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Cytokinin Metabolism in *Phaseolus* Embryos[1]

GENETIC DIFFERENCE AND THE OCCURRENCE OF NOVEL ZEATIN METABOLITES

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ABSTRACT

The metabolism of *trans*-[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 enzymic tests and GC-MS analyses. Five major metabolites were recovered from *P. vulgaris* embryo extracts: ribosylzeatin, ribosylzeatin 5′-monophosphate, an *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*-ribosyl derivatives 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*-glucosyl derivatives 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.

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 *versus* cytokinin-dependent growth) and responses to phenylurea-type cytokinins have been detected (17–20). 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[2] was examined in tissues of a number of *Phaseolus* species and genotypes. In this paper, we report the metabolism of [^{14}C]zeatin in immature embryos of *P. vulgaris* and *P. lunatus*.

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 and a 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-β-D-Glucopyranosylzeatin was a gift from Dr. R. Durley (Oregon State University). 6-Chloro[8-^{14}C]purine (24 mCi/mmol) was obtained from Amersham. [8-^{14}C]Zeatin (24 mCi/mmol) was synthesized from 6-chloro-[8-^{14}C]purine following procedures published elsewhere (9).

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 sterile condition. The embryos corresponded to late heart and early and mid cotyledonary stages at the respective length. [^{14}C]Zeatin (0.05 μCi, 0.002 μmol) dissolved in 250 μl 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 h. In addition, embryos 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 determination was made 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 2.5 parts (v/w) of cold 95% ethanol. Cell debris was removed by successive filtration through Whatman paper No. 1 and Millipore filters (0.45 μm). The ethanol extract was taken to dryness *in vacuo* at 35°C, redissolved in 4 ml of 50% (v/v) ethanol, and centrifuged at 23,500*g* for 20 min. The supernatant was condensed *in vacuo* to 100 μl with a speed vac concentrator (Savant). The sample was then analyzed directly by HPLC. A Beckman Model 110 dual pump HPLC system with a prepacked column of reversed-phase C_{18} (Ultrasphere ODS 5 μm, 4.6 × 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

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[2] Abbreviations: [^{14}C]zeatin, [8-^{14}C]zeatin; z, zeatin, *trans*-zeatin; rz, ribosylzeatin, 9-β-D-ribofuranosyl-*trans*-zeatin; Ade, adenine; Ado, adenosine; z-9-G, 9-β-D-glucopyranosyl-*trans*-zeatin; TEA, triethylamine; TBAP, tetrabutylammonium phosphate; EI, electron impact ionization; CI, chemical ionization; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine.

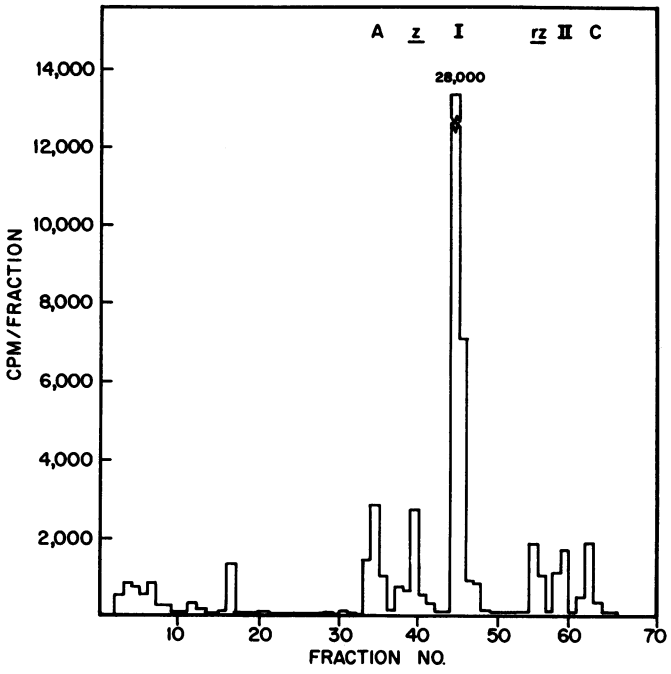

FIG. 1. HPLC profiles of [^{14}C]zeatin metabolites obtained from embryo extracts (3 mm, 4 h incubation) of *P. vulgaris* cv GN.

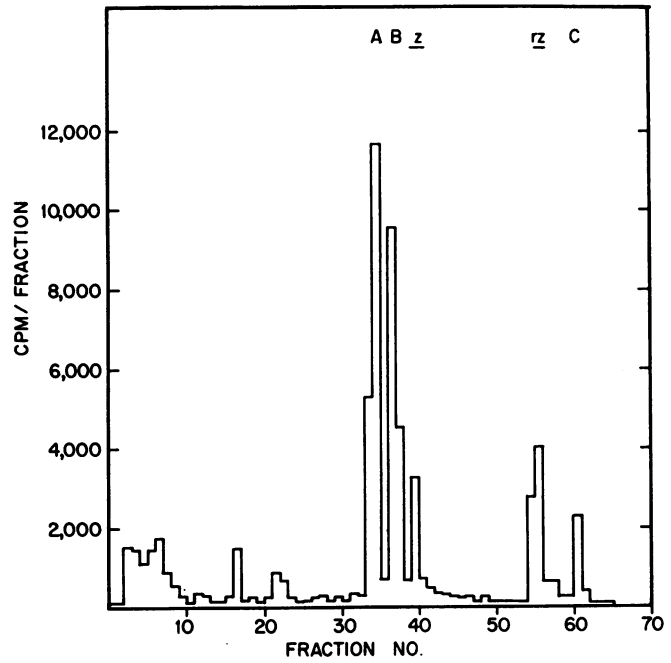

FIG. 2. HPLC profiles of [^{14}C]zeatin metabolites obtained from embryo extracts (3 mm, 4 h incubation) of *P. lunatus* cv K.

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 analyze nucleotides, paired-ion reversed-phase HPLC (as described previously [2]) 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 μl of 20% acetonitrile 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

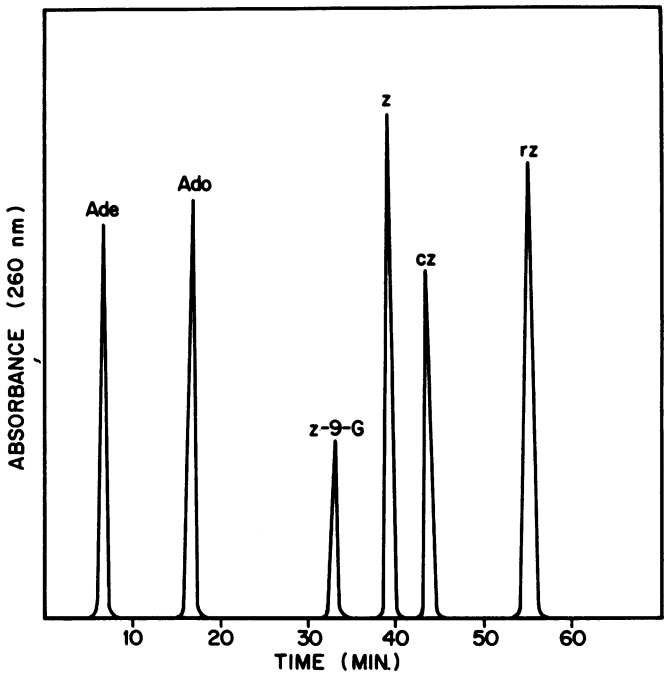

FIG. 3. HPLC profiles of standards.

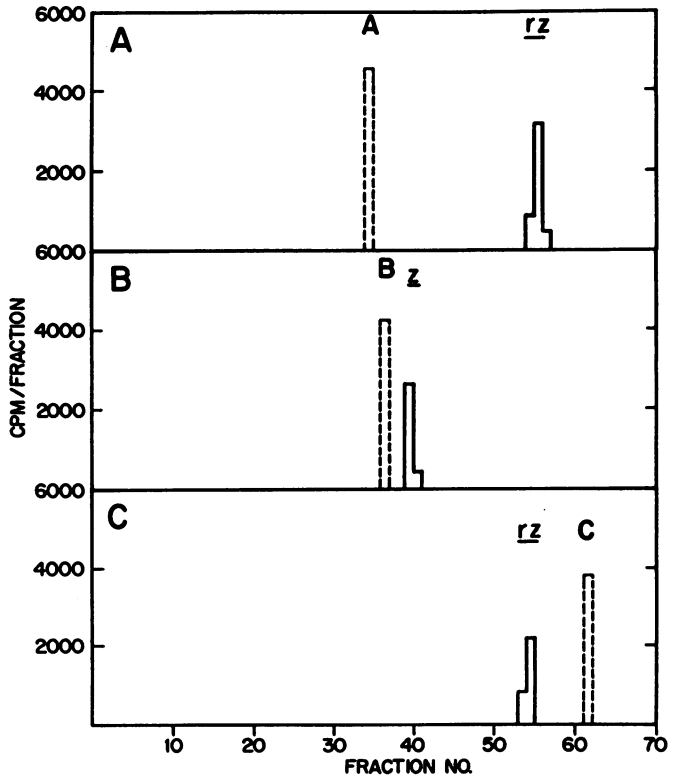

FIG. 4. Analyses by HPLC of metabolites A after treatment with 5′-nucleotidase (A), B after treatment with β-glucosidase (B), and C after treatment with β-glucosidase (C). Bars with discontinuous border indicate elution position of radioactivity before treatments.

metabolites, a reversed-phase C_{18} column was used but with a buffer solution of 0.2 M acetic acid adjusted to pH 4.8 with TEA. The metabolites were eluted by a methanol gradient of 5 to 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

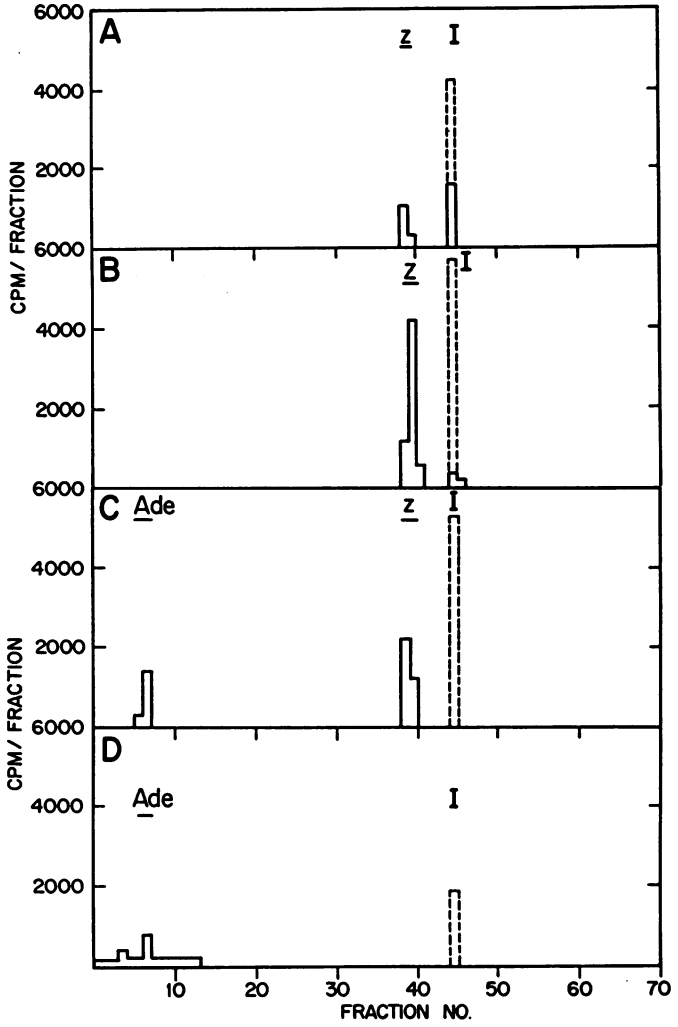

FIG. 5. Analyses by HPLC of metabolite I after treatments with β-glucosidase (A), TFA (B), periodate plus cyclohexamide (C), and permanganate (D). Bars with discontinous border indicate elution position of radioactivity before treatments.

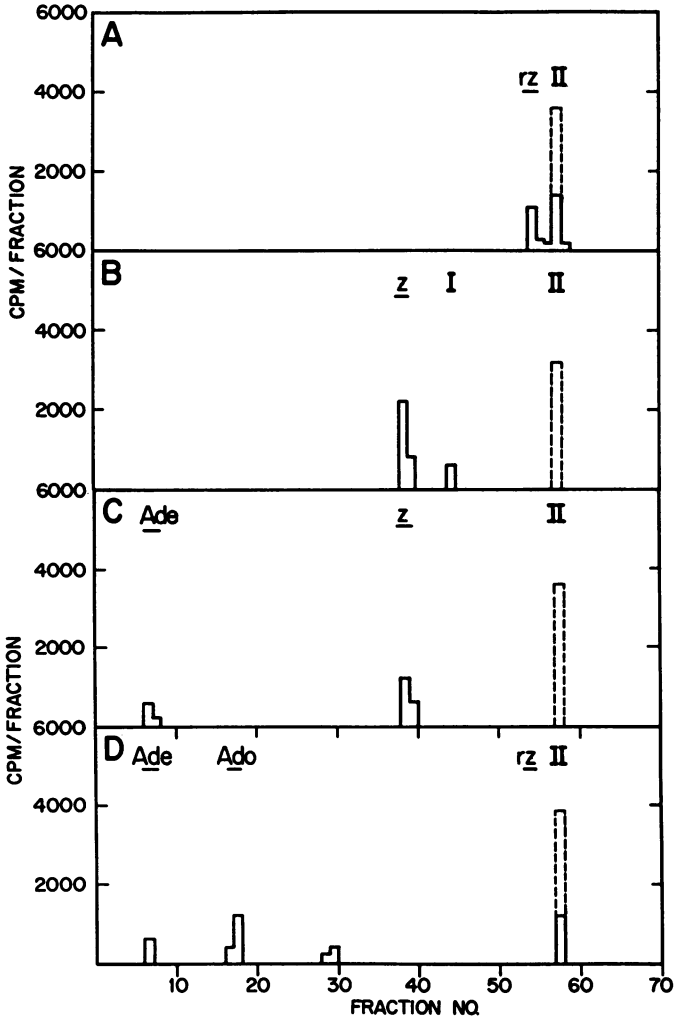

FIG. 6. Analyses by HPLC of metabolite II after treatments with β-glucosidase (A), TFA (B), periodate plus cyclohexamide (C), and permanganate (D). Bars with discontinous border indicate elution position of radioactivity before treatments.

uptake of [^{14}C]zeatin between GN and K embryos and the distribution of metabolites in embryonic tissues *versus* incubating solution, the following experiments were performed. [^{14}C]-Zeatin (0.1 μCi, 0.004 μmol) dissolved in 250 μl 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 μM of cold zeatin in 1.5 ml of H_2O. The wash solution was combined with the incubating solution, dried *in vacuo*, and analyzed 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 μl were incubated with 1 unit of 5'-nucleotidase or 3'-nucleotidase for 0.5 h at 37°C (1). 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 μl of 5% methanol, and fractionated by HPLC.

Treatment with β-Glucosidase. Fractions collected after HPLC analysis were dried and redissolved in 200 μl 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 μl of 5% methanol, and analyzed by HPLC.

Treatment with Periodate and Cyclohexamide. Fractions collected after HPLC analysis were dried and dissolved in 10 μl of periodate (10% w/v). After 3 h at room temperature, 2 μl of cyclohexamide was added (27). After 18 h, the solution was dried and the residue redissolved in 100 μl of 5% methanol and analyzed by HPLC.

Treatment with Permanganate. Fractions collected after HPLC analysis were dried and redissolved in 500 μl of H_2O, and 250 μl of 0.1 N $KMnO_4$ (w/v) was added (1, 7). 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 μl of 5% methanol and analyzed 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 (6, 11, 14) and centrifuged after addition of 1 ml H_2O. The supernatant was dried *in vacuo*,

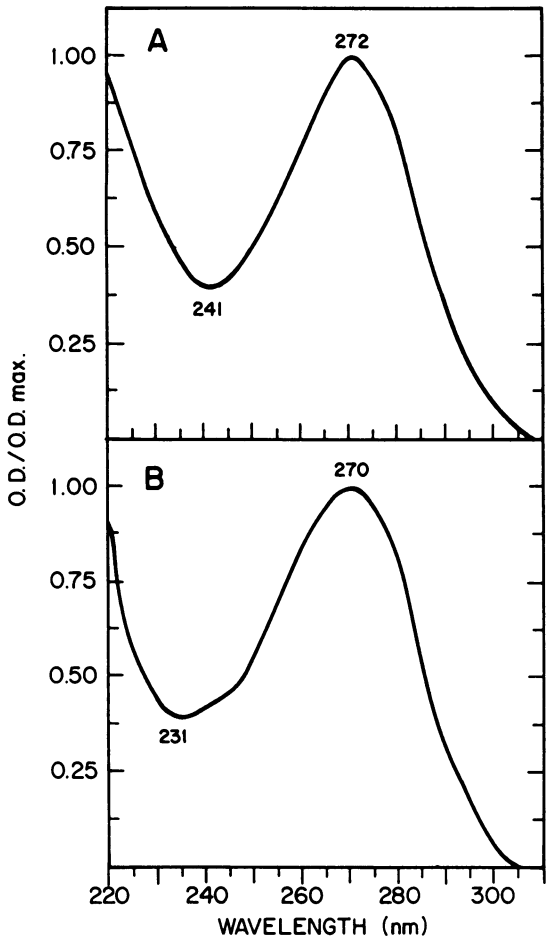

FIG. 7. UV spectra of purified metabolite I at pH 3.5 (A) and pH 8.5 (B).

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 sulfinyl 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 μl of anhydrous DMSO, followed by addition of 25 μl of methyl sulfinyl anion solution and, after 15 min, by 10 μl 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 of H_2O, dried, and redissolved in 5 μl of $CHCl_3$ prior to GC-MS analysis.

GC-MS Analysis. GC-MS analyses were performed using a Finnigan model 4023 GC-MS computer 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 0.75+ methane pressure in the source with 70 ev electrons, respectively. For GC, a glass column (250 × 2 mm i.d., 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 × 0.25 mm i.d.) 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).

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 incubation) are presented in Figures 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 coeluted with ribosylzeatin (Fig. 4A). Treatment with 3′-nucleotidase had no effect on metabolite A. These results suggest that A is a 5′-ribonucleotide 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 (2). The radioactivity co-eluted with AMP. Thus, metabolite A seems to be the 5′-monoribonucleotide 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 an *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 [6, 9, 14]) shifted the radioactivity to the position of zeatin. Therefore, metabolite C is most likely an *O*-glucoside of ribosylzeatin (3, 8). Similar results were obtained for metabolite C recovered from the two genotypes.

Metabolites I and II. Metabolites I and II were treated with β-glucosidase. About one-half of I was converted to zeatin (Fig. 5A), while one-half of the 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 a 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 acyclic derivative produced by cycloheylamine [27]). Finally, metabolites I and II were treated with $KMnO_4$ to remove the N^6sidechain (1, 7). Compound I was converted to Ade and other breakdown products (Fig. 5D) indicating that the structure modification resides on the N^6sidechain. The majority of metabolite II was converted to Ado and Ade (Fig. 6D). Based on the results of chemical and enzymic tests, it is reasonable to conclude that metabolites I and II are most likely *O*-glycosylated (but not *O*-glucosylated) derivatives of zeatin and ribosylzeatin, respectively.

To further test our interpretation, metabolite I was repurified 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 Figure 7. The absorption maxima were at 272 and 270 nm for the two conditions. The permethylated derivative of I was prepared and subjected to GC-MS analyses (Fig. 8). The mass spectrum obtained by CI (Fig. 8C)

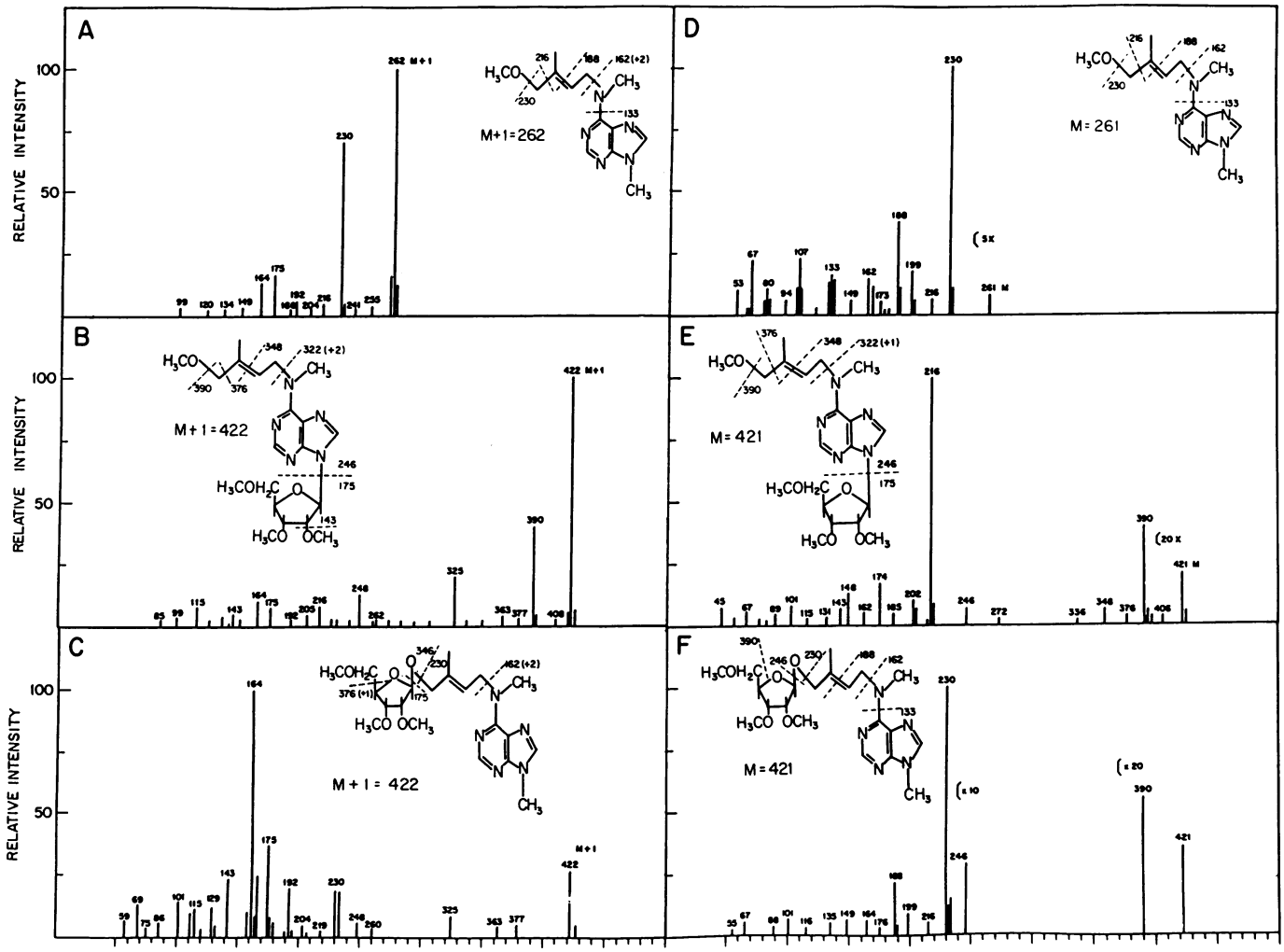

FIG. 8. Mass spectra of permethylated zeatin (A and D), ribosylzeatin (B and E), and metabolite I (C and F) obtained by CI (A, B, and C) and EI (D, E, and F) analyses.

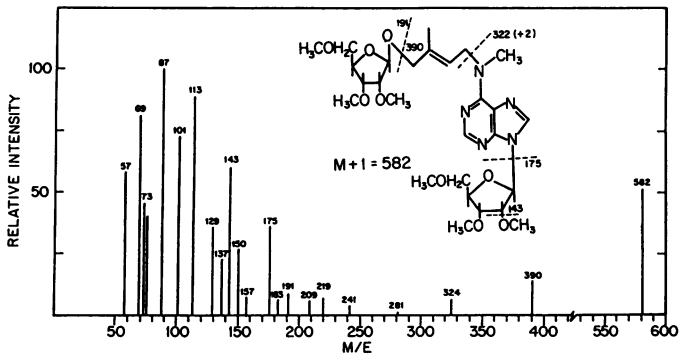

FIG. 9. Mass spectrum of permethylated metabolite II obtained by CI analysis.

indicates a mol wt of 421, identical to that of permethylated ribosylzeatin (Fig. 8B). The fragmentation pattern of lower mol wt ions was similar to that of zeatin (Fig. 8A). As mass spectral analysis by EI has the advantage of generating larger numbers of molecular fragments, permethylated zeatin, ribosylzeatin, and compound I derivatives were also subjected to this type of MS analysis (Fig. 8, D, E, and F). The spectrum obtained confirmed the mol wt of 421 for the permethylated metabolite I. The fragmentation pattern (Fig. 8F) was consistent with our interpretation that compound I could be an *O*-glycoside of zeatin. As the mol wt indicates the presence of a pentose sugar, and the higher mass ion fragmentation pattern is identical to that of ribosylzeatin, compound I is most likely an *O*-ribosylzeatin. However, other pentose sugars such as arabinose cannot be excluded at this time.

Since both metabolites I and II had the tendency of premature fragmentation under EI, much larger amounts of sample (approximately 2 μg) 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 mol wt of 581. The pattern of fragmentation was compatible with the structure of an *O*-riboside of ribosylzeatin. Nevertheless, direct synthesis of these compounds is needed to unequivocally confirm our interpretation of the structures.

Effects of Embryo Size on the Metabolism of [^{14}C]Zeatin. The proportion of radioactive metabolites (presented as per cent radioactivity at time 0) recovered from different sizes of embryos at various incubation times (2, 4, and 8 h) are presented in Figure 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

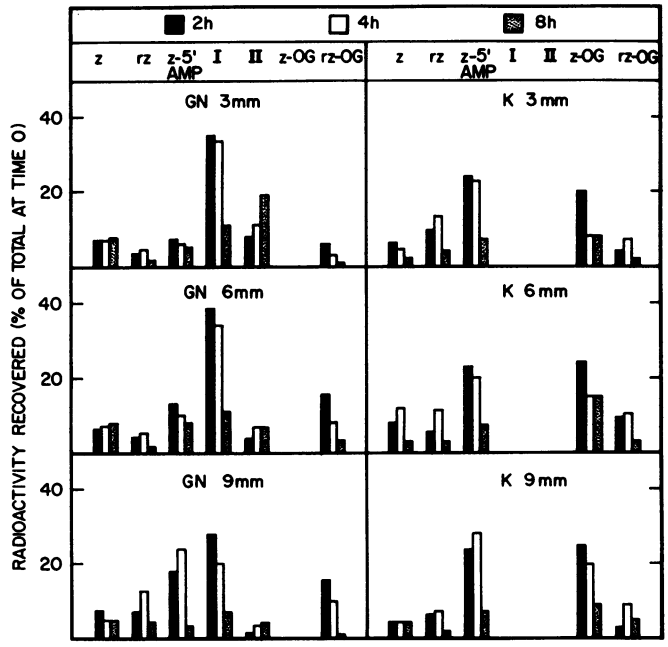

FIG. 10. Radioactive metabolites recovered from embryo extracts of *P. vulgaris* cv GN and *P. lunatus* cv K.

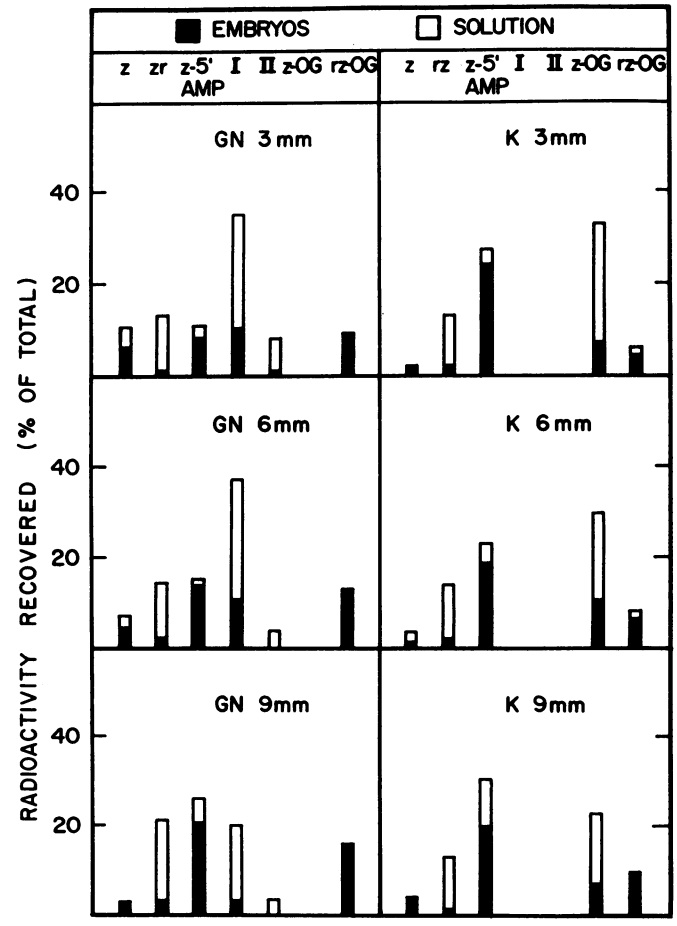

FIG. 11. Distribution of [^{14}C]zeatin metabolites between embryonic tissues and incubating solution.

from K embryos than GN embryos at all developmental stages. After 8 h, little radioactivity remained.

Distribution of Metabolites in Embryonic Tissues *versus* Incubating Solution. The distribution of metabolites in embryonic tissues *versus* incubating solution (per cent radioactivity recovered) is presented in Figure 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 predominantly found in the embryonic tissues, in contrast to *O*-glucosylzeatin which was present mainly in the solution.

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.

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, ribosylation of the N^6sidechain of zeatin has not been previously reported (12). Since earlier studies of zeatin metabolism in *P. vulgaris* axes, seeds, leaves, and roots (23, 28, 31) yielded primarily zeatin glucosides, nucleotides, and dihydrozeatin derivatives, the occurrence of metabolites I and II appears to be restricted 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 we have examined two additional genotypes each of *P. vulgaris* and *P. lunatus* and the results obtained were similar to those reported in this paper 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, it is possible 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 (30). 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 [4, 5, 8, 24], lupinic acid [11, 21, 25], glucosylribosylzeatin [29], nucleoside and nucleotides [10, 28]) are not well established. Glucosides have been suggested to be either storage forms or to be related to cytokinin transport (5, 12). Protection against cytokinin oxidase (32) attack via *O*-glucosylation and ribonucleotide formation have also been postulated (13). 9-Ribosylzeatin could be an intermediate in nucleotide formation or produced by breakdown of nucleic acids. The occurrence of *O*-ribosylation of zeatin, however, would seem to suggest additional roles of ribosylation of cytokinin bases.

We have previously examined the development of interspecific hybrid embryos of *Phaseolus* in some detail (15, 26). The arrest of embryo growth is dependent on the species combination and

the direction of the cross. For example, *P. vulgaris* × *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 (16). *P. vulgaris* × *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 cytokinins as compared with selfed embryos of both species (22). 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 some extent to the abnormal growth of the interspecific hybrid embryos. The qualitative difference in zeatin metabolism between *P. vulgaris* and *P. lunatus* embryos reported here may provide 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 metbolism. By utilizing cell culture systems, we have discovered differential structure-activity relationship between *P. vulgaris* and *P. lunatus* (17). The substantially lower activity of cytokinins with an unsaturated N^6sidechain (zeatin, i^{6}Ade) in the *P. vulgaris* callus bioassay was found to be related to a rapid degradation of the N^6sidechain of i^{6}Ado (20). Another genetic variation detected in cell cultures was cytokinin autonomous growth of *P. vulgaris* tissues which was controlled by a major locus (19). 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.

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