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Chicken Egg Yolk Antibodies for Cytokinin Analysis

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Abstract. The production, isolation, and purification of specific chicken immunoglobulins (Igs) against three main groups of naturally occurring cytokinins are reported. The specific Igs directed against, respectively, zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine are extracted from the egg yolk and used in radioimmunoassays that allow the quantification in parallel of pmol of the cytokinins in plant extracts. As little as 50 fmol of zeatin riboside, 20 fmol of isopentenyladenosine, and 40 fmol of dihydrozeatin riboside can be detected. The levels of cytokinins measured in the radioimmunoassay correlate well with physicochemical analysis methods such as high performance liquid chromatography (HPLC) with UV spectrum detection and HPLC-coupled mass spectrometric detection. Cross-reactivity studies indicate that the assay is not affected by most of the structurally related compounds. The respective antibody preparations recognized zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine and the corresponding free bases. The results obtained when analyzing crude plant extracts are expressed as zeatin riboside equivalents, dihydrozeatin riboside equivalents, and isopentenyladenosine equivalents.

Key Words. Cytokinins—Radioimmunoassay—Chicken IgG

Cytokinins by definition promote cell division in plant tissue culture and are now known to exhibit a wide range of other physiologic effects on a variety of plants and plant tissues. Most cytokinins occur as free purine bases, nucleosides, or nucleotides and as t-RNA constituents. They can be divided into the following main groups: isopentenyladenine (iP) and derivatives, zeatin (Z) and derivatives, dihydrozeatin [(diH)Z] and derivatives, and benzyladenine and derivatives, as they were listed by Letham and Palni (1983).

Their concentration in plant tissue is low relative to the concentration of interfering compounds. This fact and the various losses during the extraction procedure make the measurement of the different cytokinins and their metabolites quite difficult. Cytokinins were analyzed both qualitatively and quantitatively in a wide variety of plant tissues using bioassays (Boerjan et al. 1991, Letham 1978), paper and thin layer chromatography (Nakajima et al. 1986), HPLC (Nicander et al. 1993, Palni et al. 1983a, Wagner et al. 1993), gas chromatography (GC)- and HPLC coupled mass spectrometry (LC-MS) (Griggs et al. 1988, Imbault et al. 1993, Palni et al. 1983b), and immunoassay techniques (Badenoch-Jones et al. 1984, Eberle et al. 1986, Strnad et al. 1992, Trione et al. 1987, Weiler 1980).

Bioassays are quantitatively unreliable but are essential for the isolation of novel cytokinin-like compounds. Unequivocal identification and precise quantification can be obtained through GC- and LC-MS. The need for sophisticated equipment and the considerable sample purification form a drawback here.

The application of immunologic techniques in cytokinin quantification originates from research of their presence in nucleic acids and t-RNA, and the methods are based on earlier studies with purines and nucleosides (Erlanger and Beiser 1964). Immunoassays are already used widely to quantify the main classes of phytohormones (Weiler 1984). They have been proven to be highly sensitive and quick and easy to perform relative to physicochemical techniques.

Abbreviations: B, binding activity; B_0, maximal binding; B_1, unspecific binding; GC, gas chromatography; HPLC, high performance liquid chromatography; LC-MS, HPLC-coupled mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; RIA, radioimmunoassay; TBS, Tris-buffered saline; (diH)Z, dihydrozeatin; (diH) [9R]Z, dihydrozeatin riboside; iP, isopentenyladenine; [9R]iP, isopentenyladenosine; Z, zeatin; [9R]Z, zeatin riboside; [9G]iP, isopentenyladenine-9-glucoside; [9R-5′P]iP, isopentenyladenosine-5′-monophosphate.

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We raised specific chicken egg yolk immunoglobulins against the three main groups of naturally occurring cytokinins: zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine. We describe here their isolation, purification, and characterization and their use in radioimmunoassays (RIAs) to quantify endogenous cytokinins.

Material and Methods

Chemicals

All radioactive tracers were obtained from the Institute of Experimental Botany, Isotope Laboratory, Prague. [2-^{3}H]zeatin riboside, 0.9 TBq/μmol; *N*3-2-[2-^{3}H] isopentenyladenosine, 1.65 TBq/μmol; DL-[^{3}H]dihydrozeatin riboside, 1.27 TBq/μmol.

Zeatin, zeatin riboside ([9R]Z), dihydrozeatin, dihydrozeatin riboside ((diH) [9R]Z), isopentenyladenine, isopentenyladenosine ([9R]iP), benzylaminopurine, benzylaminopyrimidine, adenine, adenosine, adenine 2′-phosphate, adenine 3′-phosphate, kinetin, kinetin riboside, and bovine serum albumin (BSA, RIA grade) were purchased from Sigma. All other cytokinin standards were purchased from Apex (Honiton, UK). The T-gel used for further purification of the chicken antibodies was purchased from KemEnTek, Hellerup, Denmark.

Plant Material

Nicotiana tabacum L. cv. Petit Havana SR1 tissue untransformed (SR1) or transformed with gene 4 of *Agrobacterium tumefaciens* under control of the *Pisum sativum* small subunit of the ribulose-1,5-biphosphate carboxylase promoter sequence (Pssu-*ipt*) (strain pGV2488) (Beinsberger et al. 1991) was cultivated (25°C, 16 h of light, 8 h of darkness) on a Murashige and Skoog medium (Murashige and Skoog 1962) with 3% sucrose, 200 mg/liter *myo*-inositol, 10 mg/liter thiamine dichloride, 1 mg/liter pyridoxine hydrochloride, 1 mg/liter nicotinic acid, and 0.8% agar.

Extraction and Purification of the Cytokinin Fraction

The plant material was homogenized in 80% MeOH, and ^{3}H[9R]Z (1 kBq, 0.9 TBq/mmol) was added for recovery purposes. After centrifugation (24,000 ×*g*, 15 min, 4°C) the extract was purified on an RP-C18 column (Bond-Elut) and evaporated in vacuo. The samples were redissolved in phosphate-buffered saline (PBS) and analyzed by RIA.

Synthesis of the Cytokinin-BSA Conjugates

The cytokinins were coupled via periodate-oxidized vicinal hydroxy groups of the ribose to the free amino groups of BSA as described by Eberle et al. (1986). Each cytokinin (30 μmol, respectively, of [9R]Z, (diH) [9R]Z, and [9R]iP) was dissolved in 100 μL of dimethyl formamide and 0.5 mL of H_2O. Sodium periodate (60 μmol) was added over a period of 5 min. The mixture was stirred for 15 min and 25 μmol of ethylene glycol was added. After 5 min the reaction mixture was added in 10-μL aliquots to a solution of 0.6 μmol of BSA in borate buffer (25 mM $Na_2B_4O_7$, 10 mM NaCl, 1 mM $MgCl_2$, pH 9.5). The mixture was stirred for 60 min at 4°C, and 150 μmol of $NaCNBH_3$, freshly prepared in H_2O, was added in 5-μL aliquots. The mixture was stirred for an additional 30 min. The conjugates were dialyzed against PBS (0.14 M NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$) for 3 days at 4°C. The ratios of cytokinin to BSA were 6 mol of [9R]Z/mol of BSA, 5 mol of (diH) [9R]Z/mol of BSA, and 22 mol of [9R]iP/mol of BSA as obtained spectrophotometrically at 268 nm.

Immunization of the Hens

The immunogen was prepared by homogenizing 500 μL of saline (154 mM NaCl) which contained 0.5 mg of the cytokinin-BSA conjugates with an equal volume of complete Freund's adjuvant. Fourteen-week-old hens were immunized by intradermal injections in the left and right wing muscles (Kint et al. 1987). Booster injections with cytokinin conjugates emulsified in incomplete Freund's adjuvant were given after 3 and 6 weeks. Eggs were collected daily starting 6 weeks after the first injection.

Extraction of the Immunoglobulins from the Egg Yolk

Two different extraction methods were used to isolate the immunoglobulins (Igs) from the egg yolk.

Dextran Sulfate Precipitation Method

The extraction of the Igs was carried out as described by Jensenius et al. (1981). Yolk (10 mL) was added to 80 mL of TBS (0.14 M NaCl, 0.01 M Tris, pH 7.4), and the mixture was centrifuged for 30 min at 2,500 ×*g*, room temperature. The supernatant was mixed with 10 mL of dextran sulfate (10% in TBS), and after 10 min 25 mL of a 1 M $CaCl_2$ solution was added. After 30 min the mixture was centrifuged (10 min, 2,500 ×*g*, room temperature). The Igs in the supernatant were concentrated by Na_2SO_4 precipitation. The pellet was redissolved in TBS and dialyzed for 4 days against TBS at 4°C.

Chloroform Extraction

The Igs were extracted by partitioning the egg yolk by saline against chloroform at a ratio of 20 mL each/yolk (Kint et al. 1987). The mixture was stirred to form a highly viscous yellow gel. After centrifugation (24,000 ×*g*, 30 min, 4°C) the aqueous fluid contained the Igs.

The Ig preparation after chloroform extraction was still relatively crude, and a further purification by thiophilic interaction chromatography was performed (Porath et al. 1985).

The Igs were purified by selective adsorption to a mercaptoethanol derivative of divinyl sulfone-activated agarose (T-gel). The Ig extract was diluted threefold with salt buffer (0.4 M K_2SO_4, 10 mM K_2PO_4, 0.9% NaCl, pH 7) and loaded on the T-gel (10 × 2.5 cm) equilibrated with salt buffer (1 mL/min). The gel was washed with 5 column volumes of salt buffer and then eluted with 10 mM MOPS, pH 7.2. Elution was monitored by means of UV absorption (280 nm). The column material was regenerated using 0.1 N HCl and 0.1 N NaOH.

Radioimmunoassay

The RIA was performed as described by Weiler (1980). Standards and samples were tested in duplicate. The immunoreaction took place in

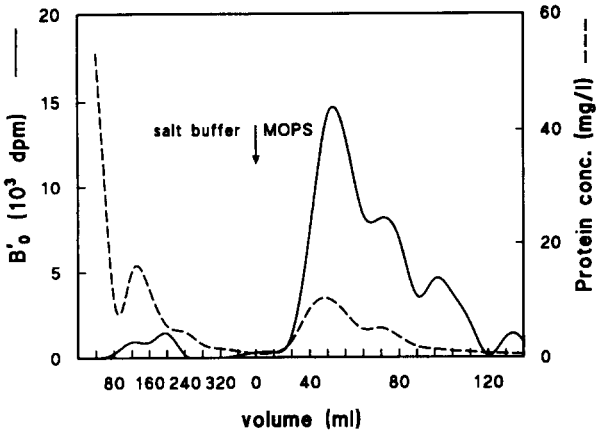

Fig. 1. Protein concentration (mg/mL, dashed line) and binding activities (B'_0, solid line), as determined in RIA, of an antiisopentenyladenosine antibody extract after purification on T-gel.

Eppendorf tubes. The reaction mixture contained 50 µL of PBS with the tritium-labeled tracer, 100 µL of sample or standard (0.1–500 pmol) in PBS, and 100 µL of antibody extract. After mixing, the tubes were incubated for 1 h at room temperature. One mL of saturated $(NH_4)_2SO_4$ solution and 0.5 mg of BSA were added to precipitate the Igs. After 1 h at 4°C and centrifugation (13,000 ×*g*, 15 min) the supernatant was aspirated, and the pellet was dissolved in 100 µL of H_2O. 1.1 mL of Instagel (Packard) was added for tritium counting (TriCarb 150, Packard). Standard curves were plotted after a logit transformation (Rodbard 1974).

HPLC-UV Spectrum Detection

Cytokinins were purified from plant material on an immunoaffinity column using monoclonal antibodies (Ulvskov et al. 1992) prior to analysis on HPLC. By using a gradient system the different isoprenic and aromatic cytokinins could be separated. Absorption at 268 nm was used for quantification; the full scale spectrum at the appropriate retention time was used for qualitative analysis. Cytokinins were separated on a Microsorb C18 column (150-mm length, 4.6-mm internal diameter, and 5-µm particle size, Rainin) with a gradient of 10% MeOH in 40 mM acetic acid, pH 3.35 (TEA) and 80% MeOH in 30 mM acetic acid buffer, flow 0.5 mL/min (Strnad, personal communication).

LC-MS

The samples were injected on HPLC, connected to a VG TRIO-2000 mass spectrometer equipped with a thermospray interface. The mobile phase was methanol, 0.1 M ammonium acetate (50:50, v/v), flow rate, 0.8 mL/min. Select ion monitoring was used for quantification. The molecules at m/z = 352 for [9R]Z and m/z = 220 for Z were monitored (Prinsen et al. 1995).

Results and Discussion

Isolation of Specific Immunoglobulins

IgG is the main class of Igs present in the yolk and this in a concentration higher than in the hen's serum (Rose et al. 1974). The transfer of IgG from the serum of the hen to the yolk is analogous to the transmission of Igs through the placenta in mammalia (Brambell 1970).

Table 1. Binding activities (B'_0 and B_1) and specific binding activities (Spec. B'_0), as determined in RIA, of the crude antibody preparation after chloroform extraction and after purification on T-gel

Antibody extract	B'_0 (dpm)	B_1 (dpm)	Spec. B'_0 (units)
[9R]ip			
Crude	36,600	11,000	136
Purified	15,000	1,400	4,100
[9R]Z			
Crude	6,500	4,000	3
Purified	9,200	1,200	4,000
(diH) [9R]Z			
Crude	13,100	5,200	51
Purified	8,800	1,000	4,400

Note. One unit = 1 dpm/µg of protein in 100 µl of antibody extract. B_0, maximal binding, as determined in RIA. B_1, unspecific binding, as determined in RIA. B'_0, $(B_0 - B_1)$.

The eggs were collected starting from 6 weeks after the first injection. The immunoresponse of the chickens remained constant during the immunization period (data not shown). This allowed us to pool the extracts obtained from the eggs coming from the most reactive chicken.

The Ig extract obtained by means of the dextran sulfate method could be used directly for the RIA.

The chloroform extraction yielded a rather crude antibody preparation, and further purification by thiophilic interaction chromatography was necessary.

The adsorption to the T-gel is based on a structural interaction between the surface area of the immunoglobulins and the C-S-C configuration of the gel. The mercaptoethanol coupled to the divinyl sulfone-activated agarose shows a specific adsorption for IgG, IgM, and IgA. The adsorption is dependent on the salt concentration (Porath et al. 1985).

Fig. 1 shows a typical elution pattern of the T-gel. The protein concentrations [determined in a Bradford assay (Bradford 1975)] and binding activities B'_0 (determined in RIA) of an anti-[9R]iP antibody extract were monitored during purification on T-gel. The salt buffer effluent contains the bulk of mainly nonimmuno proteins; the IgG are retained on the column and can be eluted at low salt concentration using a 10 mM MOPS buffer. The three subsequent peaks observed in the anticytokinin activity of the eluent are most probably the result of the presence of different IgG isotypes in the yolk, as observed by Rose et al. (1974).

The purification of the chloroform extract on the T-gel resulted in an increase of the specific binding activities (Table 1). For the anti-[9R]Z antibody extract this resulted in a 1,300-fold increase in specific binding activity; a 40-fold increase was noted for the anti-[9R]iP antibody extract and a 85-fold increase for the anti-(diH) [9R]Z antibody extract.

Table 2. Molar cross-reactivities of cytokinins and structural analogues with the [9R]iP antibodies, [9R]Z antibodies, and (diH) [9R]Z antibodies

Compound	[9R]iP antibody	[9R]Z antibody	(diH) [9R]Z antibody
cis-Zeatin	0.80	0.20	1
trans-Zeatin	0	22	1
Zeatin riboside	0.07	100	1
Zeatin *N*9-glucoside	0.02	2	4
Zeatin *N*7-glucoside	0	0.04	0
Zeatin *O*-glucoside	0	0.20	0
Zeatin *O*-glucoside-riboside	0	0.30	0
Zeatin riboside-5′-phosphate	0.04	2	2
Dihydrozeatin	0.06	11	34
Dihydrozeatin riboside	0.20	14	100
Dihydrozeatin *N*9-glucoside	0.30	0.01	10
Dihydrozeatin *N*7-glucoside	0.07	0.07	3
Dihydrozeatin *O*-glucoside-riboside	0.20	0.90	1
Dihydrozeatin riboside-5′-phosphate	0.02	0.08	60
Isopentenyladenine	3	0.30	2
Isopentenyladenosine	100	0.40	1
Isopentenyladenine *N*9-glucoside	27	0.10	2
Isopentenyladenosine 5′-phosphate	1	0.60	1
Benzylaminopurine	0.20	0.01	19
Benzylaminopyrimidine	0.50	0.05	10
Benzylaminopurine *N*9-glucoside	0.40	0.05	3
Benzylaminopurine *N*3-glucoside	0.30	0	2
Benzylaminopyrimidine phosphate	0.30	0	2
m-OH-Benzylaminopurine	0.01	0.06	0.90
m-OH-Benzylaminopyrimidine	0.04	0.20	2
o-OH-Benzylaminopurine	0.02	0.02	18
o-OH-Benzylaminopyrimidine	0.20	0.03	30
Kinetin	0.09	0.02	2
Kinetin riboside	3	0.10	9
Adenine	0	0	0
Adenosine	0	0	0
Adenine 2′-phosphate	0	0.02	0.05
Adenine 3′-phosphate	0	0	0
Inositol	0	0	0

Note. The cross-reactivities are determined from tracer displacement curves at 50% displacement, on a molar base.

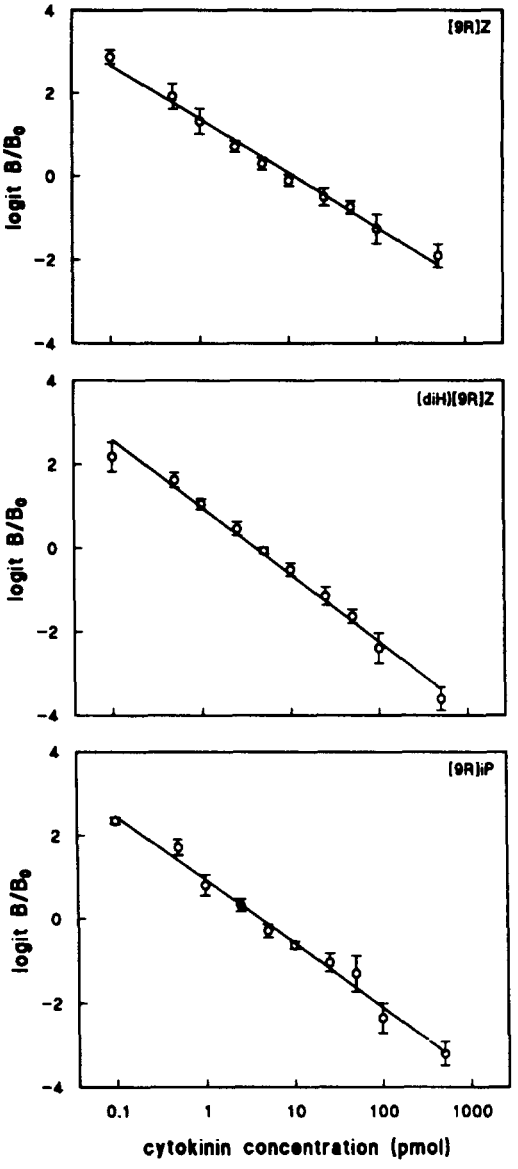

Fig. 2. RIA standard curves for zeatin riboside, dihydrozeatin riboside, and isopentenyladenosine using the anti-[9R]Z, anti-(diH) [9R]Z, and anti-[9R]iP antibody extracts, respectively. Standard curves were plotted after a logit transformation. The standard error is given ($n = 2$).

Specificity of the RIA

The specificity of the antibodies was determined by cross-reactivity studies. Table 2 shows the cross-reactivities of different structural analogues, expressed as the percentage of cross-reactivity on a molarity base (Ernst 1986). This detailed cross-reaction study revealed high selectivity of the three antibodies.

The [9R]iP antibodies are highly reactive with [9R]iP and [9G]iP. They exhibit only weak reactivity against iP and [9R-5′P]iP and almost zero cross-reactivity against the other naturally occurring cytokinins.

In addition to high reactivity for the [9R]Z and its free base the [9R]Z antibodies show some cross-reactivity with (diH)Z and (diH) [9R]Z.

The (diH) [9R]Z antibodies are highly reactive with both (diH) [9R]Z and its free base, but they show cross-reactivity with dihydrozeatin riboside-5′-phosphate, BA riboside, and derivatives.

Sensitivity of the RIA

Typical standard curves were obtained with the three different antibody preparations. The curves could be linearized over the entire measuring range by logit transformation of B/B_0 values (Fig. 2).

The [9R]Z standard curve provides a measuring range that extends from 0.1 to 500 pmol of [9R]Z. As little as

Table 3. General assay parameters

	[9R]assay	[9R]ip assay	(diH) [9R]Z assay
Specific activity tracer	0.9 TBq/mmol	1.6 TBq/mmol	1.27 TBq/mmol
Amount of tracer	0.9 pmol	0.9 pmol	0.8 pmol
Unspecific binding	8%	10%	7%
Detection limit	50 fmol	20 fmol	40 fmol
Linear range	0.1–500 pmol	0.1–500 pmol	0.1–500 pmol
Midrange (B/B_0 = 50%)	8 pmol	6 pmol	5 pmol

Note. The unspecific binding was determined in the presence of an excess of unlabeled cytokinin.

Table 4. Comparison of the cytokinin concentrations in *ipt*-transformed tobacco calli (pGV2488) and untransformed control calli (SR1) as determined by RIA in crude extracts with concentrations determined on similar tissues by HPLC-UV spectrum detection

	RIA[a] (pmol eq/g fr wt)		HPLC[b] (pmol/g fr wt)	
	pGV2488	SR1	pGV2488	SR1
[9R]Z	4,600 ± 400	12.2 ± 1.4	4,000 ± 300	7.3 ± 1.5
Z			230 ± 30	b.d.[c]
(diH) [9R]Z	1,270 ± 120	72 ± 5	1,080 ± 40	19.3 ± 0.7
(diH)Z			75 ± 5	49 ± 5
[9R]iP	8.8 ± 2.9	5.2 ± 0.8	8.3 ± 2.5	7.5 ± 2.5
iP			b.d.[c]	b.d.

Note. Data are expressed in pmol/g fresh weight.

[a] All RIA data are the mean ± S.E. of at least three sample dilutions.

[b] HPLC data are the mean ± S.E. of two replicate injections.

[c] b.d., below detection limit.

50 fmol could be detected. The [9R]iP and (diH) [9R]Z standard curves also have a measuring range from 0.1 to 500 pmol with detection limits of, respectively, 20 and 40 fmol. The results are summarized in Table 3.

The cross-reactivities of the respective antibodies with the free base and the riboside, however, are not the same. This means that the results obtained, when analyzing crude extracts, should be expressed as [9R]Z equivalents, [9R]iP equivalents, and (diH) [9R]Z equivalents, respectively. Only after fractionation, that is, by HPLC, can the free base and the riboside be quantified individually using the standard curve for the riboside and correcting for the compound cross-reactivities.

Reliability of the RIA

To test the reliability of the assay, the quantitative data obtained in the RIA were compared with two physicochemical methods for determination of cytokinins: HPLC with UV diode array detection and LC-MS.

As plant test material, *N. tabacum* L. cv. Petit Havana SR1 calli or calli transformed with gene 4 of *A. tumefaciens* (Beinsberger et al. 1991) (strain pGV2488) were chosen. Gene 4, the *ipt* gene, codes for isopentenyltransferase. This enzyme catalyzes the first step in the cytokinin synthesis. These calli contain elevated levels of cytokinins. As untransformed control tissue, SR1 calli were chosen.

RIA data were compared with HPLC-UV spectrum detection estimates (Table 4). The RIA estimates are in very good agreement with the data obtained through HPLC-UV detection.

Table 5 compares the levels of [9R]Z and Z in transgenic and control calli as estimated by RIA and LC-MS. There is good overall agreement between the RIA data and the data obtained by LC-MS.

One of our objectives was to develop a procedure by which the cytokinin contents in a large amount of plant samples could be determined in a minimum amount of time. It appears from our results that RIA requires little prepurification. Good overall agreement between the RIA data and the data obtained through physicochemical means indicates that crude extracts from *ipt*-transformed tobacco and untransformed SR1 calli can be used. However, nonspecific interference may result in overestimation or underestimation of endogenous cytokinin levels and can be due to a wide range of causes other than direct competition for antibody binding sites. The importance of testing for interference in different plant tissues used in RIA cannot be overemphasized.

Table 5. Comparison of the cytokinin concentrations in *ipt*-transformed tobacco calli (pGV2488) and untransformed control calli (SR1) as determined by RIA in crude extracts with concentrations determined on similar tissues by LC-MS

	RIA	LC-MS	
Sample	[9R]Z equivalent	[9R]Z	Z
pGV2488	1,100	510	500
SR1	270	200	80

Note. Concentrations are expressed in pmol/g fresh weight.

Conclusions

Although chicken serum and egg yolk antibodies are increasingly used for RIAs in medical research, there is no report on their use in plant science.

Eggs form a cheap and accessible source of antibodies. The Igs can be extracted easily in quantities equivalent to repeated bleedings and without any harm to the animal. Up to 20 mg of IgG/mL of egg yolk can be obtained, whereas a maximum of 10 mg is present in 1 mL of rabbit serum (Johnstone and Thorpe 1987).

We demonstrate here that it is possible to determine pmol quantities of [9R]Z, (diH) [9R]Z, and [9R]iP in plant extracts using chicken yolk antibodies.

The high concentration of [9R]Z equivalents present in the gene 4-transformed calli was confirmed with HPLC-UV diode array detection and LC-MS. These results proved this RIA to be highly sensitive and selective.

The major objective in developing this RIA was to reduce the purification procedures needed before quantification. Identification and quantification of the different cytokinins in biologic samples are essential for many physiologic studies. The data should be obtained fast in order to evaluate the kinetics of the physiologic process. The RIA combines speed, accuracy, and sensitivity.

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