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Metabolism of ^{14}C-Zeatin in *Phaseolus* Embryos[1]

OCCURRENCE OF *O*-XYLOSYLDIHYDROZEATIN AND ITS RIBONUCLEOSIDE

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DAVID W. S. MOK* AND MACHTELD C. MOK
Department of Horticulture, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

The metabolism of *trans*-[8-^{14}C]zeatin was examined in embryos of *Phaseolus acutifolius* A. Gray P.I. 321637 and *Phaseolus coccineus* Lam. cvs Scarlet Runner and Desiree. In both species zeatin was converted to ribosylzeatin, ribosylzeatin 5′-monophosphate, *O*-glucosyl-9-ribosylzeatin and the recently discovered *O*-xylosyl derivatives of zeatin and ribosylzeatin (Turner, JE, DWS Mok, MC Mok, G Shaw 1987 Proc Natl Acad Sci USA. In press). Two new metabolites, identified by enzyme degradation and gas chromatography-mass spectography analyses as *O*-xylosyldihydrozeatin and its ribonucleoside, were recovered from *P. coccineus* embryos. From this and previous studies it may be concluded that the potential to form *O*-xylosyl derivatives of zeatin is present only in embryos of three *Phaseolus* species (*P. vulgaris* L., *P. coccineus*, and *P. acutifolius*), but not in *P. lunatus* L., while the reduction of the side chain is most prominent in *P. coccineus*.

The genetic mechanisms regulating the biosynthesis and metabolism of cytokinins and other plant hormones are not well understood. We are interested in the identification and characterization of genetic differences in cytokinin metabolism using plant parts as well as tissue cultures of *Phaseolus*. Previously, we have described the genetic and developmental variations in the metabolism of ^{14}C-zeatin in embryos of *Phaseolus vulgaris* and *Phaseolus lunatus* (3). The most interesting finding was the occurrence of two new metabolites, designated as metabolites I and II, in embryos of *P. vulgaris*. These metabolites were identified as *O*-pentose derivatives of zeatin and ribosylzeatin, respectively (3). To further characterize these metabolites, we have isolated and partially purified the enzyme responsible for the formation of metabolite I (14). The enzyme mediated the conversion of zeatin to metabolite I only in the presence of UDPxylose. Both *trans*-zeatin and dihydrozeatin could serve as substrates, but *cis*-zeatin and ribosylzeatin were not recognized by the enzyme. The product of zeatin after enzymatic reaction *in vitro* in the presence of UDPxylose had identical chromatographic properties and GC-MS[2] profiles as metabolite I formed *in vivo*. Furthermore, by chemically synthesizing O-β-D-xylopyranosylzeatin (11) and comparing its chromatographic properties and its mass spectrum with that of metabolite I, we have obtained additional evidence that the novel zeatin metabolites are *O*-xylosylzeatin and its ribonucleoside.

P. lunatus embryos, however, did not convert zeatin to the *O*-xylosyl derivatives, but contained the *O*-glucosides of zeatin and ribosylzeatin (3). These results indicate that *Phaseolus* species differ pronouncedly in the metabolism of ^{14}C-zeatin, especially the type of *O*-glycosylation. It is of interest to determine the metabolism of zeatin in other *Phaseolus* species and in particular the occurrence of the xylosyl derivatives. We report here the results of studies concerning ^{14}C-zeatin metabolism in embryos of *Phaseolus coccineus* and *Phaseolus acutifolius*, which have led to the identification of two other novel metabolites, the *O*-xylosyl derivatives of dihydrozeatin and dihydroribosylzeatin.

MATERIALS AND METHODS

Plant Material. Seeds of *Phaseolus coccineus* Lam. cvs Scarlet Runner (SR) and Desiree (Des), and *Phaseolus acutifolius* A Gray P.I. 321637 (Ac2) were obtained from Northrup King, Asgrow Seed Co. and the Plant Introduction Station (Pullman, WA), respectively. Plants were grown in the greenhouse at a photoperiod of about 14 h. Immature embryos were dissected from pods. Three sizes (9, 12, and 15 mm) of *Phaseolus coccineus* embryos and one size (3 mm) of *P. acutifolius* embryos were used. The size classes represent developmental stages at which active cytokinin metabolism takes place as determined by preexperiments. It should be noted that *P. coccineus* embryos are substantially larger than those of *P. acutifolius* at the same developmental stage.

Chemicals. [8-^{14}C]Zeatin (25 μCi/μmol) was synthesized as reported earlier (2). Zeatin, dihydrozeatin, and β-D-xylosidase were purchased from Sigma. *O*-Xylosylzeatin was synthesized using recently devised methodology (11).

Metabolism of [^{14}C]Zeatin. [^{14}C]Zeatin (0.025 μCi, 1 nmol), dissolved in 250 μl H_2O, was applied to 250 μg of immature embryos. The vials were sealed and kept in the dark at 27°C for 2, 4, and 8 h. To extract metabolites, the embryos were homogenized in 2.5 parts (v/w) of cold 95% ethanol. The details of the extraction procedures and separation of metabolites by HPLC on reversed-phase C_{18} have been described earlier (3). Radioactivity in each fraction was determined using a Beckman LS7000 scintillation counter.

Identification of [^{14}C]Zeatin Metabolites. HPLC fractions presumably containing *O*-xylosyl derivatives were dried and redissolved in 200 μl of 0.03 M acetate buffer (pH 5.3). After addition of 0.1 unit of β-D-xylosidase, the solution was incubated at 37°C for 30 min. The reaction was stopped by the addition of ethanol (95%, 1.5 ml). After centrifugation at 23,500*g* for 20 min the supernatant was taken to dryness *in vacuo* and analyzed by HPLC (buffered at pH 3.5 [3]). A second HPLC protocol using

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[2] Abbreviations: GS-MS, gas chromatography-mass spectrometry; Ade, adenine; Ado, adenosine; Ino, inosine; Z, zeatin, *trans*-zeatin; DZ, dihydrozeatin; DRZ, dihydroribosylzeatin; RZ, ribosylzeatin, 9-β-D-ribofuranosyl-*trans*-zeatin; ZMP, ribosylzeatin 5′-monophosphate; OXZ, *O*-xylosylzeatin; OXRZ, *O*-xylosyl-9-ribosylzeatin; OXZMP, *O*-xylosyl-9-ribosylzeatin 5′-monophosphate; 9GZ, 9-β-D-glucopyranosyl-*trans*-zeatin; OGRZ, an *O*-glucoside of ribosylzeatin.

a TEA buffer of pH 4.8 served for further confirmation of the identity of the products, since zeatin and dihydrozeation can be clearly separated at the higher pH (4 min apart). In addition, the products were analyzed by TLC using 250 μm silica gel plates (Baker) and chloroform:methanol (9:1) as the mobile phase (4).

GC-MS Analyses of Metabolites. Purified samples of metabolites III and IV were permethylated and subjected to GC-MS analyses by chemical ionization. The procedures and conditions were identical to those used for the identification of metabolites I and II (3).

RESULTS AND DISCUSSION

Ethanol extracts of embryos of Ac2, SR, and Des incubated with ^{14}C-zeatin were fractionated by HPLC (Fig. 1). The elution profile of standards is presented in Figure 1A. The metabolites in Ac2 embryos after 4 h incubation (Fig. 1B) co-eluted with ribosylzeatin, ribosylzeatin monophosphate, *O*-xylosylzeatin, *O*-xylosyl-9-ribosylzeatin, and an *O*-glucoside of ribosylzeatin. These compounds were further identified by chemical and enzymic means as described earlier (3). Thus, the zeatin metabolism

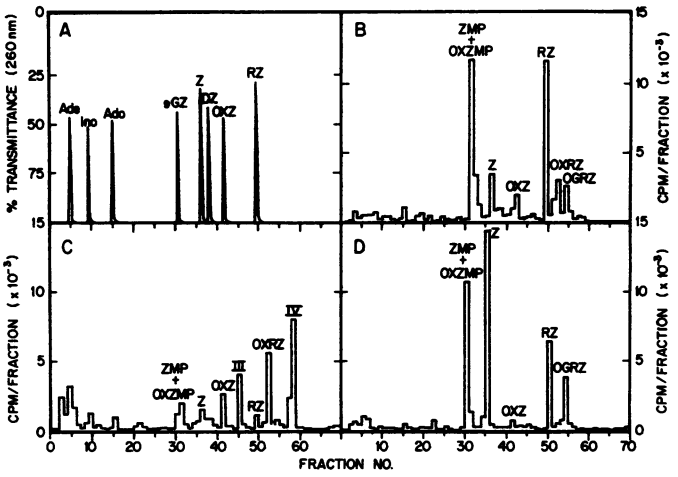

FIG. 1. Analysis of HPLC on reversed-phase C_{18} of ^{14}C-zeatin metabolites in extracts of *P. acutifolius and P. coccineus* embryos incubated for 4 h. A, Standards; B, *P. acutifolius* P.I. 321637; C, *P. coccineus* cv Scarlet Runner; D, *P. coccineus* cv Desiree. Standards and samples were eluted using a linear gradient of methanol (5–40% over 70 min) in TEA buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected and radioactivity was determined.

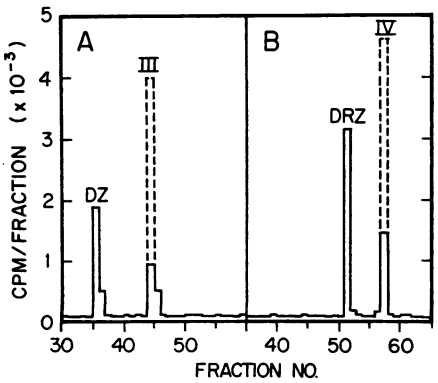

FIG. 2. Analysis of HPLC on reversed-phase C_{18} of unknown metabolites before (---) and after (——) treatment (30 min) with β-D-xylosidase. A, Metabolite III; B, metabolite IV. Samples were eluted using a linear gradient of methanol (5–40% over 70 min) in TEA buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected and radioactivity was determined.

in this *P. acutifolius* genotype was similar to that of *P. vulgaris* cv Great Northern (3). The same metabolites were isolated from embryos of SR (Fig. 1C). However, two additional metabolites were found in embryo extracts of this *P. coccineus* cultivar. These metabolites were designated as metabolites III and IV. Embryos of Des (Fig. 1D) contained only very small amounts of *O*-xylosylzeatin, while metabolites III and IV could not be detected. Although the quantities of metabolites after 2 and 8 h incubation differed from the 4 h time point, the same metabolites were recovered at these sampling times. The amounts of metabolites III and IV in SR embryos were highest after 4 and 8 h incubation, respectively.

Metabolites III and IV were identified by treatment with β-D-xylosidase followed by fractionation of the products by HPLC (Fig. 2). Treatment of metabolite III for 30 min resulted in conversion to a metabolite co-eluting with dihydrozeatin (Fig. 2A), while treatment of metabolite IV yielded a compound with similar chromatographic properties as dihydroribosylzeatin (Fig. 2B). (A longer incubation time [1 h] with β-xylosidase led to complete digestion of metabolites III and IV.) The two products also co-eluted with dihydrozeatin and dihydroribosylzeatin when the pH of the buffer was increased to 4.8. Both systems clearly separate *trans*-zeatin, *cis*-zeatin, and dihydrozeatin. In addition, the products co-chromatographed with dihydrozeatin and dihydroribosylzeatin on TLC plates.

Additional evidence concerning the identity of metabolites III and IV was provided by mass spectral analysis (positive chemical ionization). The mass ion of metabolite III (Fig. 3) was 424 which is the expected mass (+1) of a permethylated pentose derivative of dihydrozeatin. The fragment of 232 also clearly indicated the presence of dihydrozeatin. Metabolite IV had a mass ion of 584 (the expected mass [+1] of a permethylated pentose derivative of dihydroribosylzeatin) and also a prominent fragment of 232.

Third, the chromatographic properties of metabolite III were compared with those of the product obtained from incubation of dihydrozeatin (and UDPxylose) with the purified *O*-xylosyltransferase isolated from *Phaseolus vulgaris* embryos (14). In both HPLC systems (pH 3.5 and 4.8) the two compounds eluted off at identical positions. The three lines of evidence together provide strong evidence that compounds III and IV are xylosyl derivatives of dihydrozeatin and its ribonucleoside.

In addition to the genetic differences, rapid changes in the zeatin metabolism were observed during embryo development. The types and quantities of the metabolites in 9, 12, and 15 mm SR embryos are presented in Table I. While at the earlier stages large amounts of the xylosyl derivatives were found, in the 15 mm embryos only traces of these compounds could be detected. At this stage relatively large amounts of ribosylzeatin, its nucleotide, and an *O*-glucosyl derivative were present. These results indicate that the expression of the enzymes involved in cytokinin metabolism undergoes marked changes during embryo development. The metabolic transitions may occur rather swiftly, since embryo growth is rapid during this period of cotyledonary enlargement.

Thus far we have examined the ^{14}C-zeatin metabolism in four major species of *Phaseolus*. The metabolites identified in these species are listed in Table II. It is recognized that the inability to detect particular compounds in embryos of some of the genotypes does not necessarily indicate that these compounds are completely absent. Since it is apparent that the metabolic patterns are highly dependent on the developmental stage, traces of the missing compounds may be formed only at a specific stage of development. Nevertheless, careful examinations of *P. lunatus* embryos at a broad range of developmental stages failed to detect the presence of *O*-xylose compounds in this species. In addition, we were able to partially purify the enzyme (UDPxylose:zeatin-xylosyltransferase) catalyzing the formation of *O*-xylosylzeatin

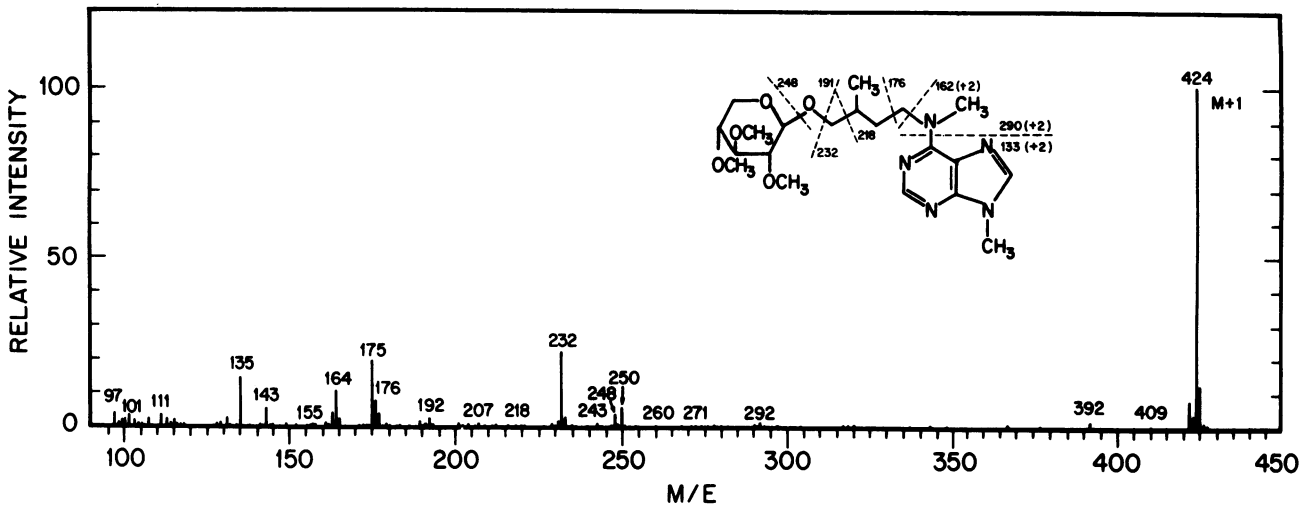

FIG. 3. Mass spectrum of permethylated metabolite III (chemical ionization).

Table I. *Major Metabolites of* ^{14}*C-Zeatin Recovered from P. coccineus cv Scarlet Runner Embryos of Three Size Classes after 4 h Incubation*

Embryo Size	Radioactivity Recovered							
	ZMP	Z	OXZ	OZDZ	RZ	OXRZ	OGRZ	OXDRZ
mm	*% of total*							
9	8	4	6	8	2	11	1	19
12	21	3	4	1	1	25	8	10
15	18	11	1	1	15	1	20	2

Table II. *Major Metabolites of* ^{14}*C-Zeatin Recovered from Embryos of Phaseolus Species*

Genotype	OXZ	OXRZ	OXDZ	OXDRZ	OGZ	OGRZ	RZ	ZMP
P. vulgaris								
cv GN	+	+				+	+	+
cv G50	+	+				+	+	+
P. lunatus								
cv K					+	+	+	+
cv JW					+	+	+	+
P. coccineus								
cv SR	+	+	+	+		+	+	+
cv Des	+	+				+	+	+
P. acutifolius								
P.I. 321637	+	+				+	+	+

from *P. vulgaris* embryos (14), but could not detect similar enzyme activity in extracts of *P. lunatus* embryos using the same isolation procedures. Therefore, the qualitative difference in the array of metabolites between *P. vulgaris* and *P. lunatus* seems to reflect a genetic difference at least at the enzyme level. Future studies of gene expression may provide insight in the regulation of cytokinin glycosylation enzymes in these two species.

The biological significance of the *O*-xylose derivatives of zeatin is uncertain at this time. The structural resemblance to *O*-glucose derivatives suggests that the two types of compounds may play similar roles. One of the roles suggested for *O*-glucosylated cytokinins is protection against cytokinin-degrading enzymes, since they seem to be resistant to cytokinin oxidases (6). Callus tissues of *P. vulgaris* have been shown to contain high level of cytokinin oxidase which degrades cytokinins with unsaturated N^6-side chains (5, 7, 9). Thus, the rapid conversion of zeatin to *O*-xylosylzeatin in embryos of *P. vulgaris* could be considered as a mechanism of protection against degradation. It should be noted, however, that the level of degradative enzymes in *P. vulgaris* embryos has not been determined. Glucosyl derivatives could possibly also serve as storage forms of active cytokinins, as supported by the large fluctuations in their levels during plant development and the ability of tissues to readily convert glucosides to aglycones (5, 7, 9). We have not yet examined the levels of *O*-xylosyl derivatives in different tissues, but have recently determined the biological activity and metabolism of *O*-xylosylzeatin in *Phaseolus* callus bioassays (17). *O*-Xylosylzeatin displayed high cytokinin activity, even in tissues which showed little response to zeatin. Moreover, while the compound was quite stable in short-term metabolism experiments using the same tissues, and the minor metabolites formed were identical to those produced by incubating tissues with ^{14}C-zeatin. These results indicate that the activity of *O*-xylosylzeatin may not be solely due to its conversion to zeatin.

The occurrence of *O*-xylosyldihydrozeatin in SR confirms the earlier reports that dihydrozeatin and derivatives are formed in *Phaseolus* tissues (12, 13, 15). As cytokinins with saturated side chains do not serve as substrates for cytokinin oxidase (6, 16), reduction of the side chain represents an effective mechanism of protection against degradation. This process appears to be prominent during early stages of embryo development and may be genotype as well as species specific. Further studies of side chain reduction, using *in vitro* assays, are in progress.

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