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Radioimmunoassay for Quantifying the Cytokinins *cis*-Zeatin and *cis*-Zeatin Riboside and Its Application to Xylem Sap Samples

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Abstract. An antiserum against the cytokinin *cis*-zeatin riboside was raised in rabbits and characterized for use in radioimmunoassays. Cross-reactivity studies demonstrated the specificity of the selected antiserum for *cis*-zeatin riboside and *cis*-zeatin in preference to a range of cytokinins and other purines. HPLC systems were developed that separated *cis*-zeatin and *cis*-zeatin riboside from zeatin/dihydrozeatin and zeatin riboside/dihydrozeatin riboside, respectively. These systems enabled the separation of these compounds in xylem sap samples of wheat and oats and their quantification using radioimmunoassay. A TLC system for the separation of *cis*-zeatin and *cis*-zeatin riboside from zeatin/dihydrozeatin and zeatin riboside/dihydrozeatin riboside, respectively, is also described.

We have previously raised and characterized antisera in rabbits against zeatin riboside ([9R]Z) (Badenoch-Jones et al. 1984a) and isopentenyladenosine ([9R]iP) and dihydrozeatin riboside ((diH)[9R]Z) (Badenoch-Jones et al. 1987). When employed in radioimmunoassays (RIAs), these antisera enable the quantification of a large number of naturally occurring cytokinins (Badenoch-Jones et al. 1987). However, cross-reactivity of these antisera with *cis*-zeatin riboside (*cis*[9R]Z) and *cis*-zeatin (*cis*Z) is generally relatively low: 0.77% and 2.1%, respectively, for the anti-[9R]Z-serum; 1.1% and 0.69%, respectively, for the anti-[9R]iP-serum; and 6.9% and 14.2%, respectively, for the anti-(diH)[9R]Z-serum. In order to detect with high sensitivity and to quantify *cis*[9R]Z and *cis*Z in biological samples, we have raised and characterized an antiserum in rabbits against *cis*[9R]Z.

Previously, the *cis* isomers of [9R]Z and zeatin (Z) have been identified by mass spectral analysis, and *cis*[9R]Z has been isolated from hydrolysates of tRNA of higher plants and of microorganisms (see Hall 1973, Letham and Palni

1983). These cytokinins have also been reported to be produced by some plant-associated bacteria, as revealed by their presence in culture media: *cis*[9R]Z (McCloskey et al. 1980) and *cis*Z (Kaiss-Chapman and Morris, 1977) in *Agrobacterium tumefaciens,* and *cis*Z in *Corynebacterium fascians* (Scarbrough et al. 1973, Murai et al. 1980). In *A. tumefaciens,* McCloskey et al. (1980) estimated the concentration of *cis*[9R]Z in the culture medium to be nearly four times that of *trans*[9R]Z.

The *trans* isomers of [9R]Z and Z have been considered to be the predominant, if not the only, isomers of these cytokinins occurring free in higher plants. However, over the last decade, there have been a number of reports of the occurrence of the *cis* isomers (mainly *cis*[9R]Z) as free cytokinins in extracts of tissues of a number of higher plants: *cis*[9R]Z in cones of the hop plant (Watanabe et al. 1978, 1981), in tobacco shoots (Kimura et al. 1978, Hashizume et al. 1978), in tissues from *Solanum tuberosum* L. (Mauk and Langille 1978), in shoot apices of monoecious *Mercurialis ambigua* (Dauphin et al. 1979), in immature seeds of *Dolichos lablab* (Yokota et al. 1981), in tubers of sweet potato (Hashizume et al. 1982), and both *cis*[9R]Z and *cis*Z in shoots, roots, and ears of rice (Takagi et al. 1985) as well as in the root exudate of the rice plant (Murofushi et al. 1983).

In some instances, it was estimated that the *cis* isomer was present in quantitatively greater amounts than the *trans* isomer, for example for [9R]Z in unfertilized hop cones (Watanabe et al. 1981); for [9R]Z in shoot, root, and ear of rice; and for Z in shoot and root of rice (Takagi et al. 1985). Tay et al. (1986), however, have cast some doubt on the occurrence of *cis*[9R]Z in tobacco shoots, with the finding that *cis*[9R]Z was considerably reduced (and barely detectable) when samples were extracted by a method known to minimize enzymic degradation. They suggested that reports of the occurrence of the *cis* isomers as free cytokinins in extracts of higher plant tissues are the result of enzymic hydrolysis of tRNA during extraction.

RIA employing the anti-*cis*[9R]Z-serum was used in the present study to examine the possible presence of *cis*[9R]Z and *cis*Z in xylem sap samples of oat and wheat in order to obtain further information to help resolve this controversy.

Materials and Methods

Chemicals

The sources of most of the chemicals used in this study have been given by Badenoch-Jones et al. (1984a,b, 1987). *cis*Z was purified as described by Badenoch-Jones et al. (1987). [^{3}H]Sodium borohydride (sp. act. 2442 GBq mmol^{-1}) for the synthesis of the *cis*[9R]Z tracer was purchased from New England Nuclear, Boston, Massachusetts.

2,5-(2-Hydroxyethyl)-3,6-dichloro-1:4-benzoquinone (QD/2) and 2,5-(6-hydroxyhexyl)-3,6-dichloro-1:4-benzoquinone (QD/1) were prepared by refluxing (10 min) an ethanol solution of 2,3,5,6-tetrachloro-1:4-benzoquinone containing an excess of ethanolamine and 6-amino-1-hexanol, respectively. The

products separated on cooling the reaction solution and were recrystallized from ethanol-petroleum. Identity was confirmed by mass spectrometry. By varying the alkyl moiety of the hydroxy amine, a series of derivatives can be readily prepared with predictable R_f values.

Plant Material

Wheat seedlings (*Triticum aestivum* cv. Isis) were grown under greenhouse conditions. Oat seedlings (*Avena sativa* cv. Coolabah) were grown in a controlled environment cabinet (25°C, 12 h/day for 2 days, and 25°C, 16 h/day for subsequent days). Seedlings were watered well 6 h prior to decapitation. They were decapitated at 2–3 cm above ground level on day 2 (oat) or day 6 (oat and wheat) after germination. At 4–6 h after decapitation, xylem sap was collected as it exuded from the cut seedlings, using a Pasteur pipette. Sap was stored at −20°C prior to analysis. Estimates of cytokinins were made on single samples of xylem sap pooled from approximately 10,000, 5000, and 3000 seedlings for the 2-day-old oat, 6-day-old oat, and 6-day-old wheat, respectively.

Sample Purification

About 19,000 dpm of the recovery markers [^{3}H]dihydrozeatin ((diH)Z) (110 GBq mmol^{-1}) and [^{3}H](diH)[9R]Z (189 GBq mmol^{-1}) were added to the xylem sap samples prior to purification. The samples were purified by either the scheme shown in Fig. 1a or that shown in Fig. 1b. Cellulose phosphate ion exchange chromatography and TLC using butan-1-ol/acetic acid/water (12:3:5 (v/v/v)) were performed as described by Badenoch-Jones et al. (1984b). HPLC on the Zorbax C_8 column (9.4 × 250 mm) (Du Pont) was as described by Badenoch-Jones et al. (1984a), except that the solvent used was 40% (v/v) methanol in 0.2 M acetic acid at a flow rate of 4.5 ml min^{-1}.

HPLC on a new Zorbax C_8 column under these conditions gave adequate resolution of [9R]Z, *cis*[9R]Z, and (diH)[9R]Z as well as Z, *cis*Z, and (diH)Z (Fig. 1a). However, an aged Zorbax C_8 column did not achieve these separations (Fig. 1b), even when the concentration of methanol in the solvent was reduced or the TLC eluate was purified using a Baker SPE C_{18} minicolumn prior to HPLC on the Zorbax column. Therefore, the subsequent HPLC procedures varied depending on whether the requirement was for separating only *cis*[9R]Z from (diH)[9R]Z and *cis*Z from (diH)Z, separations that could both be achieved with an RCM 100 module with a CN cartridge (Fig. 1a), or for the separation of *cis*[9R]Z from both [9R]Z and (diH)[9R]Z and of *cis*Z from both Z and (diH)Z (Fig. 1b). In the latter situation, these separations were achieved using an RCM 100 module with a NOVA C_{18} cartridge and a Hamilton PRP1 column, respectively.

HPLC on the RCM 100 module with a CN cartridge (8 × 100 mm) (Waters Associates) was carried out with 45% (v/v) methanol in 0.2 M acetic acid as solvent at a flow rate of 3.5 ml min^{-1}. The retention times of authentic standards in this system were 4.01 and 4.83 min for *cis*[9R]Z and (diH)[9R]Z, re-

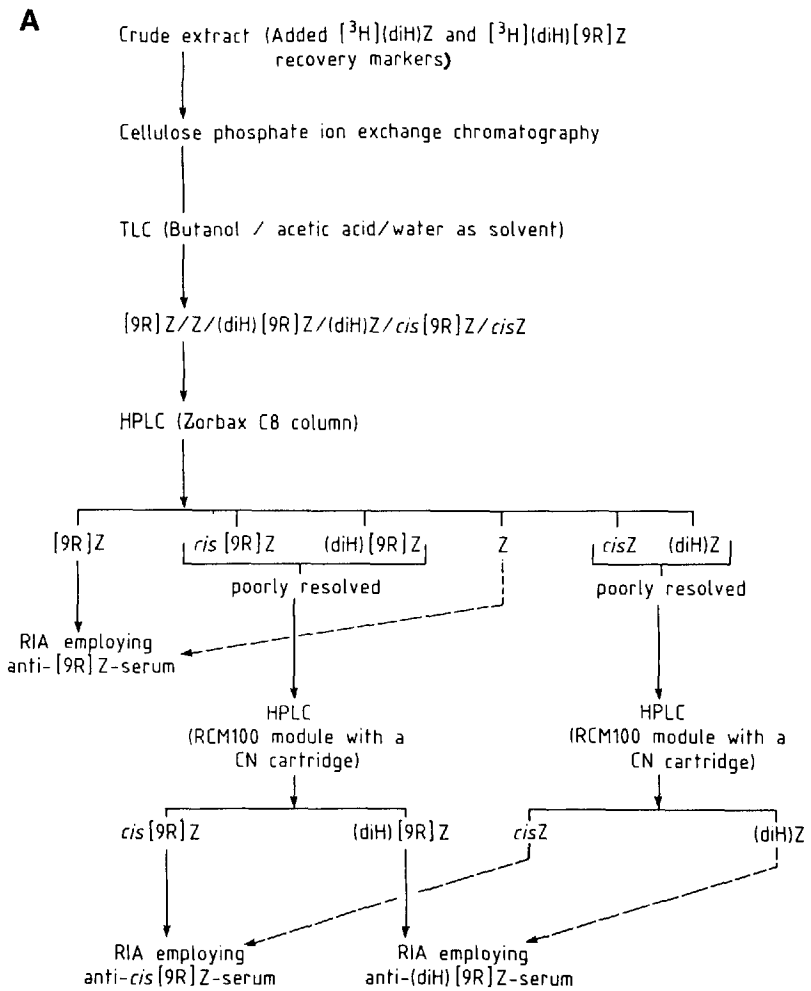

Fig. 1. (A) Scheme, using a new Zorbax C_8 column, for the separation of cytokinins in xylem sap samples of wheat, prior to RIA.

spectively, and 9.46 and 10.65 min for *cis*Z and (diH)Z, respectively. HPLC on the RCM 100 module with a NOVA C_{18} cartridge (8 × 100 mm) (Waters Associates) was carried out with 22% (v/v) methanol in 0.2 M acetic acid as solvent at a flow rate of 3 ml min^{-1}. The retention times of authentic standards in this system were 5.06 min for [9R]Z/(diH)[9R]Z and 6.56 min for *cis*[9R]Z. HPLC on the Hamilton PRP1 column (4.1 × 25 mm) (Hamilton Company, Reno, Nevada) was carried out with 35% methanol (v/v) in 0.2 M acetic acid as solvent at a flow rate of 1.5 ml min^{-1}. The retention times of authentic standards in this system were 4.91 min for Z/(diH)Z and 7.16 min for *cis*Z.

A TLC procedure (Table 1) that found limited use in the present work was a modification of the method of Playtis and Leonard (1971), which separates the *cis* and *trans* isomers of Z and [9R]Z. Dyes were used to locate the cytokinin

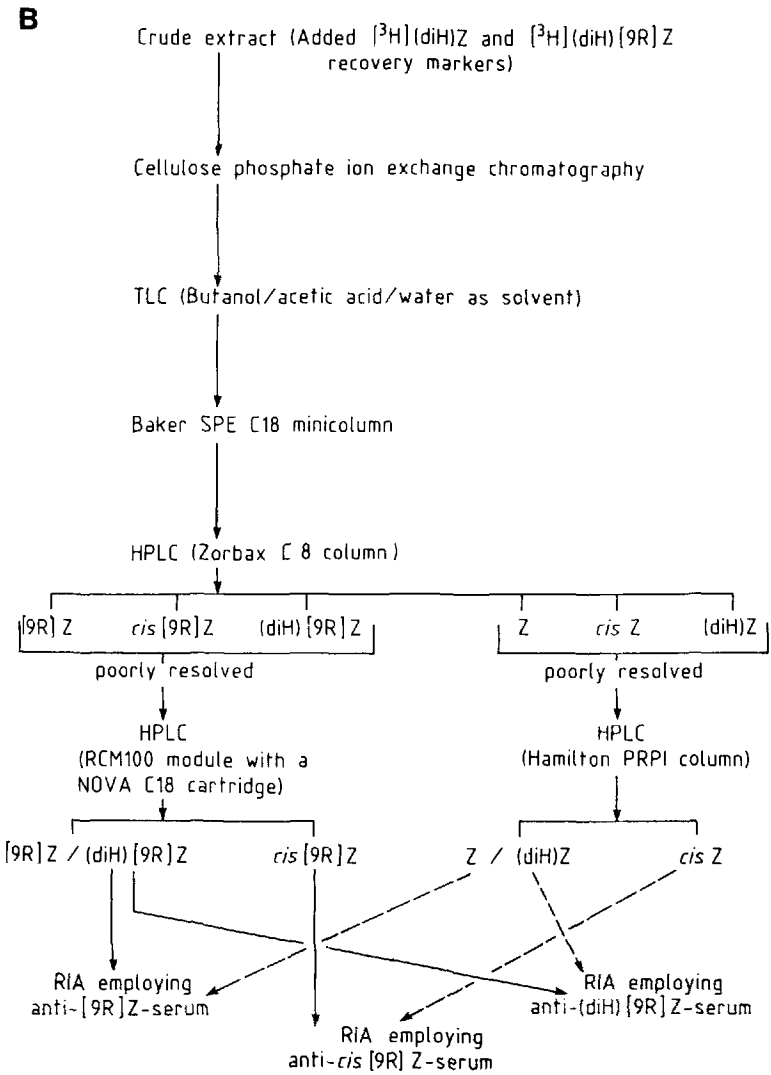

Fig. 1. (B) Scheme, using an aged Zorbax C_8 column, for the separation of cytokinins in xylem sap samples of oat, prior to RIA.

zones. This TLC step was inserted after completion of the TLC step as described in the schemes (Fig. 1a,b).

The recoveries of *cis*[9R]Z and *cis*Z following sample purification were assumed to be equal to those of the final recoveries of [^{3}H](diH)[9R]Z and [^{3}H](diH)Z, respectively. Recovery values obtained in these experiments were, respectively, 50% and 58% from 2-day-old oat, 58% and 32% from 6-day-old oat, and 29% and 22% from 6-day-old wheat. Estimates of the concentrations of the bases were corrected according to the cross-reactivity of each base with its appropriate antiserum.

Table 1. Distance traveled by authentic cytokinins and markers in a TLC system that separates the *cis* and *trans* isomers of zeatin and zeatin riboside.[a]

Compound	Distance traveled (cm)	
	Solvent I	Solvent II
QD/1	13.4	
iP	12.8	10.8
QD/2	10.9	
[9R]iP	8.7	8.5
cis-Z	8.7	6.4
Nile blue	8.2	
Z/(diH)Z	7.1	4.7
Eosin	6.5	
cis[9R]Z	4.3	4.2
[9R]Z/(diH)[9R]Z	3.2	3.2
N-2,4-Dinitrophenyl-D,L-ethionine	2.6	

[a] Layer used was 0.4 mm HF silica gel; distance from origin to top of the layer was 17.0 cm. Distance traveled was the result of developing plates twice. Solvent I was chloroform/methanol (90/10, v/v) containing 14 N NH_4OH (0.25 ml/100 ml) which was added to the tank immediately before developing. To maintain saturation the tank was lined with filter paper to which 4 drops of the 14 N NH_4OH were added immediately before developing. Solvent II (solvent I without the 14 N NH_4OH) was as used by Playtis and Leonard (1971).

Radioimmunoassay

Methods used for the preparation of the *cis*[9R]Z bovine serum albumin (BSA) conjugate and the tracer (tritiated *cis*[9R]Z dialcohol) were as reported by Badenoch-Jones et al. (1987).

Three New Zealand white rabbits were injected with *cis*[9R]Z-BSA conjugate on days 0, 28, 59, 87, 115, and 145. Freund's complete adjuvant was used on days 0 and 28, and Freund's incomplete adjuvant was used on the remaining days. The procedures for injecting and bleeding the rabbits and for screening and storing the antisera were as described by Badenoch-Jones et al. (1984a). All three rabbits developed antibodies against *cis*[9R]Z, but the best antiserum in terms of affinity and titer was selected for use in further assays. The chosen antiserum was from a bleed on day 156.

The RIA procedure was that described by Badenoch-Jones et al. (1987).

Results

Standard Curve

A mean standard curve for *cis*[9R]Z is shown in Fig. 2a. The curve could be linearized over the measuring range by logit transformation of the B/Bo values (Fig. 2b). The measuring range extended from approximately 0.15 to 150 pmol. At a final dilution of 1:9450, the antiserum bound approximately 58.0% of the tracer in the absence of unlabeled *cis*[9R]Z. Nonspecific binding (binding in the absence of antibody) was less than 1%. The antiserum contained high-affinity

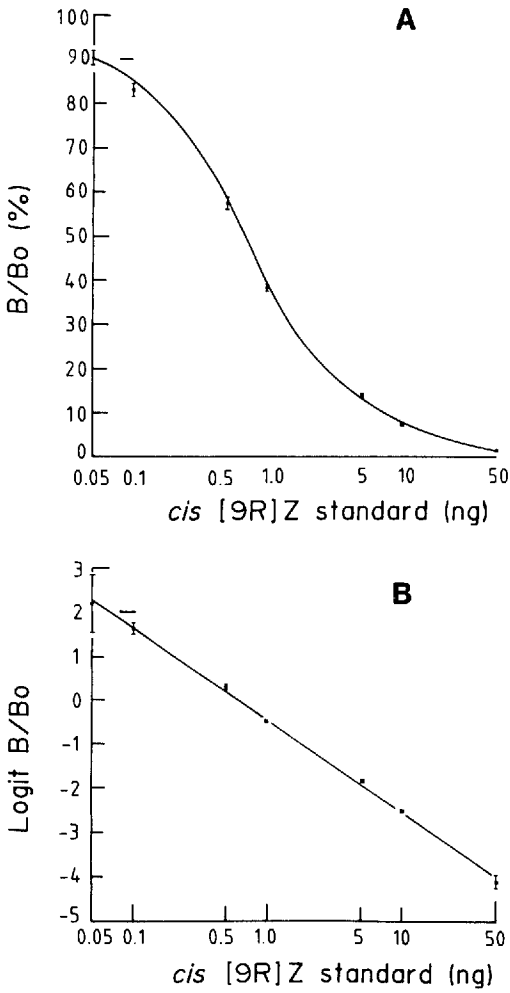

Fig. 2. (a) Mean standard curve for *cis*[9R]Z. Bars indicate SE (n = 12). B, Binding of tracer to antibody in the presence of *cis*[9R]Z standards; Bo, binding of tracer to antibody in the absence of *cis*[9R]Z. (b) Logit transformation of the mean standard curve for *cis*[9R]Z. Bars indicate SE (n = 12). B and Bo, as for Fig. 2a.

antibodies as indicated by a maximum affinity constant of 2.28×10^{-10} M determined from Scatchard plot analysis of standard curve data.

Cross-Reactivity Studies

The specificity of the antiserum was determined by cross-reactivity studies, and the results are shown in Fig. 3a,b. The slopes of the logit transformation curves for most of the compounds tested were similar to, although almost invariably lower than, the slope of the logit transformed standard curve for the antigen. [9R]Z 5′-monophosphate ([9R-5′P]Z), [9R]iP 5′-monophosphate ([9R-5′P]iP), and kinetin (K) had slopes very significantly lower than that for the antigen. Molar cross-reactivities were calculated and are presented in Table 2. No cross-reactivity was found for adenine, adenosine, or adenosine 5′-

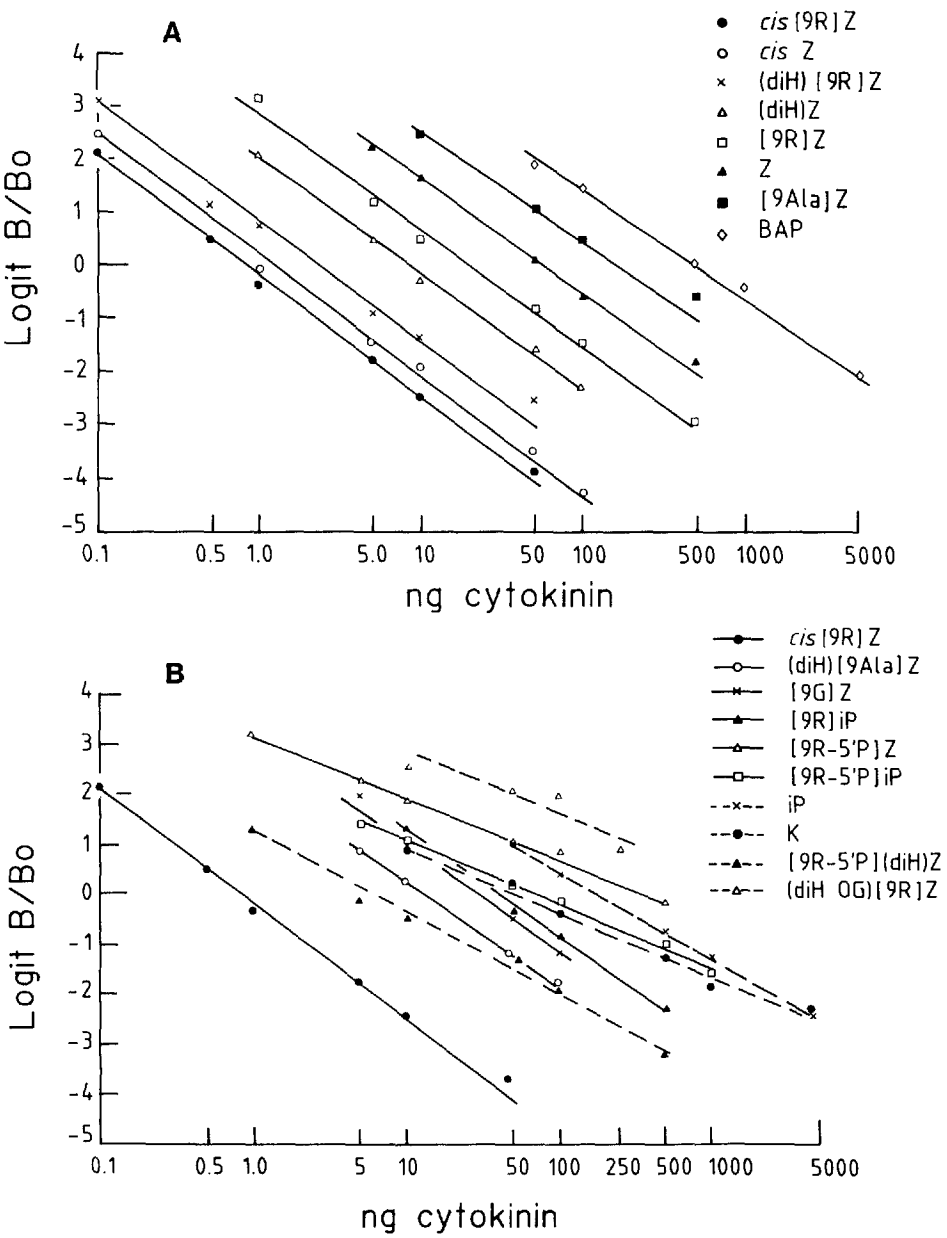

Fig. 3. Logit transformation curves for *cis*[9R]Z and compounds cross-reacting with the anti-*cis*[9R]Z-serum. Results are for representative experiments. B, binding of tracer to antibody in the presence of compound; Bo, binding of tracer to antibody in the absence of compound.

monophosphate (AMP) when tested in amounts up to 5000 ng per assay tube. No cross-reactivity was found for zeatin-7-glucoside ([7G]Z) or O-glucosyl zeatin riboside ((OG)[9R]Z), when tested in amounts up to 1000 ng per assay

Table 2. Percentage cross-reactivities of various purines with anti-*cis*[9R]Z-serum.[a]

Compound	Molar cross-reactivity
cis[9R]Z	100
cis-Z	42.9
(diH)[9R]Z	36.1
(diH)Z	6.0
[9R]Z	3.9
Z	0.90
[9R]iP	2.0
iP	0.26
[9G]Z	2.6
[7G]Z	0
[9Ala]Z	0.40
(diH)[9Ala]Z	0.55
(OG)Z	0
(diH OG)Z	0
(OG)[9R]Z	0
(diH OG)[9R]Z	<0.01
Adenosine	0
Adenine	0
Kinetin	0.56
6-Benzylaminopurine	0.10
Adenosine 5′-monophosphate	0
[9R-5′P]Z	0.26
[9R-5′P](diH)Z	1.6
[9R-5′P]iP	1.4

[a] Results are expressed as 100 times the inverse of the concentration, required to produce the same effect.

tube, or for O-glucosyl zeatin ((OG)Z), when tested in amounts up to 500 ng per assay tube. Cross-reactivity data for anti-*cis*[9R]Z-serum taken from one of the other rabbits were similar to those shown in Table 2. *cis*Z, followed by (diH)[9R]Z, (diH)Z, [9R]iP, and [9R]Z had the highest cross-reactivity. Percentage molar cross-reactivity values with these respective compounds were 48.7%, 15.9%, 5.8%, 3.1%, and 2.6%, respectively, compared with 42.9%, 36.1%, 6.0%, 2.0%, and 3.9% for the antiserum selected for use in this study.

Separation of cis*[9R]Z and* cis*Z from Other Cytokinins*

On TLC (butanol/acetic acid/water), *cis*[9R]Z, *cis*Z, [9R]Z, Z, (diH)[9R]Z, and (diH)Z all chromatographed in a narrow zone. Considerable effort was expended in an attempt to develop a TLC system for separating *cis*[9R]Z and *cis*Z from each other and from the other above-mentioned cytokinins, but none was found to be entirely adequate with the xylem sap fractions used.

The TLC system described in Table 1 separates *cis*Z and *cis*[9R]Z from each other and from mixtures of Z/(diH)Z and [9R]Z/(diH)[9R]Z when authentic standards are used. Because of the specificity of the [9R]Z and (diH)[9R]Z antisera for the unsaturated and saturated compounds, respectively, this one

Table 3. Concentration of cytokinins in xylem sap samples as estimated by RIA (ng ml^{-1} sap)

Cytokinin	Wheat xylem sap (6 day)	Oat xylem sap (2 day)	Oat xylem sap (6 day)
cis[9R]Z	0.61	0.84	0.52
cis-Z	0.32	0.05	0.26
(diH)[9R]Z	0.87	—[a]	—
(diH)Z	0.70	—	—
[9R]Z	1.61	—	—
Z	0.75	—	—

[a] Not assayed.

TLC separation would enable all six cytokinins to be quantified by RIA. Unfortunately, however, the separation of the ribosides was found to be subject to interference when xylem sap fractions were used. This TLC system did achieve adequate separation of the bases in the extract samples used. It is not possible to quantify each of the bases following TLC with the solvent of Playtis and Leonard (1971), because Z/(diH)Z and *cis*[9R]Z overlap in their system.

Because of the limitations of the TLC systems, the HPLC procedures described (Fig. 1a,b) were developed particularly to resolve the *cis* and dihydro compounds, which tend to overlap in HPLC systems. The baseline separation of these is particularly important because of the cross-reactivities noted in Table 2. The HPLC systems devised gave excellent separations of *cis*Z from (diH)Z and of *cis*[9R]Z from (diH)[9R]Z, so that intermediate fractions could be collected and monitored by RIA. Such separations were not achieved by numerous other HPLC systems tried. The HPLC methods described in Fig. 1b may be generally useful, as many chromatographic systems yield a base fraction containing (diH)Z and the isomers of Z plus a riboside fraction with the corresponding 9-ribosyl derivatives.

Cytokinins in Xylem Sap Samples

In Table 3 are presented the concentrations of *cis*[9R]Z, *cis*Z, (diH)[9R]Z, (diH)Z, [9R]Z, and Z in wheat xylem sap as estimated by RIA. Values for the *cis* compounds in oat sap at two stages of development are also included. Following the cellulose phosphate step, the xylem sap sample from 2-day-old oat plants was divided into two aliquots for duplicate analysis, and mean values are presented in Table 3. Reproducibility of cytokinin estimates was found to be satisfactory (difference $< 10\%$). Of particular interest in the present study was the occurrence of *cis*[9R]Z and *cis*Z in xylem sap of both wheat and oats. Further evidence to verify the occurrence of *cis*[9R]Z in the xylem sap sample of 2-day-old oat was obtained as follows: an aliquot of the putative *cis*[9R]Z fraction from the NOVA C_{18} cartridge was further subjected to a TLC step, as described in Table 1, which separates the *cis* and *trans* isomers of [9R]Z. Following this TLC step, the anticipated level of activity in the *cis*[9R]Z RIA was detected at the R_f expected for *cis*[9R]Z.

Discussion

To our knowledge antibodies against *cis*[9R]Z have not previously been elicited. This study therefore provides the first detailed cross-reactivity data for an anti-*cis*[9R]Z-serum. These data reveal that, as with other cytokinin antisera (see Badenoch-Jones et al. 1987), there is a marked selectivity for the N^6-prenyl side chain. The base, *cis*Z, is strongly cross-reactive (42.9%). This cross-reactivity of the anti-*cis*[9R]Z-serum with *cis*Z is almost identical to the cross-reactivity of the anti-[9R]Z-serum with Z (42%) (Badenoch-Jones et al. 1984a). Unfortunately, *cis*Z nucleotide was not available to us for cross-reactivity testing, but it is also likely to be strongly cross-reactive. The only other cytokinins that showed notable cross-reactivity with the anti-*cis*[9R]Z-serum were (diH)[9R]Z (36.1%) and (diH)Z (6.0%). It is noteworthy that correspondingly, *cis*[9R]Z and *cis*Z had measurable cross-reactivity with the anti-(diH)[9R]Z-serum (6.9% and 14.2%, respectively) (Badenoch-Jones et al. 1987).

We have previously emphasized the need to separate each of the naturally occurring cytokinins in a biological sample prior to their quantification by RIA (Badenoch-Jones et al. 1984a, 1987). The procedures shown in Fig. 1 for the separation of *cis*[9R]Z and *cis*Z from other cytokinins can be readily incorporated into the larger scheme of Badenoch-Jones et al. (1987), in which the separation of a wide range of cytokinins is described.

In both schemes the major steps are the same. In the scheme of Badenoch-Jones et al. (1987), if *cis*[9R]Z and *cis*Z are present in a sample, then (diH)[9R]Z and (diH)Z may be slightly overestimated because of the small cross-reactivity of the anti-(diH)[9R]Z serum with *cis*[9R]Z and *cis*Z.

The scheme shown in Fig. 1b is slightly less satisfactory than that shown in Fig. 1a because of the need to cross-assay the fraction containing putative [9R]Z and (diH)[9R]Z and the fraction containing putative Z and (diH)Z—i.e., to assay the same fraction with both the anti-[9R]Z- and the anti-(diH)[9R]Z-sera. For each pair, unless one cytokinin is present in considerably greater amounts than the other, cross-assaying should result in little inaccuracy, as the cross-reactivities of (diH)[9R]Z and (diH)Z with the anti-[9R]Z-serum are low (1.9% and 1.3%, respectively; Badenoch-Jones et al. 1984a), and the cross-reactivities of [9R]Z and Z with the anti-(diH)[9R]Z-serum are low (3.6% and 0.69%, respectively; Badenoch-Jones et al., 1987). In the scheme shown in Fig. 1b, an extra purification step on a Baker SPE C_{18} minicolumn was incorporated prior to HPLC on the Zorbax C_8 column. Since this step requires minimal time and effort and results in cytokinin recoveries of close to 100% but improves the quality (in particular the consistency) of the following HPLC step, we consider its routine use at this stage in the purification scheme to be worthwhile.

In the present study we have obtained evidence for the occurrence of *cis*[9R]Z and *cis*Z in xylem sap samples. Xylem sap was chosen for analysis in preference to tissue in order to obviate the need for an extraction step, as this is the basis of the controversy over the occurrence of free *cis*[9R]Z or *cis*Z in higher plant tissues. Our results provide reasonable evidence for the natural

occurrence in two monocots of *cis*[9R]Z and *cis*Z that is not due to the breakdown of tRNA during extraction. These results are in accord with those of Murofushi et al. (1983), who detected *cis*[9R]Z and *cis*Z in rice root exudate. In our samples, the concentrations of the *trans* isomers were generally greater than those of the *cis* isomers.

The availability of RIA (employing the anti-*cis*[9R]Z-serum developed in this study) should enable further studies to clarify the controversy over the occurrence of *cis*[9R]Z and *cis*Z as free cytokinins in higher plants to be carried out more readily than previously. RIA will also readily enable other investigations of physiological problems that require detection and quantification of *cis*[9R]Z and *cis*Z (including further studies of production of these cytokinins by bacteria and their quantification in tRNA). RIA is particularly useful for the quantificaiton of the *cis* isomers of [9R]Z and Z because these isomers are substantially less active in cytokinin bioassays than the respective *trans* isomers (Vreman et al. 1974, Kaminek et al. 1979).

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