Concentration Gradients of *trans*-Zeatin Riboside and *trans*-Zeatin in the Maize Stem

MEASUREMENT BY A SPECIFIC ENZYME IMMUNOASSAY

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ABSTRACT

A sensitive, specific enzyme immunoassay (EIA) for *trans*-zeatin riboside (ZR) and *trans*-zeatin (Z) in the 0.3 to 30 picomole range has been described. The reliability of the method for measuring ZR + Z in partially purified extracts of *Zea mays* L. tissues was verified by high-performance liquid chromatography. EIA measurements showed that there was a concentration gradient of ZR + Z along the length of the *Zea* stem. The topmost internodes, internodes 7 and 8 counting from the coleoptilar node, had the highest concentration ($\simeq$130 picomoles per gram fresh weight). Moving basipetally, the concentration dropped $\simeq$10-fold to a minimum at internode 4, and then increased slightly in internodes 2 and 3. There were also gradients within each internode. The five lowest internodes contained the highest concentrations toward their apical end, the region which included the node; this asymmetry was less pronounced near the top of the plant.

The cytokinins Z,[1] DHZ, and the corresponding ribosides and glucosides have been found in immature maize kernels (7, 9, 15). Although there is evidence that the most abundant of these cytokinins, Z and ZR, are produced in the root tip (4, 14), little is known about the distribution of cytokinins in maize tissues at different stages of development. In this report, we describe a sensitive, specific EIA for ZR + Z and use the assay to measure cytokinin concentrations along the stem of individual maize plants.

The principle underlying EIAs of the type we have developed is reviewed by Schuurs and Van Weemen (12). In brief, the method depends upon binding specific, anti-ZR antibody to a plastic surface and allowing the antibody to react sequentially with an unknown amount of ZR in a plant extract, and then with ZR coupled to alkaline phosphatase. Because the free and coupled ZR compete for the same sites, the amount of enzyme activity bound to the plastic surface is inversely related to the amount of ZR in the sample.

We will show that EIA provides a reliable assay for ZR + Z and that there is a striking concentration gradient of these cytokinins along the length of the stem and within individual internodes of the maize plant.

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[1] Abbreviations: Z, *trans*-zeatin; DHZ, DL-dihydrozeatin; ZR, *trans*-zeatin riboside; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; IgG, immunoglobulin G; CZ, *cis*-zeatin; CZR, *cis*-zeatin riboside; KIN, kinetin; IPA, isopentenyladenosine; IP, isopentenyladenine; RIA, radioimmunoassay.

MATERIALS AND METHODS

Chemicals. *Trans*-zeatin riboside, DL-dihydrozeatin and *cis*-zeatin riboside were purchased from Calbiochem-Behring; kinetin, *trans*-zeatin, isopentenyladenine, isopentenyladenosine, N^6-benzylaminopurine, adenine, adenosine, guanosine, BSA ("essentially globulin free" grade), and *p*-nitrophenylphosphate ("Sigma 104" grade) were purchased from Sigma; alkaline phosphatase from calf intestine (Grade I, sp. activity >2,500 U mg^{-1}) was purchased from Boehringer; Freund's complete- and incomplete-adjuvant were purchased from Difco; protein A-sepharose CL-4B was purchased from Pharmacia; and silicone antifoam was purchased from Fluka. All other chemicals and reagents were of the highest purity available.

Plant Materials. *Zea mays* L. cv Seneca 60 seed was purchased from Stokes Seeds Ltd. (St. Catharines, Ont. Canada); seed of the inbred line A188 was kindly provided by Funk Seeds International (Bloomington, IL). Seneca 60 plants were grown from seed in clay pots of loam soil for 7 weeks in a greenhouse and then for 1 week in a controlled-environment chamber. After removal of roots, leaves, and leaf sheaths, plant stems were photocopied and the internodes (numbered consecutively beginning with the coleoptilar node) were cut into 2 to 4 pieces, weighed, and immediately used to prepare tissue extracts.

Immature kernels of field-grown A188 maize were obtained from ears harvested 12 to 18 d after pollination and stored at $-20°C$.

ZR Conjugates of BSA and Alkaline Phosphatase. ZR-BSA conjugate was prepared by a modification of the method described by Erlanger and Beiser (3) for coupling nucleosides to protein amino groups. Ten mg of ZR in 10 ml of halogen-free 0.1 M sodium citrate, pH 7.0, was incubated with 0.5 ml of 56 mM NaIO$_4$ in the dark under N$_2$ for 2 h at 4°C. Unreacted NaIO$_4$ was removed by adding 0.5 ml of 40% (v/v) ethylene glycol and incubating for an additional 15 min. A solution of 100 mg of BSA in 20 ml of 0.1 M Na$_2$CO$_3$ plus one drop silicone antifoam was added dropwise with stirring to the reaction mixture maintained at pH 9.5. The reaction was continued for 60 min in the dark under N$_2$ at 4°C. The resultant Schiffs base was reduced by adding 1.5 ml of 1 M NaBH$_4$ in 0.1 M Na$_2$CO$_3$ to the reactive mixture and incubating overnight in the dark at room temperature. The solution was adjusted to pH 7.0 with 1 N HCl and then to 8.5 with saturated Na$_2$CO$_3$, was dialyzed overnight at 4°C three times against 2 L of 1 M NaCl plus 0.1 M NaHCO$_3$ and three times against 10^{-3} M NaCl, and then was lyophilized. The lyophilized conjugate, which contained 8 mol ZR/mol BSA when assayed by absorbance at 268 nm, was stored at $-20°C$.

A ZR-alkaline phosphatase conjugate was prepared by essentially the same method except that 3 mg of protein were used and the volumes and other reagents were scaled down propor-

tionally. After dialysis, the solution of conjugate was stored at 4°C in the presence of 0.02% (w/v) NaN_3; 50% of the enzyme activity was lost during the conjugation procedure. The conjugate contained $\simeq 3$ mol ZR/mol alkaline phosphatase.

Anti-ZR Immunoglobulin. Antibody against ZR was elicited by immunizing 6- to 12-week-old, male, New-Zealand White rabbits with ZR-BSA conjugate. One mg of conjugate dissolved in 0.5 ml of PBS and emulsified with an equal volume of Freund's complete adjuvant was injected subcutaneously in divided doses at 20 different sites. Subsequent monthly injections were administered using Freund's incomplete adjuvant. Blood was collected from marginal ear veins 10 d after each injection and the serum from the 30-ml blood collections were stored at −20°C. IgG was purified by affinity chromatography on protein A-sepharose as described by Goding (5), lyophilized, and stored at −20°C. Each bleeding provided approximately 200 mg IgG, sufficient for $\simeq 100,000$ assays.

Extraction of Tissues. Partially purified cytokinin extracts were prepared from stem segments and immature kernels by a modification of the method of Carnes *et al.* (1). Depending upon amount, tissues in three volumes of ice-cold 95% (w/v) ethanol were homogenized at room temperature for three 1-min intervals at half-maximum speed with either a Polytron PTA 10S homogenizer (Kinematica, Luzern, Switzerland) or a Sorvall Omnimixer. The homogenate was filtered in a Büchner funnel, taken to dryness at 50°C in a rotatory evaporator, and the residue dissolved in water. After clarifying by centrifugation and adjusting to pH 9.0 with 1 N NaOH, the solution was extracted twice with equal volumes of *n*-butanol. The *n*-butanol phases were pooled, evaporated to dryness, and dissolved in PBS or water as indicated.

HPLC. The *n*-butanol fraction was purified further on a 200 × 46 mm column containing 10 μm particle-size Nucleosil C18 by a modification of the method of Horgan and Kramers (6). The column was eluted with a 25-min, concave-hyperbolic gradient of 10 to 40% (v/v) acetonitrile in 20 mM acetic acid adjusted to pH 5.6 with triethylamine. The gradient was generated using setting 9 of a model 660 solvent programmer (Waters Associates). The flow rate was 1.5 ml/min; fractions were collected at 1-min intervals, evaporated to dryness at 50°C in a rotatory evaporator, and dissolved in PBS.

Immunoassay of ZR. Assays of ZR were carried out in flat bottom, Immulon M129A Micro ELISA plates (Dynatech Laboratories, South Windham, ME) using a modification of the EIA for auxin described by Weiler *et al.* (17). The wells were filled with 200 μl of 10 μg/ml anti-ZR IgG in 50 mM $NaHCO_3$, pH 9.6, incubated overnight at 4°C with gentle shaking, and washed thoroughly with PBS. To saturate the remaining protein-binding sites, the wells were incubated an additional 30 min at room temperature with 250 μl of 0.1% (w/v) BSA in PBS. After rinsing with PBS, the coated wells were filled with 100 μl of PBS plus 50 μl of sample or ZR standard, and incubated for 30 min at 4°C. Fifty μl of a 3000-fold dilution of the ZR-alkaline phosphatase conjugate (approximately 20 ng of protein) was added to each well, the incubation was continued for an additional 30 min, and unbound conjugate was removed by rinsing repeatedly with PBS. Alkaline phosphatase activity was measured by incubating each well for 1 h at room temperature with 200 μl of a freshly prepared solution of 4.24 mM *p*-nitrophenylphosphate in 50 mM $NaHCO_3$, pH 9.6, and then measuring the absorbance at 405 nm in a Titertek Multiscan MC spectrophotometer (Flow Laboratories, Irvine, UK). When samples were not measured immediately, the reaction was stopped by adding 50 μl of 5 N KOH to each well.

As recommended by Rodbard (11), the per cent maximal binding, B/B_o, was corrected for nonspecific binding (*NS*)—usually less than 1% of the total binding—using the relationship:

$$B/B_o = \frac{A - A_{NS}}{A_o - A_{NS}} \times 100$$

where A is the absorbancy at 405 nm obtained with the sample, A_o is the absorbancy obtained with the complete reaction mixture in the absence of ligand (*e.g.*, ZR), and A_{NS} is the absorbancy obtained with the complete reaction mixture plus saturating amounts of ligand. A linear standard curve was obtained by plotting the logit transform of the binding values, *i.e.*

$$\text{Logit } B/B_o = \ln \frac{B/B_o}{100 - B/B_o}$$

versus the amount of ligand added to the reaction mixture (10). The slope and *y*-intercept of the standard curve were estimated by the least squares method and used to calculate the amount of ZR in unknown samples.

Measurement of ZR in Plant Extracts. Ethanol, *n*-butanol, and HPLC-purified fractions of plant tissues containing 1 to 6 pmol of ZR in 150 μl of PBS were assayed by the EIA method described above. Because the antibody cross reacts with Z, values obtained with extracts in which Z and ZR have not been separated are expressed as ZR + Z measured in ZR equivalents. Recovery of ZR after the extraction and purification procedures was estimated by adding a known amount of ZR to the tissue homogenate and comparing the ZR content of samples with and without internal standard. Because the recovery values obtained with upper and lower internodes were essentially the same, 80.1 and 80.4% respectively, the estimates of ZR content in stem segments are corrected for a recovery of 80%.

RESULTS

Sensitivity and Specificity of the EIA. Plots of B/B_o *versus* the natural logarithm of amount ZR gave a sigmoidal curve typical of competitive EIAs (11); with increasing amounts of ZR there was decreasing alkaline phosphatase activity (Fig. 1). There are several lines of evidence to indicate that enzyme activity reflects the specific binding of the ZR-alkaline phosphatase conjugate to anti-ZR antibody. First, all but 1 to 2% of the enzyme activity is lost when the assay is run in the presence of saturating amounts of ZR, *i.e.* 500 pmol/assay. Second, there was no appreciable enzyme activity when uncoupled alkaline phosphatase was used; binding of the enzyme depends on the covalent linkage of the enzyme to ZR. Finally, there was no appreciable enzyme activity using IgG from rabbits before immunization.

The data shown in Figure 1, when plotted as the logit transformation, gave a linear standard curve from 0.3 to 30 pmol ZR (Fig. 2). Thus, the effective range of the assay is over two orders of magnitude with a sensitivity of $\simeq 6$ nM ZR in a sample volume of 50 μl.

To measure the specificity of the anti-ZR antibody, we ran standard curves with various purines structurally related to ZR and calculated the amount of ligand needed to inhibit binding
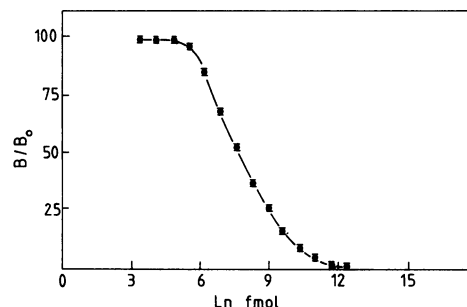

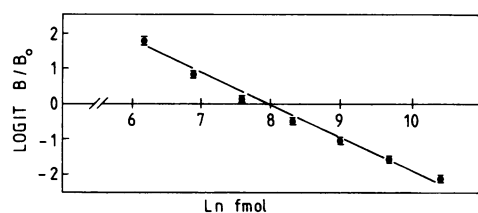Fig. 1. EIA dose response curve for ZR. Error bars: ±SE, 4 replicates.

FIG. 2. EIA standard curve for ZR. Logit transformed data from Figure 1. (——), Least squares fit of the data points; error bars, ±SE, 4 replicates.

Table I. Cross-Reactivity of Anti-ZR Antibody

Compounds	Amount Needed for 50% Inhibition	Cross-Reactivity
	pmol	%
trans-Zeatin riboside	2.3	100
trans-Zeatin	6.8	33.8
DL-Dihydrozeatin	107	2.14
Isopentenyladenosine	270	0.84
cis-Zeatin riboside	294	0.78
Isopentenyladenine	1,008	0.23
N^6-Benzyladenine	1,870	0.12
N^6-Furfuryladenine	2,430	0.09
Adenosine	>56,000	<0.004
Guanosine	>53,000	<0.004
Adenine	>110,000	<0.002

of ZR-alkaline phosphatase by 50%. The results summarized in Table I are expressed as per cent cross-reactivity, the 50% inhibition value for ZR divided by the 50% inhibition value for the ligand. The only appreciable cross-reactivity, 33.8%, was observed with Z. The low cross-reactivity observed with the other cytokinins tested shows that the antibody is extremely specific for the N^6-side chain of the purine moiety. Adenosine, adenine, and guanosine, which are abundant components of plant-tissue extracts, exhibited no detectable cross-reactivity.

Validation of the Assay. The results described in the preceding section show that EIA provides a sensitive, specific assay for ZR in samples of known chemical composition. To verify that the EIA can also be used to measure ZR + Z in extracts of plant materials, we prepared extracts from immature maize kernels known to contain these cytokinins (7, 9), purified the extracts by n-butanol-water partition, fractionated the cytokinins by HPLC, and assayed each fraction by EIA. Approximately 97% of the immunoreactive material applied to the column was recovered in two peaks, one with a retention time corresponding to ZR, the other with a retention time corresponding to Z (Fig. 3). By this criterion, essentially all of the material detected by EIA in the n-butanol-water purified extracts was either ZR or Z.

Because the EIA is a competitive assay, components in the tissue extract that inhibit alkaline phosphatase activity or the reaction between the antibody and ZR could give an overestimate of ZR content. To detect this type of interference, we compared the ZR contents of the tissue obtained by assaying extracts at different stages of purification (Table II). Using the total amount of immunoreactive material in the ZR + Z peaks from HPLC as a standard, crude ethanol extracts gave a 25% overestimate of ZR + Z. On the other hand, virtually all of the material detected in assays of the n-butanol fraction was accounted for by ZR + Z, indicating that the n-butanol-water partition step is sufficient to eliminate substances present in maize kernels that interfere with the EIA procedure.

Distribution of ZR + Z in the Maize Stem. We measured the distribution of cytokinins in different internodes along the length
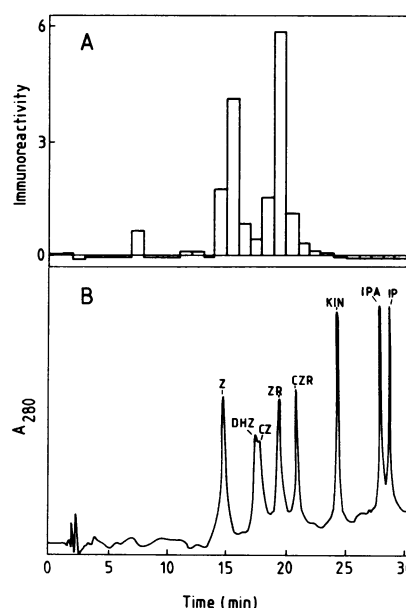

FIG. 3. Distribution of ZR immunoreactivity in HPLC fractions of extracts from immature maize kernels (A). Retention times of cytokinin standards (B). Absorbance expressed in arbitrary units, and immunoreactivity in pmol ZR equivalents.

Table II. Recovery and ZR + Z Content of Extracts of Immature Maize Kernels

Extracts assayed by EIA for ZR + Z content at the stage of purification indicated.

Stage of Purification	Recovery	ZR + Z Content
	%	nmol/ g fresh wt[a]
Crude ethanol extract	63.0	1.60
After n-butanol-water partition	56.4	1.22
After HPLC[b]	42.4	1.24

[a] Expressed in ZR equivalents and corrected for recovery.

[b] Immunoreactive material in peaks corresponding to the retention times for ZR and Z.

of 8-week-old plants. EIA was performed on tissue extracts purified by n-butanol-water partition after first verifying by HPLC that essentially all the immunoreactive material remaining after this treatment was either ZR or Z. Figure 4 shows the results obtained with six plants in different experiments. Expressed as ZR equivalents per g fresh weight, there was a striking concentration gradient of ZR + Z along the length of the plant. The highest concentrations, ≃130 pmol/g, were found in the topmost internodes (VII and VIII), which were the least expanded of the internodes assayed. Moving basipetally, the concentration dropped ≃10-fold to a minimum at internode IV, and then increased slightly in internodes II and III. Figure 4 also shows the relative concentrations of ZR + Z in the upper and lower halves of the internodes. The data are expressed as a ratio: the concentration in the upper half of the internode, including the next higher node, divided by the concentration in the lower half of the internode. Near the bottom of the plant (internodes II–V), the ZR + Z concentrations were from 1.6- to 2-fold higher in the upper half than in the lower half. This ratio decreased toward the top of the plant; and, in internodes VII and VIII the concentrations in the two halves were approximately equal. Thus, there is a polar distribution of cytokinin within each internode which varies with the position of the internode in the
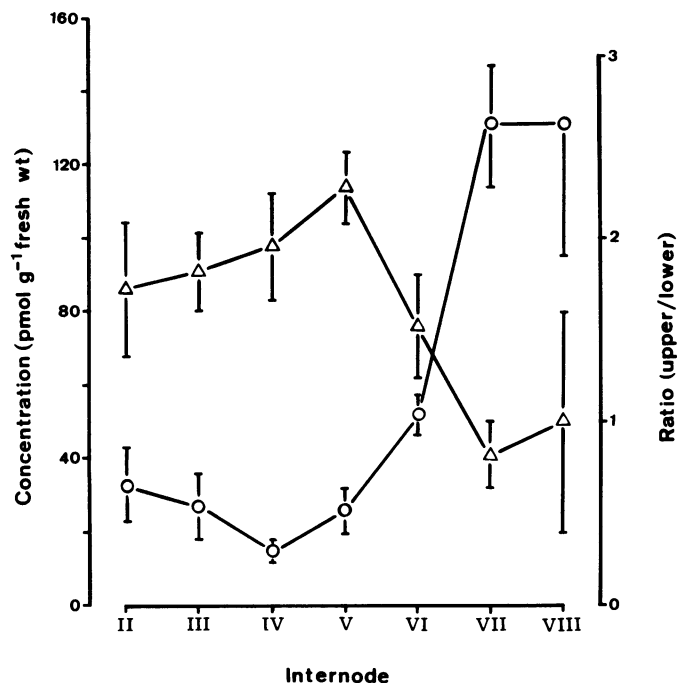
FIG. 4. Distribution of ZR + Z in stem segments from 8-week-old maize plants. The ZR + Z concentration in each internode (O——O). The ratio of ZR + Z concentrations in the upper and lower halves of the internodes (△———△). ZR + Z expressed as pmol ZR equivalents/g fresh weight; error bars, ±SE for 3 to 5 replicates and for 2 replicates of the ratio value obtained with internode VIII.
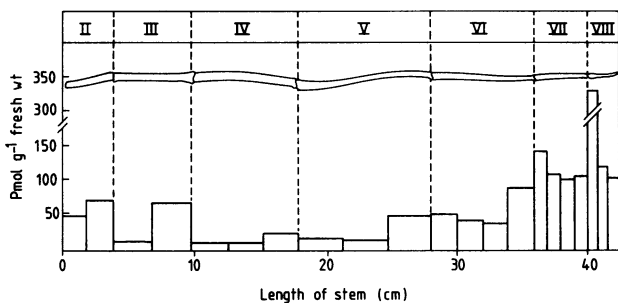

FIG. 5. Distribution of ZR + Z in stem segments from an 8-week-old maize plant. The positions of the tissues assayed are indicated on the tracing of the stem shown in the upper half of the figure. Internodes were divided from nodes just above the leaf insertion. ZR + Z content is expressed in ZR equivalents.

stem.

In our most detailed experiment, internodes II–VIII were cut into two to four segments of equal length, which were then extracted and assayed. Because internode length varied considerably among plants of comparable height and the same age, it was not possible to pool the results. The distributions obtained with a single, representative plant are shown in Figure 5. In every case where the concentration of ZR + Z was higher in the upper half than in the lower half of the internode, the increased concentration was due to high value in the uppermost segment assayed. In this particular plant, the polar distribution was inverted in internodes near the top of the plant, i.e. the ZR + Z content was higher near the base of the internodes. This result was obtained in two of the four plants assayed; in the remaining plants the upper to lower ratio was ≃ 1.0.

DISCUSSION

The EIA we have developed for ZR has a slightly lower sensitivity and roughly the same specificity as the RIAs for Z previously described (8, 16). The main advantages of our method are that it does not require the preparation of high-specific activity, radioactive cytokinins, and that it can be automated allowing the measurement of several hundred samples over a 3-h period.

The limiting step in immunoassaying cytokinins is the time required for sample preparation. Although there are substances in crude ethanol extracts of maize tissues that interfere with the EIA, reliable results were obtained with extracts purified by *n*-butanol-water partition. When it is necessary to distinguish the free base from the riboside, the immunoassay can be combined with HPLC as recommended by MacDonald *et al.* (8). This was not done in our study because samples from individual plants were too small for HPLC analysis. Internode length varied in the plants we assayed and it was not desirable to pool samples from different plants.

There appears to be a parallel between the distribution of ZR + Z and the anatomy of the maize stem. The stem consists of a stack of polar structures, each with leaves developing at the apical end and root initials at the basal end, arranged in a head-to-foot fashion along the axis of the plant (13). Each of the polar units has its own ZR + Z gradient, and each differs in its stage of development and cytokinin content. The fact that the ZR + Z concentration is high in immature regions of the stem near the top of the plant and is inversely correlated with internode length ($n = 18$ internodes, $p < 0.01$) suggests that actively expanding internodes contain the highest cytokinin concentrations.

Although it is generally believed that cytokinins are produced in the root system and transported into the aerial parts of the plant via the xylem (14), there is direct evidence that cytokinins are produced in shoots of tobacco (2). The high levels of ZR + Z in stem segments that include the node and in the stem near the top of the maize plant suggest that leaves and the shoot apex may be additional sites of cytokinin production. A direct test of this hypothesis will require measuring cytokinin synthesis in the different regions using labeled precursors.

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