyl-serine as donor of the alanine moiety and converted zeatin to 9-alanyl-zeatin (Murakoski et al., 1977 and Entsch et al., 1983). Such an enzyme may also be classified as a C-N-ligase (Entsch et al., 1983).

D. Analysis of Cytokinins

Cytokinins and their metabolites are usually extracted with

organic solvent or acids and purified and analyzed by chromatography. Thin layer paper and liquid chromatography have been used extensively to separate and identify cytokinins.

Carnes et al. (1975) first introduced HPLC for the fractionation and analysis of cytokinins. The method is suitable in separating cytokinin bases, nucleosides, glucosides and nucleotides (Horgan and Kramers, 1979 and Scott and Horgan, 1982). The cis- and trans-forms of zeatin and ribosylzeatin can be separated by increasing the concentrations of the organic phase. The analysis time using HPLC is shorter and the resolution is much higher than low pressure column chromatography such as polystyrene-base ion exchange resins and Sephadex LH-20 columns. The final HPLC fractions are often pure enough for direct GC-MS analysis.

Mass spectrometry has been used to determine the molecular weights, mass ion composition and quantity of cytokinins. Generally there are two ways of converting the purified metabolites to volatile derivatives via methylation: trimethylation (TMS) and permethylation. Some metabolites are more amenable to one or the other method of methylation. For example: MacLeod et al. (1976) compared the suitability of TMS and permethylated derivatives of glucosides of zeatin and N^6-benzyladenine for GC-MS and mass spectral studies. Mass spectra of the TMS derivatives show more significant isomeric differences than the corresponding permethylated compounds and this method of derivatization also gave better results for N-glucosides. However, the permethylation has the advantage of being hydrolytically

stable and the derivatized products are lower in molecular weight than the TMS derivatives. Scott and Horgan (1984b) recently compared the mass spectra of cytokinin metabolites from tobacco crown gall tissue using both derivatizations.

Both electron impact (EI) ionization and chemical ionization (CI) mass spectrometry can be used for the identification of purified metabolites. The CI technique is useful for cytokinins having a sugar moiety linked through the side chain oxygen atom (Summons et al., 1980). But CI-MS gives less fragmentations compared with EI-MS. An ideal approach would be using both EI and CI spectro-analyses. Usually, direct synthesis is performed to unequivacally confirm the structure of new metabolites identified.

E. Cytokinin Metabolism in Phaseolus Species

Studies of cytokinin metabolism in intact plant tissue of Phaseolus species have been examined (Wang and Horgan, 1978; Palmer et al., 1981 and Scott and Horgan, 1984a). The major cytokinin identified in P. vulgaris leaves and decapitated plants was DZ-O-G and the minor cytokinin was RDZ (Wang et al., 1977 and Wang and Horgan, 1978). The quantity of DZ-O-G was determined using the isotope dilution method employing a deuterium-labelled internal standard and combined GC-MS (Palmer et al., 1981). The accumulation of cytokinin appeared to parallel the gradual increase in fresh weight of leaves of intact plants, or in leaves of plants that were decapitated but not disbudded. When secondary lateral buds were allowed to grow out

from decapitated plants the levels of DZ-O-G in the primary leaves rapidly declined to a value similar to or lower than that found in leaves of intact plants. The major cytokinins in stems of decapitated, disbudded bean plants have been identified as RZ, RDZ, ZMP and DZMP. The stability of cytokinin has been examined (Palmer, et al., 1981). The order of the stability appears to be DZ-O-G>Z-O-G>DZ>Z. These results suggest that side-chain glucosylation may confer some degree of metabolic stability on cytokinins in bean leaves although it is not clear whether this effect might be due to an inhibition of enzymic cleavage of the side-chain or to compartmentation of the glucoside away from the site of metabolism.

Callus cultures have been used to study the genetic variations in cytokinin metabolism. Studies on the structure-activity relationships of tissue cultures derived from P. vulgaris cv. Great Northern and P. lunatus cv. Kingston revealed dramatic differences in the responses of these callus tissues to cytokinins bearing unsaturated isoprenoid side chain [zeatin and i^6Ado] (Mok et al., 1978). In P. vulgaris cultures, the presence of a double bond in the cytokinin side chain resulted in a marked reduction in cytokinin activity. The growth responses of callus of the interspecific hybrid were intermediate between the parental tissue (Mok et al., 1982b). The differential structure-activity relationship was related to a rapid degradation of unsaturated cytokinins in P. vulgaris tissue (Mok et al., 1982b), presumably due to a higher level of cytokinin oxidase.

In order to determine if genetic variations in cytokinin metabolism also occur in whole plant tissues of Phaseolus, [^{14}C]zeatin metabolism was examined in a variety of tissues. The research described in this dissertation concerns [^{14}C]zeatin metabolism in immature embryos of P. vulgaris and P. lunatus. The results are presented in manuscript format.

III. MATERIALS AND METHODS

Plant Materials

Seeds of *Phaseolus vulgaris* L. cv. Great Northern (GN) and *P. lunatus* cv. Kingston (K) were originally obtained from Dr. Dermot Coyne (University of Nebraska) and Dr. Don Grabe (Oregon State University). Plants were grown in the greenhouse at 25°C with the photoperiod of 14 h. Immature embryos, 3, 6, and 9 mm in length were collected.

Chemicals

Trans-zeatin, *trans*-ribosylzeatin, Ade, Ado, 5'-nucleotidase (*Crotalus adamanteus* venom) and β-glucosidase (almond) were obtained from Sigma. 9-Glucosylzeatin was a gift from Dr. R. Durley (Oregon State University). 6-Chloro[8-^{14}C]purine (24 mCi/mmole) was obtained from Amersham. [8-^{14}C]Zeatin (24 mCi/mmole) was synthesized from 6-chloro-[8-^{14}C]purine following procedures published elsewhere. (Kadair *et al.*, 1984).

Metabolism of [^{14}C]zeatin

Immature embryos at three developmental stages (measuring 3, 6, and 9 mm in length) were dissected from the pods under sterial condition. The embryos corresponded to late heart, and early and mid

cotyledonary stages at the respective length. [^{14}C]Zeatin (0.05 uCi, 0.002 umol) dissolved in 250 ul of H_2O was applied aseptically to 250 mg of immature embryos. The vials were sealed and maintained at 27°C in the dark for 2, 4, and 8 hours. In addition, embyos were incubated with radioactively labeled zeatin dissolved in 0.05 M Tris-HCl (pH 6.0) buffer with all other conditions identical to those described above. To determine the amount of radioactivity recovered at time 0, [^{14}C] zeatin was applied and metabolites extracted immediately. This estimate was taken for the three sizes of embryos of both genotypes. Each experiment was repeated at least once and averages of two experiments are presented.

To extract metabolites, the embryos were homogenized with a Tissuemizer equipped with a Microprobe Shaft (Tekmar) in 2ml of cold 95% ethanol. Cell debris was removed by successive filtration through Whatman paper (No. 1) and Millipore filters (0.25 um). The ethanol extract was taken to dryness in vacuo at 35°C, redissolved in 2 ml of 50% (v/v) ethanol and centrifuged at 23,500 g for 20 min. The supernatant was condensed in vacuo to 100 ul with a speed vac concentrator (Savant). The sample was then analysed directly by HPLC. A Beckman Model 110 dual pump HPLC with a prepacked column of reversed-phase C_{18} (Ultrasphere ODS 5 um, 4.6 x 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid, adjusted to pH 3.5 with TEA. Samples were eluted with a linear gradient of methanol (5-50% over 90 min) in TEA buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted in Ready-Solve

MP Scintillation fluid (Beckman) with a Beckman LS 7000 Scintillation counter. To purify and further analyse nucleotides, paired-ion reversed-phase HPLC (Capelle et al., 1983) was used. The buffer consisted of 0.3% (w/v) TBAP and 0.65% (w/v) KH_2PO_4, adjusted to pH 5.8 with NH_4OH, and acetonitrile was used as the organic phase. The sample was applied in 100 ul of 20% aetonitrile in buffer and eluted with a linear gradient of acetonitrile (20-60% over 20 min) in buffer at a flow rate of 1.5 ml/min. Fractions of 1 ml were collected and counted as described above. To purify other metabolites, a reversed-phase C_{18} column was used but with a buffer solutions of 0.2 M acetic acid adjusted to pH 4.8 with TEA. The metabolites were eluted by a methanol gradient of 5-50% over 90 min at a flow rate of 1 ml/min.

Uptake of [^{14}C]zeatin and distribution of metabolites in embryos and solution

To determine if there are differences in the uptake of [^{14}C] zeatin between GN and K embryos and the distribution of metabolites in embryonic tissues vs. incubating solution, the following experiments were performed. [^{14}C]Zeatin (0.1 uCi; 0.004 umol) dissolved in 250 ul of H_2O was incubated with 250 mg of immature embryos. (The amount of radioactively labeled zeatin was twice that of other experiments in order to obtain sufficient radioactivity in HPLC fractions when embryo and solution portions are analyzed separately). After 2 h, the embryos were removed and subsequently washed with 1 uM of cold zeatin in 1.5 ml of H_2O. The wash solution

was combined with the incubating solution, dried *in vacuo* and analysed with reversed-phase HPLC. The embryos were homogenized and zeatin metabolites were extracted and analyzed as described above. The same experiment was performed for embryos of each size class.

Identification of [^{14}C]zeatin metabolites

Treatment with 5'-nucleotidase: Fractions collected after HPLC analysis were dried and redissolved in 0.05 M Tris-HCl buffer (pH 6.8) containing 5 mM $MgCl_2$, and aliquots of 30 ul were incubated with one unit of 5'-nucleotidase or 3'-nucleotidase for 0.5 h at 37 ^{o}C (Aung, 1978). Ethanol (1.5 ml) was added and the solution was centrifuged at 23,500 g for 20 min. The supernatant was taken to dryness *in vacuo* at room temperature, redissolved in 100 ul of 5% methanol and fractionated by HPLC.

Treatment with ß-glucosidase: Fractions collected after HPLC analysis were dried and redissolved in 200 ul of 0.03 M acetate buffer (pH 5.3). After adding 0.5 units of the enzyme, the solution was incubated at 37°C for 1 h. Ethanol (95%, 1.5 ml) was added and the solution was centrifuged at 23,500 g for 20 min. The supernatant was taken to dryness *in vacuo* at room temperature, redissolved in 100 ul of 5% methanol and analysed by HPLC.

Treatemnt with periodate and cyclohexamide: Fractions collected after HPLC analysis were dried and dissolved in 10 ul of periodate (10% w/v). After 3 h at room temperature, 2 ul of cyclohexamide was

added (Robins et al., 1967). After 18 h the solution was dried and the residue redissolved in 100 ul of 5 % methanol and analysed by HPLC.

Treatment with permanganate: Fractions collected after HPLC analysis were dried and redissolved in 500 ul of H_2O, and 250 ul of 0.1 N $KMnO_4$ (w/v) was added (Aung, 1978 and Hall, 1971). After 5 min, 1 ml of 95% ethanol was added and the solution was left for 24 h at room temperature. After centrifugation at 23,500 g, the supernatant was dried in vacuo. The residue was redissolved in 100 ul of 5% methanol and analysed by HPLC.

Acid hydrolysis with TFA: Fractions collected from HPLC analysis were dried and redissolved in 1 ml of 0.6 M TFA. The solution was incubated at 95°C for 3 h (Hall, 1964; Letham el al., 1979 and Miura and Hall, 1973) and centrifuged after addition of 1 ml H_2O. The supernatant was dried in vacuo, redissolved in 5% methanol and chromatographed by HPLC.

Permethylation of metabolites for GC-MS analysis: Anhydrous DMSO was obtained by treating DMSO with activated molecular sieves over night. Methyl sufinyl anion (4% w/v) was prepared by reacting NaH (200 mg) with 5 ml of DMSO under nitrogen. Standards and/or metabolites were dried and dissolved in 100 ul of anhydrous DMSO, followed by addition of 25 ul of methyl sulfinyl anion solution after 15 minutes by 10 ul of CH_3I. After 1.5 h, the reaction was terminated by adding 1 ml of H_2O. Permethylated derivatives were extracted with 1 ml of $CHCl_3$. The organic layer was washed three

times with 1 ml H_2O, dried and redissolved in 5 ul of $CHCl_3$ prior to GC-MS analysis.

GC-MS analysis: GC-MS analyses were performed using a Finnigan model 4023 GC-MS computor system with model 4500 source retrofit and pulsed positive-negative chemical ionization. The source temperature was 190°C. The conditions for EI and CI were 70 eV electron energy and 0.75t methane pressure in the source with 70 eV electron energy and 0.75t methane pressure in the source with 70 eV electrons respectively. For gas chromatography, a glass column (250 mm x 2 mm ID, Pyrex) packed with 7% OV-101 on Supelcoport (100-120 mesh) was used. The column temperature was programmed to run from 200 to 320°C (8°/min). The injector and detector temperature was 275°C. The capillary (15 m x 0.25 mm ID) contained fused silica (J and W DB-1). For combined GC-MS analyses, the temperature was held at 50°C for 1 min, raised to 225°C (25°C/min) and then to 320°C (8°C/min).

IV RESULTS

[^{14}C]Zeatin metabolism in Phaseolus vulgaris L. cv. GN. and P. lunatus cv. K.

Ethanol extracts of embryos of P. vulgaris (GN) and P. lunatus (K) were fractionated by HPLC. Representative elution profiles (3 mm embryos, 4 h incubations) are presented in Figs. 1 and 2 respectively for the two genotypes. In both genotypes [^{14}C]zeatin was rapidly metabolized. Five major radioactive peaks in addition to zeatin were recovered in extracts of GN (Fig. 1). One of the peaks coeluted with ribosylzeatin (see Fig. 3 for the elution positions of standards); the remaining four peaks were designated as A (35-36), I (45-47), II (58-59) and C (62-63). Large amounts of compound I were formed in embryos of this genotype. In K extracts, however, metabolites I and II were absent (Fig. 2). Of the four major metabolites recovered from this genotype, three had elution positions identical to those of ribosylzeatin, A and C. However, the fourth metabolite (fractions 37-38) was not found in GN extracts and was designated as B.

Identification of major radioactive metabolites:

Metabolite A:

Metabolite A from both tissues was treated with 5'-nucleotidase and rechromatographed on HPLC. The radioactivity co-eluted with ribosylzeatin (Fig. 4A). Treatment with 3'-nucleotidase had no effect on metabolite A. These results suggest that A is a 5'-

FIGURE 1. HPLC profiles of [^{14}C] zeatin metabolites obtained from embryo extracts (3 mm, 4 h incubation) of P. vulgaris cv. GN.

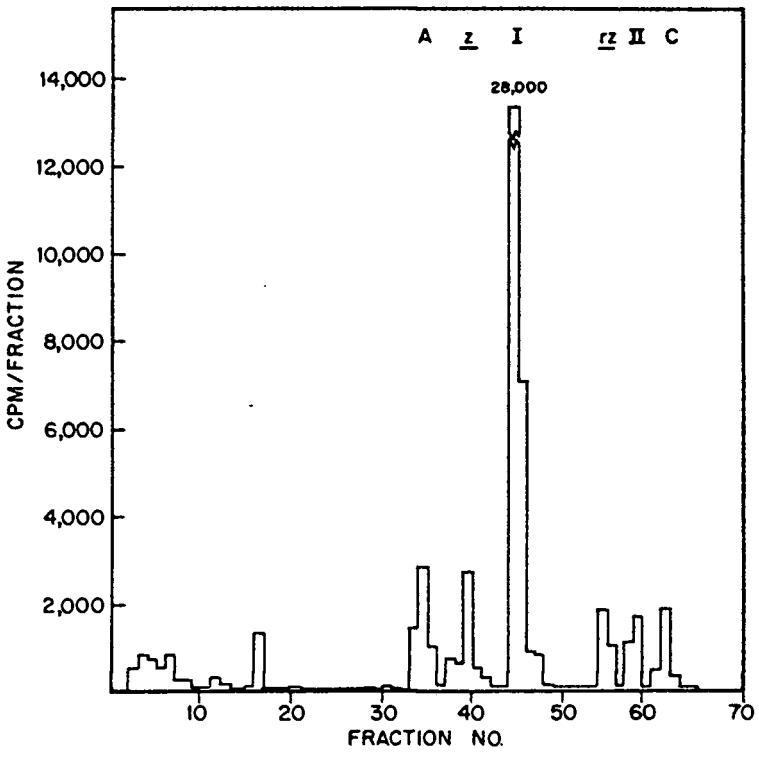
A z I rz II C
28,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
CPM/FRACTION
10
20
30
40
50
60
70
FRACTION NO.

Figure 1

FIGURE 2. HPLC profiles of [^{14}C]zeatin metabolites obtained from embryo extracts (3 mm, 4 h incubation) of *P. lunatus* cv. K.

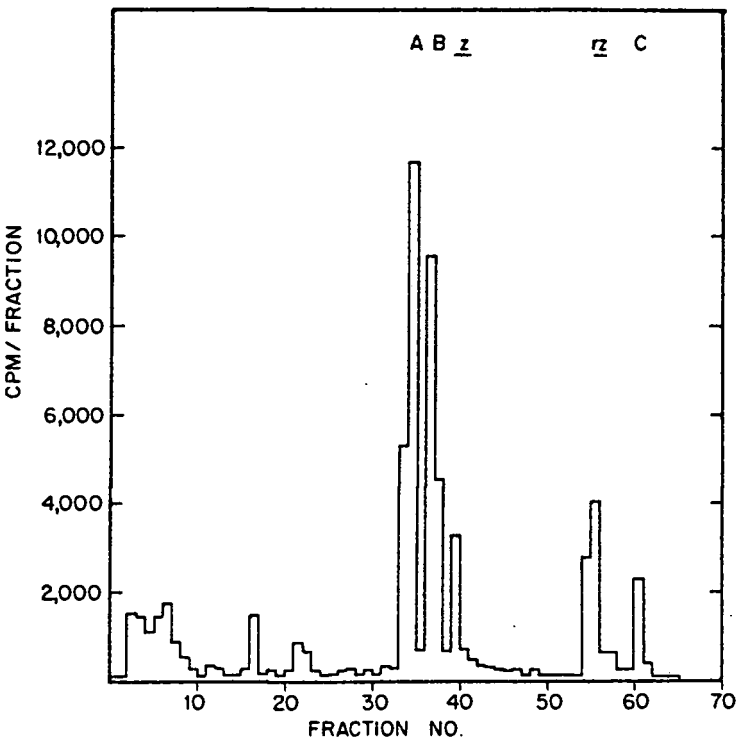
A B
C
12,000
10,000
8,000
6,000
4,000
2,000
CPM/ FRACTION
10
20
30
40
50
60
70
FRACTION NO.

Figure 2.

FIGURE 3. HPLC profiles of Ade, Ado and zeatin standards.

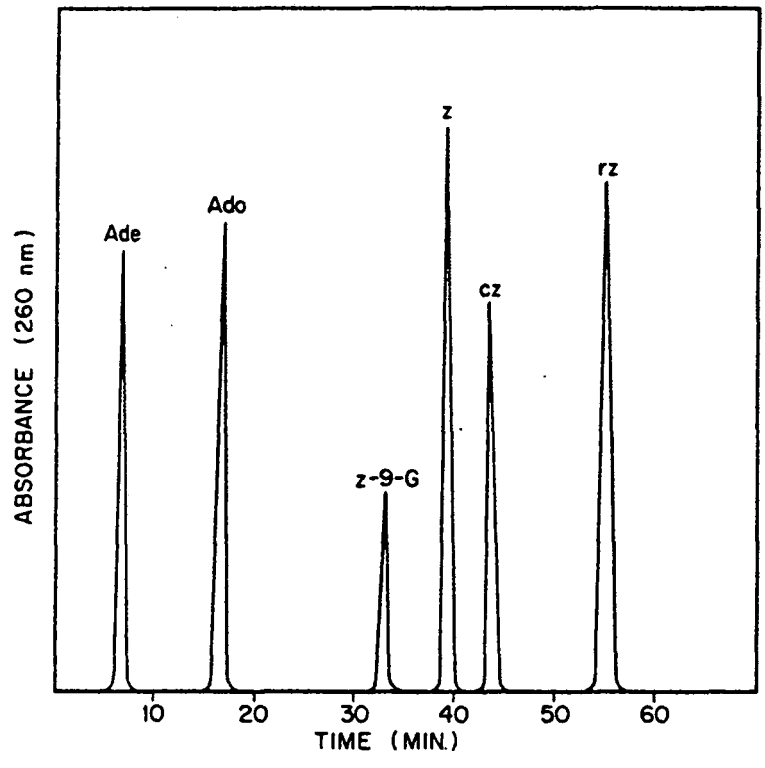

Figure 3.

FIGURE 4. Analyses by HPLC of metabolites A after treatment with 5'-nucleotidase (A), B after treatment with β-glucosidase (B), and C after treatment with β-glucosidase (C). Bars with discontinuous border indicate elution position of radioactivity before treatments.

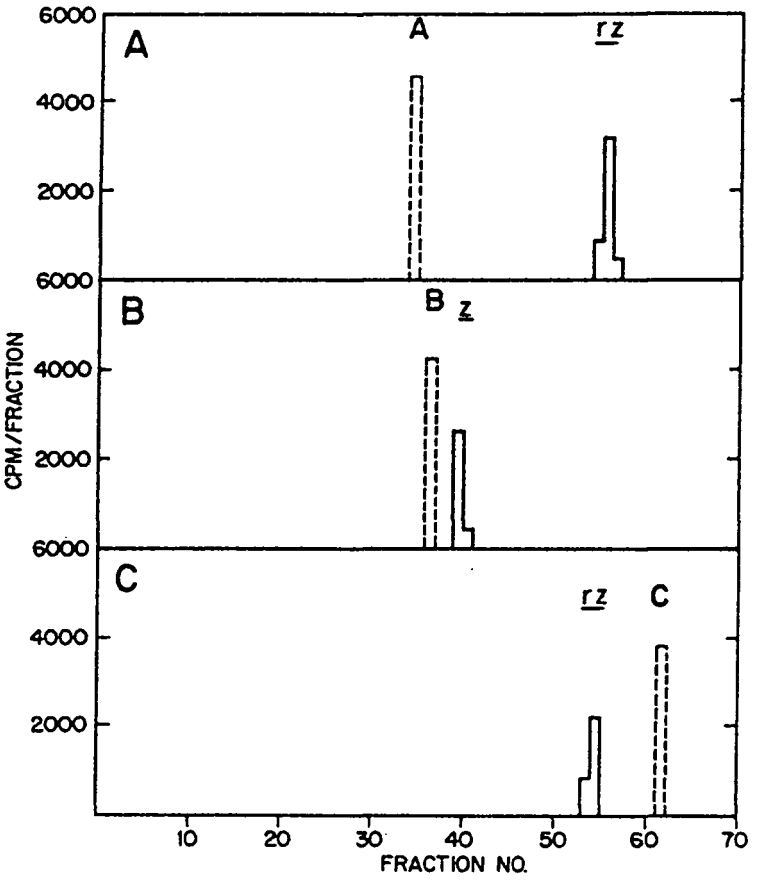
A
A
rz
B
B z
C
rz
C
6000
4000
2000
CPM/FRACTION
10
20
30
40
50
60
70
FRACTION NO.

Figure 4.

nucleotide of zeatin. To determine if A is the mono-, di- or trinucleotide, it was treated with $KMnO_4$ and rechromatographed on paired-ion reversed-phase HPLC (Capelle et al., 1983). The radioactivity co-eluted with AMP. Thus metabolite A seems to be the 5'-mononucleotide of zeatin.

Metabolite B:

Metabolite B from tissue extracts was treated with β-glucosidase. After fractionation by HPLC the radioactivity coincided with the elution position of zeatin (Fig. 4B). Thus this metabolite seems to be the O-glucoside of zeatin.

Metabolite C:

Treatment of metabolite C with β-glucosidase resulted in a shift of the elution position to that of ribosylzeatin after HPLC fractionation (Fig. 4C). Acid hydrolysis with TFA (removing sugar moieties (Hall, 1964; Kadair et al., 1984 and Miura and Hall, 1973)) shifted the radioactivity to the position of zeatin. Therefore, metabolite C is most likely the O-glucoside of ribosylzeatin (Entsch et al., 1980 and Horgan, 1975). Similar results were obtained for metabolite C recovered from the two genotypes.

Metabolites I and II:

Meabolites I and II were treated with β-glucosidase. About one-half of I was converted to zeatin (Fig. 5A), while one-half of the

FIGURE 5. Analyses by HPLC of metabolite I after treatments with β-glucosidase (A), TFA (B), periodate plus cyclohexamide (C) and permanganate (D). Bars with discontinuous border indicate elution position of radioactivity before treatments.

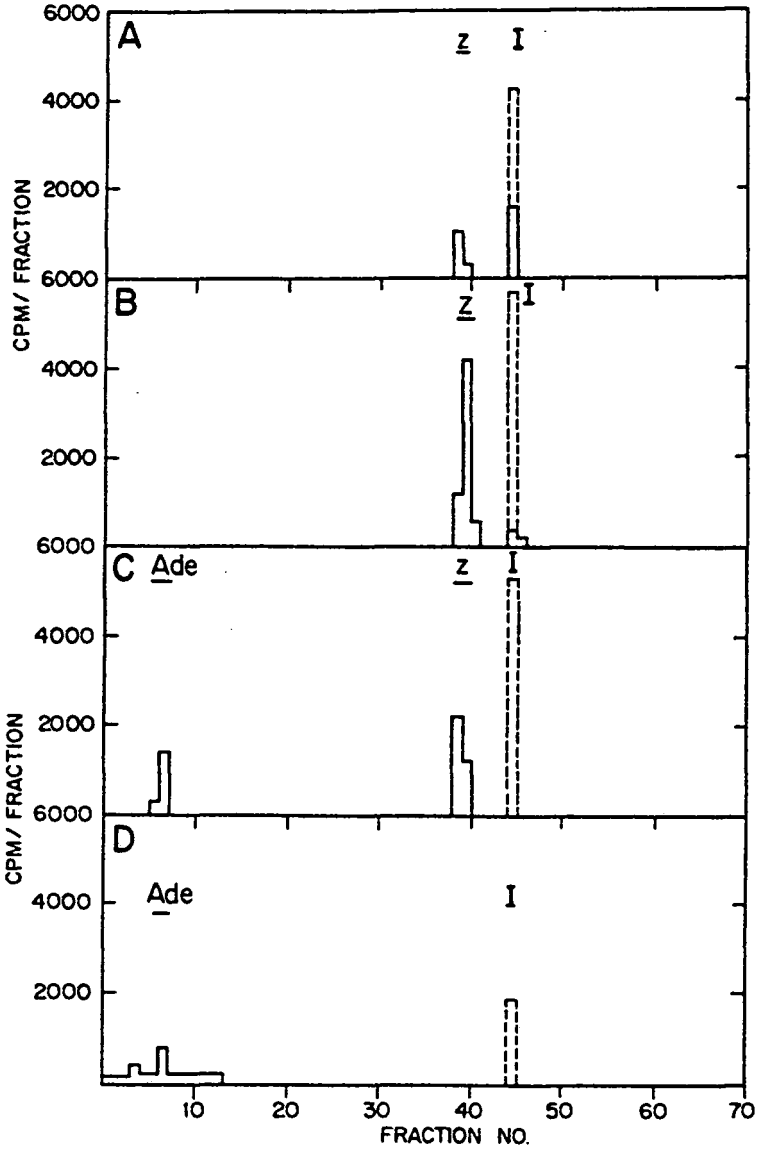
A
Z
I
B
Z
I
C Ade
Z
I
D
Ade
I
6000
4000
2000
CPM/ FRACTION
10
20
30
40
50
60
70
FRACTION NO.

Figure 5.

original radioactivity of II shifted to the position of ribosylzeatin (Fig. 6A). These results suggest that portions of metabolites I and II probably involve glycosylation of zeatin and ribosylzeatin. The difference between I and II may reside in the ribosylation of II at the 9 position. Acid hydrolysis with TFA converted the majority of I to zeatin (Fig. 5B), whereas identical treatment of II resulted in the formation of zeatin and small radioactive peak eluting off at the position of I (Fig. 6B). Treating metabolites I and II with periodate and cyclohexamide resulted in the recovery of zeatin in both cases (Figs. 5C and 6C). (Periodate opens ring structures of sugar moieties at the 2-3 position allowing subsequent removal of the open ring by cyclohexamine (Robins et at., 1967)). Finally, metabolities I and II were treated with $KMnO_4$ to remove the N^6-sidechain (Aung, 1978 and Hall, 1971). Compound I was converted to Ade and other breakdown products (Fig. 5D) indicating that the structure modification resides on the N^6-sidechain. The Majority of metabolite II was converted to Ado and Ade (Fig. 6d). Based on the results of chemical and enzymatic tests, it is reasonable to conclude that metabolites I and II are most likely O-glycosylated (by not O-glucosylated) derivatives of zeatin and ribosylzeatin respectively.

To further test our interpretation, metabolite I was re-purified by HPLC with paired-ion reversed-phase (pH 5.8) and with reversed-phase C_{18} (pH 4.8) columns. The UV spectra of the purified compound at acidic (pH 3.5) and alkaline (pH 8.5) conditions are presented in Fig. 7. The absorption maxima were at 272 and 270 nm for the two

FIGURE 6. Analyses by HPLC of metabolite II after treatments with β-glucosidase(Fig. A), TFA (Fig. B), periodate plus cyclohexamide (Fig. C) and permanganate (Fig. D). Bars with discontinuous border indicate elution position of radioactivity before treatments.

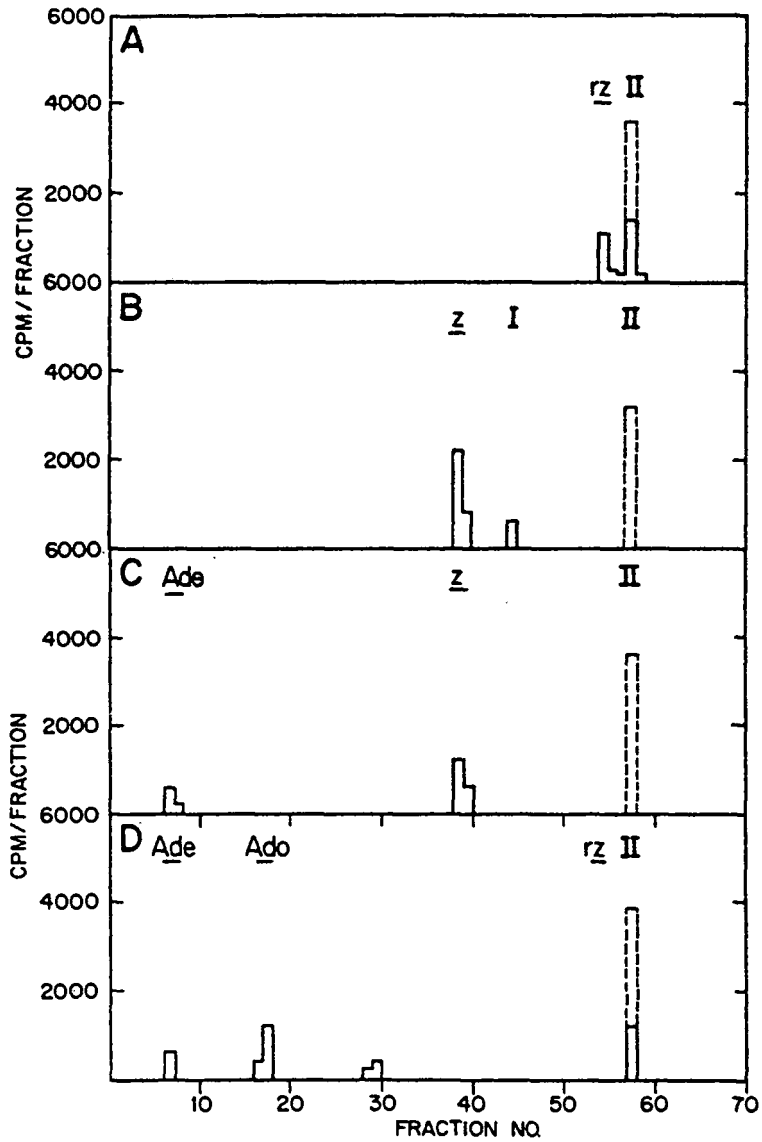
A
rz II
B
z I II
C Ade
z II
D Ade Ado
rz II
6000
4000
2000
CPM/FRACTION
10 20 30 40 50 60 70
FRACTION NO.

Figure 6.

FIGURE 7. UV spectra of purified metabolite I at pH 3.5 (A) and pH 8.5 (B).

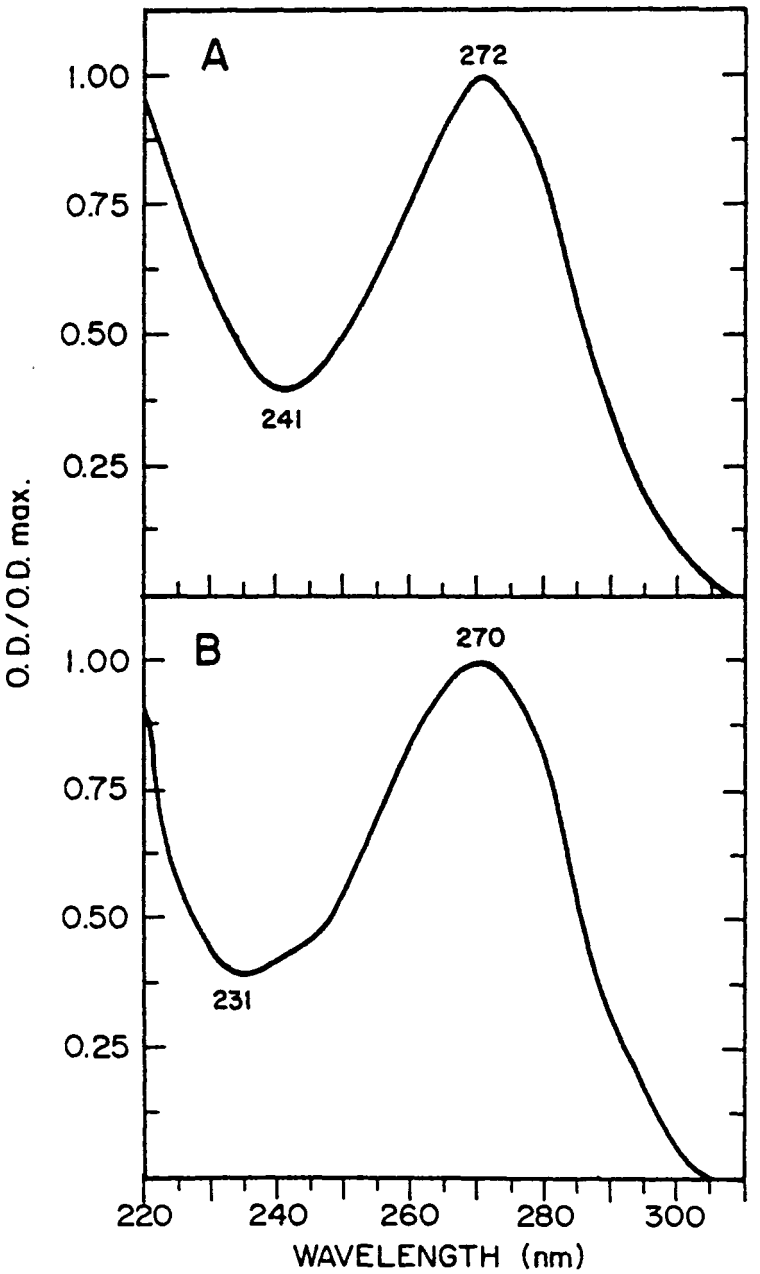
A
272
241
B
270
231
1.00
0.75
0.50
0.25
O.D./O.D. max.
220
240
260
280
300
WAVELENGTH (nm)

Figure 7.

conditions. The permethylated derivative of I was prepared and subjected to GC-MS analyses (Fig. 8). The mass spectrum obtained by CI (Fig. 8C) indicates a molecular weight of 421, identical to that of ribosylzeatin (Fig. 8B). The fragmentation pattern of lower molecular weight ions was similar to that of zeatin (Fig. 8A). As mass spectral analysis by EI has the advantage of generating larger number of molecular fragments, permethylated zeatin, ribosylzeatin and compound I derivatives were also subjected to this type of MS analysis (Figs. 8D, E and F). The spectrum obtained confirmed the molecular weight of 421 for metabolite I. The fragmentation pattern (Fig. 8F) was consistent with our interpretation that compound I could be an O-glycoside of zeatin. As the molecular weight indicates the presence of a pentose sugar, compound I is most likely O-ribosylzeatin.

Since both metabolites I and II had the tendency of premature fragmentation under EI, much larger amounts of sample (approximately 2 ug) were required to obtain a complete spectrum. The relatively small amount of metabolite II obtained dictated an analysis by CI only (Fig 9). The GC-MS analyses revealed a molecular weight of 581. The pattern of fragmentation was compatible with the structure of the O-riboside of ribosylzeatin. Nevertheless, direct synthesis of these compounds is needed to unequivocally confirm our interpretation of the structures.

FIGURE 8. Mass spectra of permethylated zeatin (A and D), ribosylzeatin (B and E) and metabolite I (C and F) obtained by CI (A, B and C) and EI (D, E and F) analyses.

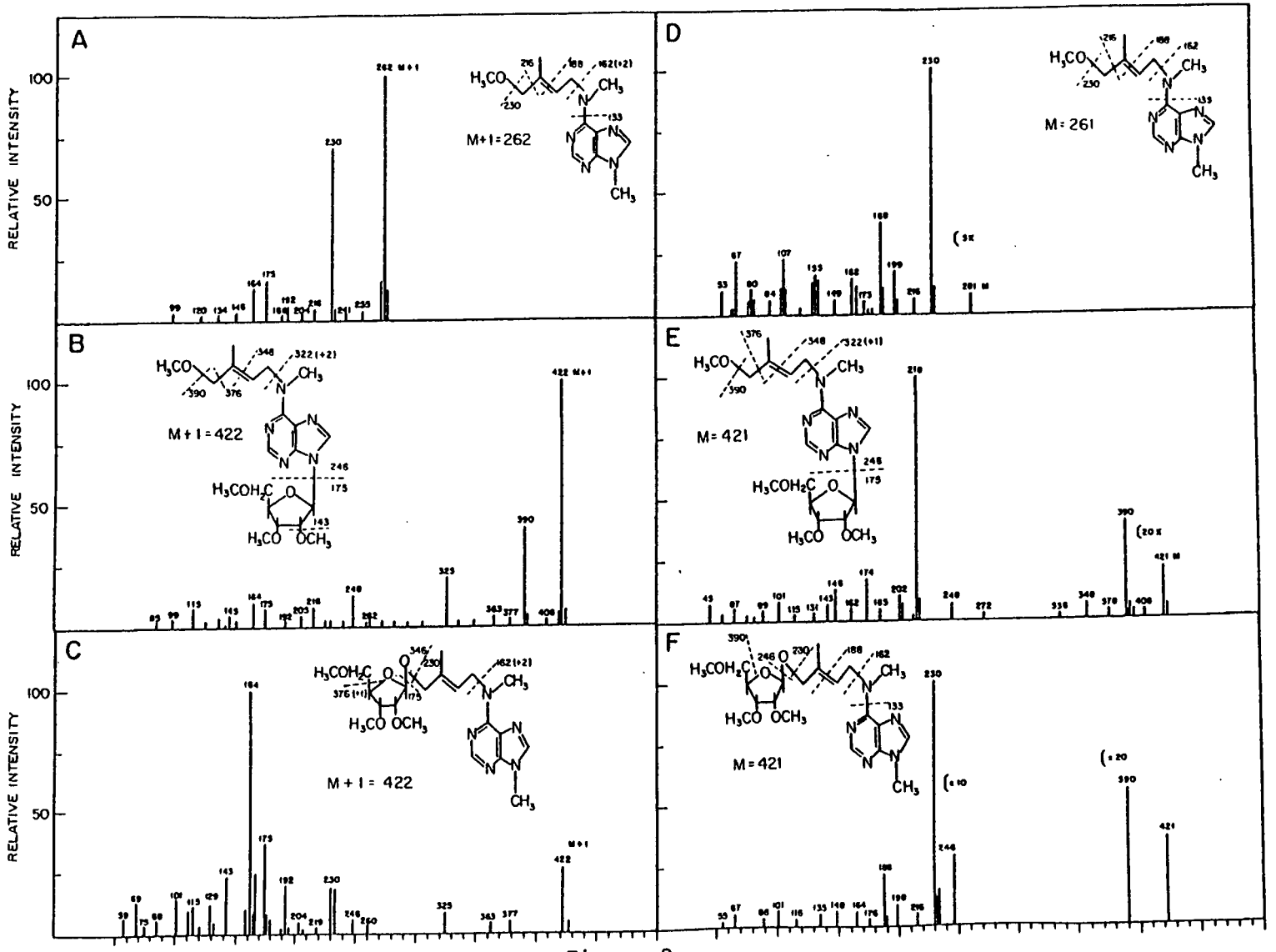
A
B
C
D
E
F
RELATIVE INTENSITY
M+1=262
M+1=422
M+1=422
M=261
M=421
M=421

Figure 8.

FIGURE 9. Mass spectrum of permethylated metabolite II obtained by CI analysis.

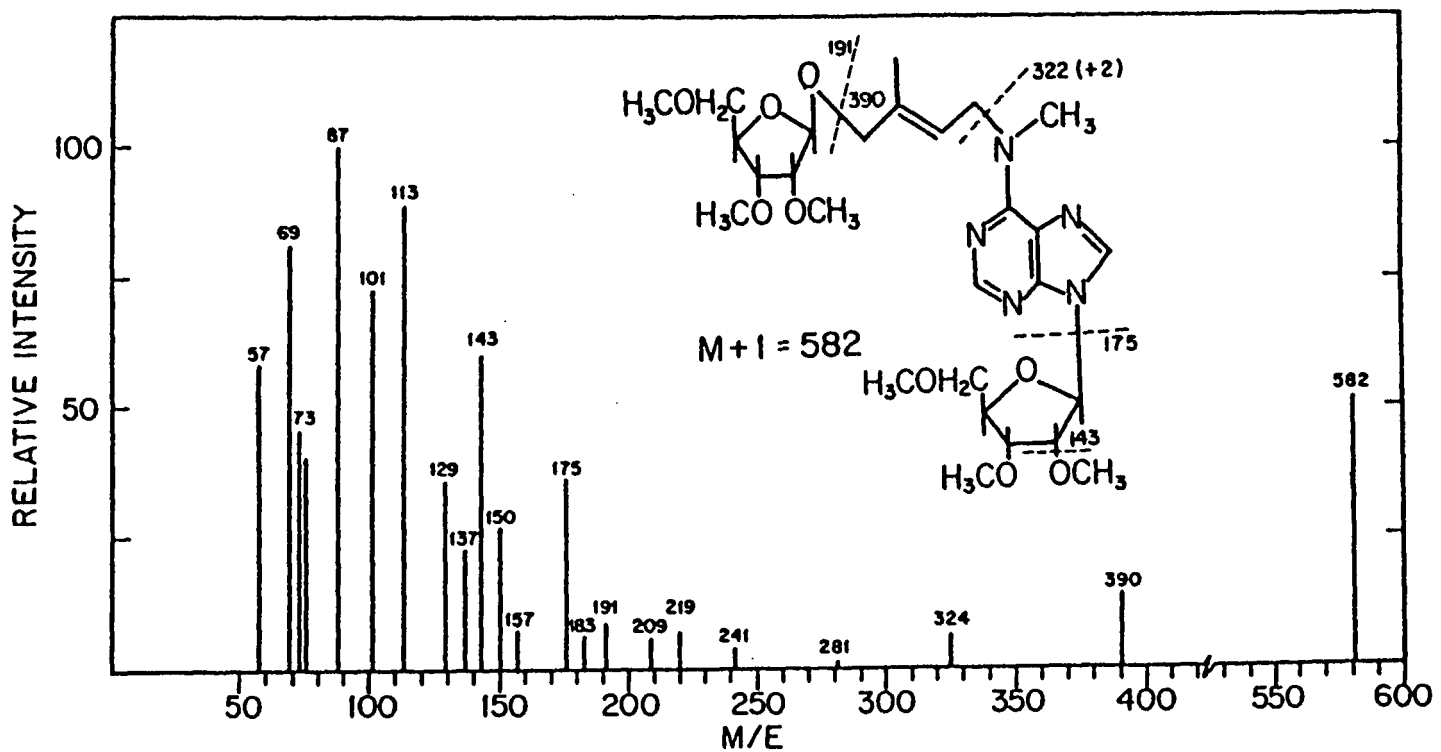
RELATIVE INTENSITY
100
50
M/E
50
100
150
200
250
300
350
400
550
600
57
69
73
87
101
113
129
137
143
150
157
175
183
191
209
219
241
281
324
390
582
M+1 = 582
191
390
322 (+2)
175
143

Figure 9.

Effects of embryo size on the metabolism of [^{14}C]zeatin

The proportion of radioactive metabolites (presented as % radioactivity at time 0) recovered from different sizes of embryos at various incubation times (2, 4 and 8 h) are presented in Fig. 10. No qualitative differences were observed between the 3, 6 and 9 mm embryos. However, some differences in the quantities of metabolites were observed between developmental stages. Younger embryos (3 and 6 mm) of GN appeared to convert larger proportions of zeatin to compounds I and II. Moreover, generally higher levels of zeatin mononucleotide were recovered from K embryos than GN embryos at all developmental stages. After 8 h, little radioactivity remained.

Distribution of metabolites in embryonic tissues vs. incubating solution

The distribution of metabolites in embryonic tissues vs. incubating solution (% of radioactivity recovered) is presented in Fig 11. Most of the zeatin metabolites were recovered from the embryos as well as the incubating solution, indicating that most likely the metabolites (and possibly also some of the enzymes) are released from the embryos to the solution. However, the majority of the zeatin nucleotide was retained in the embryonic tissues which is consistent with the reported properties of nucleotides. Also ribosylzeatin-O-glucoside was predominatly found in the embryonic tissues, in contrast to zeatin-O-glucoside which was present mainly in the solution.

FIGURE 10. Radioactive metabolites recovered from embryo extracts of *P. vulgaris* cv. GN and *P. lunatus* cv. K.

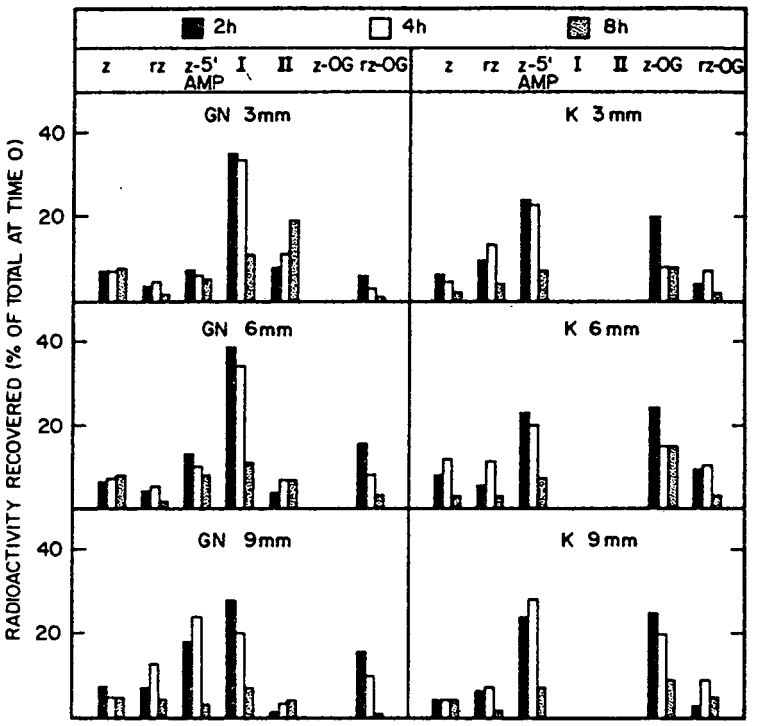
2h
4h
8h
z
rz
z-5' AMP
I
II
z-OG
rz-OG
GN 3mm
K 3mm
GN 6mm
K 6mm
GN 9mm
K 9mm
RADIOACTIVITY RECOVERED (% OF TOTAL AT TIME 0)
40
20

Figure 10.

FIGURE 11. Distribution of[^{14}C]zeatin metabolite between embryonic tissues and incubating solution.

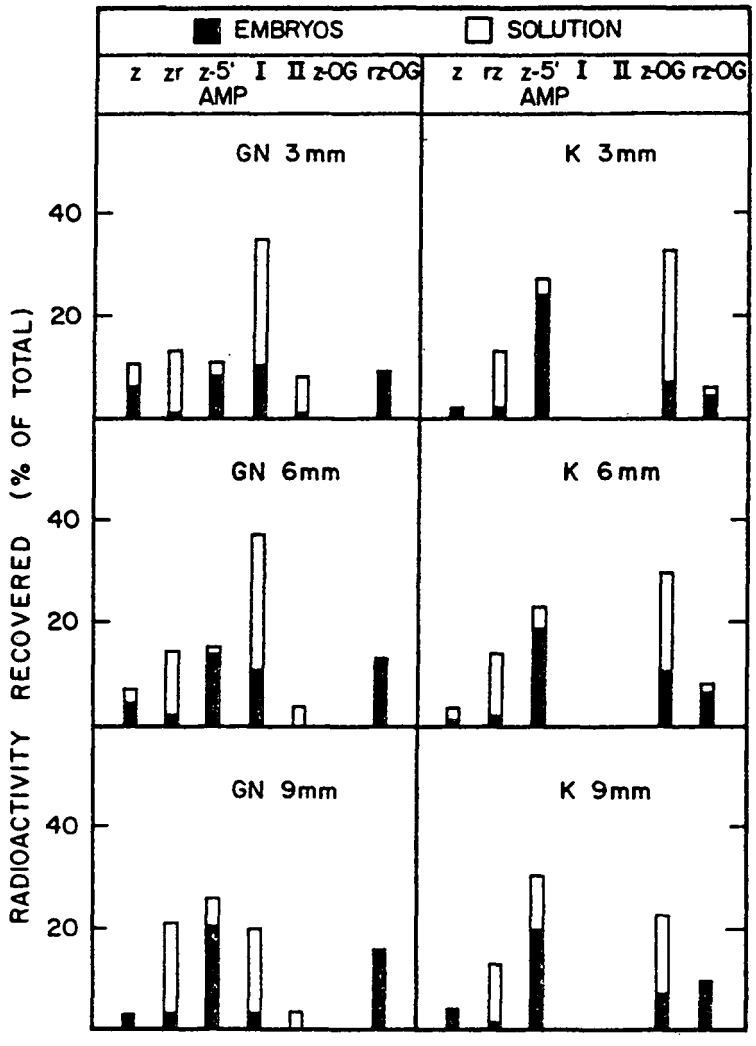
EMBRYOS
SOLUTION
z zr z-5' AMP I II z-OG rz-OG
z rz z-5' AMP I II z-OG rz-OG
GN 3mm
K 3mm
GN 6mm
K 6mm
GN 9mm
K 9mm
RADIOACTIVITY RECOVERED (% OF TOTAL)
40
20

Figure 11.

Incubating embryos with radioactive zeatin dissolved in water and Tris-HCl buffer (in selected samples) gave essentially the same results regarding the types and proportion of metabolites recovered as well as the distribution of metabolites between embryonic tissues and incubating solutions.

V. DISCUSSION

The most striking difference in zeatin metabolism between P. vulgaris and P. lunatus embryos resides in the occurrence of metabolites I and II in P. vulgaris only. These compounds were tentatively identified as the O-ribosides of zeatin and ribosylzeatin. As far as we know, the ribosylation of the N^6-sidechain of zeatin has not been previously reported (Letham and Palni, 1983). Since earlier studies of zeatin metabolism in P. vulgaris axes, seeds, leaves and roots (Palmer et al., 1981; Sondheimer and Tzou, 1971 and Wang et al., 1977) yielded primarily zeatin glucosides, nucleotides, and dihydrozeatin derivatives, the occurrence of metabolites I and II appears to be restriced to embryonic tissues. Unpublished results obtained in our laboratories using other plant parts of cv. GN also substantiate the embryo-specific nature of these compounds. In addition, their presence is also likely to be species-specific, since two additional genotypes of P. vulgaris and P. lunatus have been examined and the results obtained were similar to those reported in this study for GN and K respectively.

The biological significance of O-ribosylation is presently unknown. However, the conversion of close to 30% of the exogenously supplied zeatin to compounds I and II in relatively short periods of time must represent a rather active process. Moreover, the enzyme system(s) associated with the ribosylation of the sidechain must be

quite specific since glucosylation of the sidechain and the ribosylation at the 9 position of the purine ring occur in GN as well as K. Therefore, intuitively, one would tend to speculate that the occurrence of these metabolites may be important to some aspects of embryonic growth in P. vulgaris. The immature embryos used in this study correspond to late heart (3 mm), early and mid-cotyledonary (6 and 9 mm) stages, with the younger embryos undergoing rapid growth (Walbot et al., 1972). It is of interest to note that younger embryos were also more active in the formation of metabolite I.

The biological functions of zeatin metabolites (such as O- and N-glucosides (Fox et al., 1974; Horgon, 1975 and Parker et al., 1973), lupinic acid (Letham et al., 1979; Murakoshi et al., 1977 and Parker et al., 1978), glucosyl-ribosylzeatin (Taylor et al., 1984), nucleoside and nucleotides (Laloue et al., 1974 and Sondheimer and Tzou, 1971)) are not well established. Glucosides have been suggested to be either storage forms or to be related to cytokinin transport (Gordon et al., 1974 and Letham and Palni, 1983). Protection against cytokinin oxidase (Whitty and Hall, 1974) attack via O-glucosylation and ribonucleotide formation have also been postulated (McGaw and Horgan, 1983b). Zeatin-9-riboside could be an intermediate in nucleotide formation or breakdown of nucleic acids. The occurrence of O-ribosylation of zeatin, however, would seem to suggest additional roles of ribosylation of cytokinin bases.

The development of interspecific hybrid embryos of Phaseolus has previously been examined in some detail (Mok et al., 1978 and

Rabakoarihanta et al., 1979). The arrest of embryo growth is dependent on the species combination and the direction of the cross. For example, P. vulgaris x P. lunatus embryos develop only to the preheart stage. The reciprocal cross gives embryos which cease to divide at the four-celled stage, but can be stimulated to progress to the preheart stage by supplying cytokinin to the female parent via hydroponic culture (Mok et al., in press). P. vulgaris x P. acutifolius crosses result in embryos which develop to the cotyledonary stage but also do not reach maturity. It was reported that the slower growth rate of P. vulgaris-P. acutifolius hybrid embryos was correlated with lower amount of extractable cytokinis as compared with selfed embryos of both species (Nesling and Morris, 1979). As the largest differences in cytokinin metabolism usually occur between Phaseolus species, it is conceivable that an imbalance of cytokinin utilization or metabolism in the hybrid tissues could have contributed to the abnormal growth of the interspecific hybrid embryos. The qualitative difference in zeatin metabolism between P. vulgaris and P. lunatus embryos reported here provides a useful basis for further research into the relationship between abnormal embryonic development and hormonal derangement.

Identification of genetic variations is essential for studies of the genetic regulation of metabolic processes. Callus cultures have proven to be a versatile screening system for genetic variations in cytokinin metabolism. By utilizing cell culture systems, it has been discovered that there is a differential structure-activity relation-

ship between P. vulgaris and P. lunatus (Mok et al., 1978). The substantially lower activity of cytokinins with an unsaturated N^6-sidechain (zeatin, i^6Ade) in the P. vulgaris callus bioassay was found to be related to a rapid degradation of the N^6-sidechain of i^6Ado (Mok et al., 1982). Another genetic variation detected in cell cultures was cytokinin autonomous growth of P. vulgaris tissues which was controlled by a major locus (Mok et al., 1980). The results described in this paper indicate that intrinsic genetic differences can also be detected at the whole plant level. Since these differences are qualitative and tissue-specific, analyses of both organized (intact) and unorganized (callus) tissues of the same genotype may provide an opportunity to address the problem of differential expression during development of the genes governing cytokinin metabolism.

VI. BIBLIOGRAPHY

Aung, T. 1978. Metabolic studies of N^6-(Δ^2-isopentenyl)adenosine in normal and autonomous tissues of Nicotiana tabacum L. Ph.D. Thesis. MacMaster University, Hamilton.

Brownlee, B. G., R. H. Hall and C. D. Whitty. 1975. 3-Methyl-2-butenal: an enzymatic product of the cytokinin, N^6-(Δ^2-isopentenyl) adenine. Can. J. Biochem. 53:37-41.

Bruce, M. I. and J. A. Zwar. 1966. Cytokinin activity of some substituted ureas and thioureas. Proc. R. Soc. London Ser. B. 165:245-265.

Bruce, M. I. J. A. Zwar and N. P. Kefford. 1965. Chemical structure and plant kinin activity: The activity of urea and thiourea derivatives. Life Sci. 4:461-466.

Burrows, W. J. and K. J. Fuell. 1981. Cytokinin biosynthesis in cytokinin-autonomous and bacteria-transformed tobacco callus tissues. In: Metabolism and molecular activities of cytokinins. ed. Guern, J. and C. Peaud-Lenoel, pp. 44-55. Berlin, Springer.

Capelle, S. C., D. W. S. Mok, S. C. Kirchner and M. C. Mok. 1983. The effects of Thidiazuron on cytokinin autonomy and the metabolism of N^6-(Δ^2-isopentenyl) [8-^{14}C]adenosine in callus tissues of Phaseolus lunatus L. Plant Physiol. 73:796-802.

Carnes, M. G., M. L. Brenner and C. R. Anderson. 1975. Comparison of reversed phase high pressure liquid chromatography with Sephadex LH-20 for cytokinin analysis of tomato root pressure exudate. J. Chromatogr. 108:95-106.

Chatfield, J. M. and D. J. Armstrong. 1984. Cytokinin oxidase activity in Phaseolus callus tissue. Plant Physiol. 75:60. (Abstract)

Chen, C. M. and R. L. Eckert. 1977. Phosphorylation of cytokinin by adenosine kinase from wheat germ. Plant Physiol. 59:445-447.

Chen, C. M. and S. M. Kristopeit. 1981a. Metabolism of cytokinin: Dephosphorylation of cytokinin ribonucleotide by 5'-nucleotidases from wheat germ cytosol. Plant Physiol. 67:494-498.

Chen, C. M. and S. M. Kristopeit. 1981b. Metabolism of cytokinin: Deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ cell. Plant Physoil. 68:1020-1023.

Chen, C. M., D. K. Melitz and F. W. Clough. 1982. Metabolism of cytokinin: Phosphoribosylation of cytokinin bases by adenine phosphoribosyltransferase from wheat germ. Arch. Biochem. Biophys. 214:634-641.

Chen, C. M. and B. Petschow. 1978. Metabolism of cytokinin: Ribosylation of cytokinin bases by adenosine phosphorylase from wheat germ. Plant Physiol. 62:871-874.

Cowley, D. E., C. C. Duke, A. J. Liepa, J. K. MacLeod and D. S. Letham. 1978. The structure and synthesis of cytokinin metabolites I. The 7- and 9-β-D-glucofurranosides and pyranosides of zeatin and 6-benzylaminopurine. Aust. J. Chem. 31:1095-1111.

Entsch, B., D. S. Letham, C. W. Parker, R. E. Summons and B. I. Gollnow. 1980. Metabolism of cytokinins. In F. Skoog, ed., Plant Growth Substances 1979. Springer-Verlag, Berlin, pp. 109-118.

Entsch, B., C. W. Parker and D. S. Letham. 1983. An enzyme from lupin seeds forming alanine derivatives of cytokinins. Phytochemistry 22:375-381.

Entsch, B., C. W. Parker, D. S. Letham and R. E. Summons. 1979. Preparation and characterization, using high-performance liquid chromatography of an enzyme forming glucosides of cytokinins. Biochem. Biophys. Acta. 570:124-139.

Fox, J. E., J. Cornette, G. Deleuze, W. Dyson, C. Giersak, P. Niu, J. Zapata and J. McChesneey. 1973. The formation, isolation and biological activity of a cytokinin 7-glucoside. Plant Physiol. 52:627-632.

Fox, J. E. and J. D. McChesney. 1974. Cytokinin-7-glucosides: some properties of synthetic analogs of a naturally occurring cytokinin metabolite in: Plant Growth Substance 1973, Hirokawa, Tokyo, pp. 468-479.

Gordon, M. E., D. S. Letham and C. W. Parker. 1974. The metabolism and translocation of zeatin in intact radish seedling. Ann. Bot. 38:809-825.

Hall, R. H. 1964. Isolation of N^6-(aminoacyl) adenosine from yeast ribonucleic acid. Biochemistry 3:769-773.

Hall, R. H. 1971. The modified nucleoside. In: Nucleic Acid. Columbia University Press, New York.

Hecht, S. M., N. J. Leonard, R. Y. Schmitz and F. Skoog. 1970. Cytokinins: Influence of side-chain planarity of N^6- substituted adenines and adenosines on their activity in promoting cell growth. Phytochemistry 9:1907-1913.

Horgan, R. 1975. A new cytokinin metabolite. Biochem Biophys Res Commun 65:358-363.

Horgan, R. and M. R. Kramers. 1979. High-performance liquid chromatography of cytokinins. J. Chromatogr. 173:263-270.

Horgan, R., L. M. S. Palni, I. M. Scott and B. A. McGaw. 1981. Cytokinin biosynthesis and metabolism in Vinca rosea crown gall tissue. In: Metabolism and molecular activities of cytokinins. pp. 56-65. Guern, J., Peaud-Lenoel, C., eds. Springer, Berlin Heidelberg New York.

Isogai, Y. 1981. Cytokinin activities of N-phenyl-N-(4-pyridyl) ureas. In: J. Guern, C. Peaud-Lenoel, eds., Metabolism and molecular activities of cytokinins. springer-Verlag, Berlin, pp. 115-128.

Isogai, Y., K. Shudo and T. Okamoto. 1976. Effect N-N'-(4-pyridyl) urea on shoot formation in tobacco pith disk and callus cultured in vitro. Plant Cell Physiol. 17:591-600.

Kadair, K., S. Gregson, G. Shaw, M. C. Mok and D. W. S. Mok. 1984. (in press) Purines, pyrimidines and imidazoles. Part 61. A convenient synthesis of trans-zeatin and [8-^{14}C]trans-zeatin. J. Chem Res

Laloue, M., C. Terrine and M. Gawer. 1974. Cytokinins: Formation of the nucleoside-5'-triphosphate in tobacco and Acer cells. FEBS Lett 46:45-50.

Leonard, N. J. 1974. Chemistry of the cytokinins. Recent Adv. Phytochem. 7:21-56.

Lethem, D. S. 1963. Zeatin, a factor inducing cell division isolated from Zea mays. Life Sci. 2:569-573.

Letham, D. S. 1966. Purification and probable identity of a new cytokinin in sweet corn extracts. Life Sci. 5(6):551-554.

Letham, D. S. and C. O. Miller. 1965. Identify of kinetin-like factors from Zea mays. Plant Cell Physiol. 6:355-359.

Letham, D. S. and L. M. S. Palni. 1983. The biosynthesis and metabolism of cytokinins. Ann. Rev. Plant. Physiol. 34:163-197.

Letham, D. S., C. W. Parker, C. C. Duke, R. E. Summons and J. K. MacLeod. 1976. O-glucosylzeatin and related compounds - A new group of cytokinin metabolites. Ann. Bot. 40:261-263.

Letham, D. S., J. S. Shannon and I. R. McDonald. 1964. The structure of zeatin, a factor inducing cell division. Proc. Chem. Soc. 230-231.

Letham, D. S., R. E. Summons, C. W. Parker and J. K. MacLeod. 1979. Identification of an amino-acid conjugate of 6-benzylaminopurine formed in Phaseolus vulgaris seedlings. Planta 146:71-74.

Letham, D. S., G. Q. Tao and C. W. Parker. 1982. An overview of cytokinin metabolism. In: Plant Growth Substance 1982. ed. P. F. Wareing, pp. 143-153.

MacLeod, J. K., R. E. Summons, C. W. Parker and D. S. Letham. 1975. Lupinic acid, a purinyl amino acid and a novel metabolite of zeatin. J. Chem. Soc. Chem. Commun. 809-810.

MacLeod, J. K., R. E. Summons and D. S. Letham. 1976. Mass spectrometry of cytokinin metabolites, Per (trimethylsilyl) and permethyl derivatives of glucosides of zeatin and 6-benzylaminopurine. J. Chem. 41:3959-3967.

Matsubara, S. 1980. Structure - activity relationships of cytokinins. Phytochemistry. 19:2239-2253.

McGaw, B. A. and R. Horgan. 1983a. Cytokinin catabolism and cytokinin oxidase. Phytochemistry. 22:1103-1105.

McGaw, B. A. and R. Horgan. 1983b. Cytokinin oxidase from Zea mays kernels and Vinca rosea crown-gall tissues. Planta 159:30-37.

Miller, C. O. 1961. A kinetin-like compound in maize. Proc. Natl. Acad. Sci. U.S.A. 47:170-174.

Miller, C. O., F. Skoog, F. S. Okumura, M. H. Von Saltza and F. M. Strong. 1955. Structure and synthesis of kinetin. J. Am. Chem. Soc. 77:2662-2663.

Miller, C. O., F. Skoog, F. S. Okumura, M. H. Von Saltza and F. M. Strong 1956. Isolation and synthesis of kinetin, a substance promoting cel division. J. Am. Chem. Soc. 78:1375-1380.

Mok, M. C., S. G. Kim, D. J. Armstrong and D. W. S. Mok. 1979. Induction of cytokinin autonomy by N. N'-diphenylurea in tissue cultures of Phaseolus Lunatus L. proc. Natl. Acad. Sci. U.S.A. 76:3880-3884.

Mok, M. C., D. W. S. Mok and D. J. Armstrong. 1978. Differential cytokinin structure - activity relationships in Phaseolus. Plant Physiol. 61:72-75.

Mok, M. C., D.W.S. Mok, D. J. Armstrong, A. Rabakoarihanta and S. G. Kim. 1980. Cytokinin autonomy in tissue cultures of Phaseolus: A genotype specific and heritable trait. Genetics 94:675-686.

Mok, M. C., D. W. S. Mok, D. J. Armstrong, K. Shudo, Y. Isogai and T. Okamoto 1982a. Cytokinin activity of N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (Thiodiazuson). Phytochemistry 21:1509-1511.

Mok, M. C., D. W. S. Mok, S. C. Dixon, D. J. Armstrong and G. Shaw. 1982b. Cytokinin structure-activity relationships and the metabolism of N^6-(Δ^2-isopentenyl)adenosine-8-[^{14}C]in Phaseoulus callus tissues. Plant Physiol. 70:173-178.

Mok, D. W. S., M. C. Mok and A. Rabakoarihanta. 1978. Interspecific hybridization of Phaseolus vulgaris with P. lunatus and P. acutifolius. Theor. Appl. Genet. 52:209-215.

Mok, D. W. S., M. C. Mok, A. Rabakoarihanta and C. T. Shii. (in press). Phaseolus-Wide hybridization through embryo culture. In Y.P.S. Bajaj, ed, In Vitro Improvement of Crops. Springer-Verlag, Berlin.

Morris, R. O. 1977. Mass spectroscopic identification of cytokinins. Plant Physiol. 59:1029-1033.

Morris, R. O., D. A. Regier and E. M. S. MacDonald. 1981. Analytical procedures for cytokinins: application to Agrobacterium tumefaciens. In: Metabolism and Molecular Activities of Cytokinins. ed. J. Guern and C. Peaud-Lenoel. pp. 3-16.

Murakoshi, I., F. Ikegami, N. Ookawa, J. Haginiwa and d. S. Letham 1977. Enzymic synthesis of lupinic acid, a novel metabolite of zeatin in higher plants. Chem. Pharm. Bull. Tokyo. 25:520-522.

Nesling, F. A. V. and D. A. Morris. 1979. Cytokinin levels and embryo abortion in interspecific Phaseolus crosses. Z Pflanzenphysiol 91:345-358.

Nishinari, N. and K. Syono. 1980. Cell-free biosynthesis of cytokinins in cultured tobacco cells. Z. Pflanzenphysiol. 99:383-392.

Paces, V., E. Werstiuk and R. H. Hall. 1971. Conversion of N^6-(Δ^2-isopentenyl)adenosine to adenosine by enzyme activity in tobacco tissue. Plant Physiol. 48:775-778.

Palmer, M. V., I. M. Scott and R. Horgan. 1981. Cytokinin metabolism in Phaseolus Vulgaris L. II. Comparative metabolism of exogenous cytokinins by detached leaves. Plant Sci. Lett. 22:187-195.

Palni, L. M. S. and R. Horgan. 1983. Cytokinins in transfer RNA of normal and crown-gall tissue of Vinca rosea. Planta 159:178-181.

Parker, C. W. and D. S. Letham. 1973. Metabolism of zeatin by radish cotyledons and hypocotyls. Planta 114:199-218.

Parker, C. W. and D. S. Letham. 1974. Regulators of cell division in plant tissue. XVIII. Metabolism of zeatin in Zea mays seedlings. Planta 115:337-344.

Parker, C. W., D. S. Letham, B. I. Gollnow, R. E. Summons, C. C. Duke and J. K. MacLeod. 1978. Metabolism of zeatin by lupin seedlings. Planta 142:239-251.

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

Parker, C. W., M. M. Wilson, D. S. Letham, D. E. Cowley and J. K. MacLeod 1973. The glucosylation of cytokinins. Biochem. Biophys. Res. Commun. 55:1370-1376.

Rabakoarihanta, A., D. W. S. Mok and M. C. Mok. 1979. Fertilization and early embryo development in reciprocal interspecific crosses of Phaseolus. Theor. Appl. Genet. 54:55-59.

Robins, M. J., R. H. Hall and R. Thedford. 1967. N^6-(Δ^2-isopentenyl)adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissues. Methods of isolation and characterization. Biochemistry 6:1837-1848.

Scott, I. M. and R. Horgan. 1982. High-performance liquid chromatography of cytokinin ribonucleoside-5'-monophosphates. J. Chromatogr. 237:311-315.

Scott, I. M. and R. Horgan. 1984a. Root cytokinins of Phaseolus vulgaris L. Plant Sci. Letters 34:81-87.

Scott, I. M. and R. Horgan. 1984b. Mass-spectrometric quantification of cytokinin nucleotides and glycosides in tobacco crown-gall tissue. Planta 161:345-354.

Scott, I. M., R. Horgan and B. A. McGaw. 1980. Zeatin-9-glucoside, a major endogenous cytokinin of Vinca rosea crown gall tissue. Planta 149:472-475.

Scott, I. M., B. A. McGaw, R. Horgan and P. E. Williams. 1982. Biochemical studies on cytokinins in Vinca rosea crown gall tissue. In: Plant growth substance. 1982 pp. 165-174, Wareing, P. F. ed., Academic Press, London, New York.

Shantz, E. M. and F. C. Steward. 1955. The identification of compound A from coconut milk as 1,3-diphenylurea. J. Amer. Chem. Soc. 77: 6351-6353.

Shaw, G. and D. V. Wilson. 1964. Synthesis of zeatin. Proc. Chem. Soc. 231.

Skoog, F. and D. J. Armstrong. 1970. Cytokinins. Ann. Rev. Plant Physiol. 21:359-384.

Skoog, F., Strong, F. M. and C. O. Miller. 1965. Cytokinins. Science 148:532-533.

Sondheimer, E. and D. S. Tzou. 1971. The metabolism of hormones during seed germination and dormancy II. The metabolism of [8-^{14}]C zeatin in bean axes. Plant Physiol. 47:516-520.

Strong, F. M. 1958. Kinetin and kinins. In: Topics in Microbial Chemistry. J. Wiley and Sons, Inc., New York, pp 98-157.

Summons, R. E., B. Entsch, D. S. Letham, B. I. Gollnow and J. K. MacLeod. 1980. Regulators of cell division in plant tissues. XXVIII. Metabolites of zeatin in sweet-corn kernels: purification and identification using high-performance liquid chromatography and chemical-ionization mass spectrometry. Planta 147:422-434.

Taylor, J. S., M. Koshioka, R. P. Pharis and G. B. Sweet. 1984. Changes in cytokinins and gibberellin-like substances in Pinus radiata buds during lateral shoot initiation and the characterization of ribosyl zeatin and a novel ribosyl zeatin glycoside. Plant Physiol. 74:626-631.

Walbot, V., M. Clutter and I. M. Sussex. 1972. Reproductive development and embryogency in Phaseolus. Phytomorphology 22:59-68.

Wang, T. L. and R. Horgan. 1978. Dihydrozeatin riboside, a minor cytokinin from the levaes of Phaseolus vulgaris. Planta 140:151-153.

Watanabe, N., T. Yokota and N. Takahashi. 1981. Variations in the levels of cis and trans-ribosylzeatins and other minor cytokinins during development and growth of cones of the Hop plant. Plant & Cell Physiol. 22(3):489-500.

Wang, T. L., A. G. Thompson and R. Horgan. 1977. A cytokinin glucoside from the elaves of Phaseoulus vulgaris L. Planta 135:285-288.

Whitty, C. O. and R. H. Hall. 1974. A cytokinin oxidase in Zea mays. Can. J. Biochem. 52:789-799.