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Enzyme Immunoassays of N^6-Benzyladenine and N^6-(*meta*-Hydroxybenzyl)adenine Cytokinins

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Abstract. Enzyme-linked immunosorbent assays (ELISAs) were developed for determination of N^6-benzyladenosine, N^6-(*meta*-hydroxybenzyl)adenosine, and structurally related cytokinins. The use of the ELISAs allowed detection over the range of 0.05–70 pmol for N^6-benzyladenine and 0.01–20 pmol for the N^6-(*meta*-hydroxybenzyl)adenine cytokinins. Polyclonal antibodies used in the assays were specific for N^6-benzyladenine and N^6-(*meta*-hydroxybenzyl)adenine and their corresponding N^9-substituted derivatives. By the use of internal standardization, dilution assays, authentic [2-^{3}H]cytokinin recovery markers, and immunohistograms, the ELISAs have been shown to be applicable for the estimation of N^6-benzyladenine and N^6-(*meta*-hydroxybenzyl)adenine-type cytokinins in plant tissues. For the analysis of cytokinins in the tissues of young poplar leaves and *Solanum* teratoma shoot culture, the extracts were fractionated by high performance liquid chromatography (HPLC) and the fractions analyzed by ELISAs. Immunohistogram ELISA analysis of fractions from different HPLC systems indicated major peaks of immunoreactivity co-chromatographing with the labeled and unlabeled standards of N^6-benzyladenine, N^6-*meta*-hydroxybenzyl)adenine, and their N^9-glycosides in these tissues.

Key Words. N^6-Benzyladenosine—Cytokinins—Enzyme immunoassay—N^6-(*meta*-Hydroxybenzyl) adenosine—*Populus* × *Robusta* leaves—*Solanum* shoots (transformed and normal plants)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FW, fresh weight; (mOH)[9*R*]BAP, N^6-(*meta*-hydroxybenzyl)adenosine; HPLC, high performance liquid chromatography; TBS, Tris-buffered saline; TEAA, triethylammonium acetate; [9*R*]BAP, N^6-benzyladenosine.

For the major groups of naturally occurring cytokinins, polyclonal and monoclonal antibodies of high quality have been developed (see Weiler 1984, Strnad et al. 1992a). Only a few studies have been carried out with cytokinins bearing an aromatic ring as the side chain. Constantinidau et al. (1978) described the production and immunologic characteristics of an antiserum against N^6-benzyladenosine, a synthetic cytokinin, which has already been identified in an old *Pimpinella anisum* cell culture (Ernst et al. 1983b) and *Solanum* crown gall tumors (Nandi et al. 1989a). For analysis of synthetic N^6-benzyladenine levels in plant tissue cultures, several workers have used the antibodies against isopentenyladenosine, which are known to cross-react strongly with [9*R*]BAP-type cytokinins (Ernst et al. 1983b, Vaňková et al. 1987, Label and Sotta 1988). We have already described an ELISA based on antibodies specific for N^6(*ortho*-hydroxybenzyl)adenosine, an aromatic cytokinin present at very high endogenous levels in *Populus* × *Robusta* leaves (Strnad et al. 1992b). In this paper I report the development of the enzyme immunoassays for detection and quantification of N^6-benzyladenosine ([9*R*]BAP), N^6-(*meta*-hydroxybenzyl)adenosine ((mOH)[9*R*]BAP), and related compounds. Using the ELISAs in conjunction with different reversed phase HPLC separations, it was possible to detect several immunoreactive compounds coeluting with authentic labeled and unlabeled standards of aromatic cytokinins in young poplar leaves (*Populus* × *canadensis* Moech., cv. *Robusta*) and teratoma shoot culture derived from *Solanum* leaf discs transformed by T-DNA gene 4 (*ipt*).

Materials and Methods

Chemicals and Reagents

Unlabeled cytokinins were from Apex Organics (Leicester, UK); isopentenyladenosine, isopentenyladenine, zeatin, zeatin riboside, dihydrozeatin; dihydrozeatin riboside, kinetin, N^6-benzyladenosine, N^6-

benzyladenine, and *N,N'*-diphenylurea were from Sigma (St. Louis, MO, USA); N^6-(hydroxybenzyl)adenines and their ribosides were kindly supplied by Dr. Tomáš Vaněk, Institute of Organic Chemistry and Biochemistry, Prague. N^6-(*ortho*-Hydroxybenzylamino)-N^9-β-D-glucopyranosylpurine, N^6-(*meta*-hydroxybenzylamino)-N^9-β-D-glucopyranosylpurine, N^6-[2-^{3}H]benzyladenine, N^6-[2-^{3}H]benzyladenosine, N^6-[2-^{3}H](*meta*-hydroxybenzyl)adenine, and N^6-[2-^{3}H](*meta*-hydroxybenzyl)adenosine (specific activity approximately 1.0 TBq · mmol^{-1}) were synthesized by Dr. J. Hanuš, Isotopic Laboratory, Institute of Experimental Botany, Prague, by an unpublished method. Before use for syntheses, analyses, and cross-reactivity studies, the purity of labeled and unlabeled cytokinins was checked by HPLC. Alkaline phosphatase for enzyme immunoassay (2,500 units · mg^{-1}) and *p*-nitrophenylphosphate were from Boehringer (Mannheim, FRG); acetonitrile for chromatography was from Merck (Darmstadt, FRG); Tris, bovine serum albumin, and acid phosphatase (0.4 units · mg^{-1}) were from Sigma, DEAE-cellulose, a reversed phase column (Separon SGX C_{18}), and C_{18} cartridges were from Tessek (Prague, Czech Republic). All other chemicals were obtained from Lachema (Brno, Czech Republic).

Plants

The growing leaves of poplar (*P.* × *canadiensis* Moench., cv. *Robusta*), collected from the field on June 10, were used for cytokinin analysis. The first four young leaves without petioles were cut just 1 h after daybreak, dropped immediately into liquid nitrogen, and extracted. Potato shoots (*Solanum tuberosum* L., cv. *Oreb*) grown on Murashige and Skoog (1962) medium without cytokinin were either control plants or teratoma shoots (clone 1). Clone 1 was selected after transformation of *Solanum* leaf discs by pTi C58 T-DNA gene 4 (*ipt*) and formed moss-like teratomas (Ondřej et al. 1990). The shoots were collected 4 weeks after subcultivation and then either extracted or stored at −70°C until use.

Immunologic Reagents

N^6-Benzyladenosine and N^6-(*meta*-hydroxybenzyl)adenosine were coupled to bovine serum albumin by a modification of the method of Erlanger and Beiser (1964). Cytokinin (30 μmol) was dissolved in a solution of 200 μl of dimethyl sulfoxide and 2 mL of bidistilled water, and 2 mL of 0.03 M $NaIO_4$ solution (60 μmol) was added dropwise over a period of 5 min. The solution was stirred for 15 min in the dark at room temperature. The excessive periodate was destroyed by adding 15 μL of 1.8 M ethylene glycol (30 μmol). After 5 min the reaction mixture was added in portions of 50 μL to bovine serum albumin dissolved in carbonate buffer (10 mM K_2CO_3, 10 mM $KHCO_3$, pH 9.6). The solution was stirred at 4°C for 60 min in the absence of light. During this period the pH was kept between 9.3 and 9.5 with 5% K_2CO_3. The conjugates were stabilized overnight at 4°C with an excess of $NaBH_4$ (5 mg, 132 μmol), then dialyzed against 6 × 2 liters of phosphate-buffered saline (50 mM, $NaHPO_4$, 0.15 M NaCl, 0.4 g liter^{-1} NaN_3, pH 7.4), lyophilized, and stored at −20°C. From the UV spectra of the conjugates a coupling ratio of 9 mol of (mOH)[9*R*]BAP and 7 mol of [9*R*]BAP/mol of bovine serum albumin was determined. The immunization schedule and purification of antibodies are described in detail in our previous papers (Strnad et al. 1990, 1992b).

Extraction and Purification of Cytokinins from Plant Tissues

The procedure for tissue extraction and purification is a modification of the method described previously by MacDonald et al. (1981). Frozen plant tissues were ground to a fine powder under liquid nitrogen. The powder was divided into three aliquots corresponding to 2 g fresh weight (FW). Each aliquot was extracted in ice-cold 80% methanol(10 mL · g^{-1} FW) containing sodium diethyldithiocarbamate as antioxidant (400 μg · g^{-1} FW). About 420 Bq (25,000 dpm) of [2-^{3}H](mOH)[9*R*]BAP, [2-^{3}H][9*R*]BAP, and corresponding tritiated free bases were added to the extracts to monitor for losses during purification steps and to validate the chromatographic data. After a 2-h extraction, the homogenate was centrifuged (15,000 × g, 4°C) and pellets reextracted the same way. The combined extracts were concentrated to approximately 1.0 mL by rotary evaporation under vacuum at 35°C. The samples were diluted to 20 mL with ammonium acetate buffer (40 mM, pH 6.5) containing sodium diethyldithiocarbamate (5 mM) and then incubated with wheat germ acid phosphatase (0.05 units · mL^{-1}) for 30 min in the dark (25°C) to dephosphorylate cytokinin 5′-phosphates. For the immunoassay dilution analysis, the 2-mL eluates were dried in vacuo and redissolved in Tris-buffered saline (TBS, 50 mM Tris, 10 mM NaCl, 1 mM $MgCl_2$, pH 7.5). Aliquots of these solutions were either analyzed in serial dilutions or mixed with known amounts of cytokinin standards and then analyzed by ELISA.

The extracts were purified using a combined DEAE-cellulose (1.0 × 5.0 cm)-octadecylsilica (0.5 × 1.5 cm) column as described in MacDonald et al. (1981). Cytokinins were loaded onto a reversed phase C_{18} column cartridge that was then washed with 10 mL of H_2O and eluted in 7 mL of 70% (v/v) methanol in triethylammonium acetate buffer (TEAA, 40 mM, pH 3.35). The eluates were evaporated to dryness, dissolved in 0.5 mL of 70% methanol in TEAA (7:3 v/v), and filtered through a Millipore filter (0.22 μm).

High Performance Liquid Chromatography

The equipment consisted of a Spectra Physics SP 8800 solvent delivery system coupled to an SP 100 UV-vis detector and SP 4400 computing integrator. The injection was performed by a Rheodyne 7010 injection loop (100 μL). Two different gradient systems on a Separon SGX C_{18} column (250 × 4 mm inner diameter, 7 μm particle size; Tessek) were used to separate different aromatic cytokinins. In system I solvent A was 20% methanol in TEAA buffer (v/v, 40 mM, pH 3.35); solvent B 80% methanol in 40 mM acetic acid (v/v, pH 3.65). Initial conditions were 90% A, 10% B; then a linear gradient to 60%A, 40% B at 15 min; a linear gradient to 40% A, 60% B at 24 min; 100% methanol for 10 min (column wash); and 90% A, 10% B at 10 min (regeneration). The flow rate was 1.0 mL/min. In system II, the column was eluted at 1.2 mL/min with acetonitrile and TEAA buffer (40 mM, pH 3.35) according to the following gradient profile: 0 min of 5% acetonitrile, 10 min of 7%, 30 min of 10%, 40 min of 15%, 50 min of 14%, 10 min of 100% (washing). Timed fractions (0.5 min) were collected by a FRAC 100 fraction collector (Pharmacia, Uppsala, Sweden), dried in vacuo, and redissolved in 500 μL of TBS buffer. Fifty-μl aliquots were investigated in duplicate by scintillation counting and ELISA. The content of individual cytokinins in the appropriate immunoreactive fractions was assessed using a series of different ELISAs including dilution and internal standardization (Weiler 1982, Badenoch-Jones et al. 1984).

Enzyme-linked Immunosorbent Assay (ELISA)

The assays were performed using a modification of the ELISA protocol described by Weiler et al. (1981). The microtiter plates (Gama, České Budějovice, Czech Republic) were coated with 150 μL of rabbit anti-[9*R*]BAP or anti-(mOH)[9*R*]BAP antibodies (5 μg · mL^{-1} 50 mM $NaHCO_3$, pH 9.6). The wells were washed with distilled water, filled with 200 μL of bovine serum albumin solution (0.04 g · L^{-1}), and incubated for 1 h at 25°C. After decanting and two washes with dis-

Table 1. Assay parameters of N^6-benzyladenosine and N^6-(*meta*-hydroxybenzyl)adenosine enzyme immunoassay.

Parameter	[9R]BAP assay	(mOH)[9R]BAP assay
Amount of tracer/assay	5 ng	2 ng
Unspecific binding	3.5%	2.2%
Detection limit	76 fmol, 27 pg	19 fmol, 7 pg
Linear average of logit/log plot	0.05–70 pmol	0.01–20 pmol
Midrange (50% binding)	1.5 pmol, 0.54 ng	0.5 pmol, 190 pg
Intraassay variance[a]	4.2%	3.5%
Interassay variance[b]	6.8%	6.1%

[a] Eight replicates.
[b] Twenty assays.

tilled H_2O, the wells were filled in the following sequence: 50 µL of TBS, 50 µL of standard or sample in TBS, and 50 µL of cytokinin-alkaline phosphatase tracer diluted in TBS-bovine serum albumin buffer (0.04 $g \cdot L^{-1}$). Nonspecific binding was determined by adding an excess (200 pmol) of cytokinin standard; for maximum tracer binding, TBS was used instead of standard. After 1 min of shaking, the plates were incubated for 1 h at 25°C. The decanted plates were then washed four times with TBS and filled immediately with 150 µL of a *p*-nitrophenylphosphate solution (1 $mg \cdot mL^{-1}$ 50 mM $NaHCO_3$, pH 9.6). The reaction was stopped after a 1-h incubation at 25°C by adding 50 µL of 5N KOH and the absorbance measured at 405 nm in a Titertek Multiscan MCC 340 (Flow Laboratories, Irvine, UK). A Wia-Calc computer program (LKB, Bromma, Sweden) was used for assay evaluation and computation of results. Sigmoidal curves for standards, cross-reacting compounds, and dilution analysis were linearized by log-logit transformation as follows (Weiler 1980): logit $B/B_0 = \ln [(B/B_0)/(100 - B/B_0)]$ (see *insets*, Fig. 1).

The cytokinin value obtained by the ELISA of the fraction(s) containing 2-^{3}H-labeled cytokinin was corrected by the appropriate cross-reactivity and recovery values to obtain estimates of cytokinin levels in plant tissue (expressed as [9*R*]BAP or (mOH)[9*R*]BAP equivalent). If there was any spread of radioactivity into a second fraction, the cytokinin content was estimated from the fractions containing radioactivity. Levels of N^9-glucosides were calculated from immunoactivity (in the appropriate ELISA) of fraction(s) collected at the retention time of authentic standards and on the assumption that recovery for these cytokinins was same as the recovery of 2-^{3}H-labeled ribosides.

Results

Assay Characteristics

All immunized rabbits produced antisera to the cytokinin conjugates, but serum titers differed considerably and reflected the reaction of the individual animal. Because of its high selectivity characteristics, antibody 474 specific for [9*R*]BAP and antibody 754 specific for (mOH)[9*R*]BAP were selected and used routinely for cytokinin analysis. Some of the assay parameters are summarized in Table 1. The mean standard curves and their log/logit plots are shown in Fig. 1. The dilutions of antisera required to give 50% binding of an appropriate 2-^{3}H-labeled cytokinin were 1:28,000 and 1:150,000 for [9*R*]BAP and (mOH)[9*R*]BAP, respectively. As little as 76 fmol of [9*R*]BAP and 19 fmol of (mOH)[9*R*]BAP could be detected by the ELISAs. Within the measuring range, the standard curves were almost linear over 3 orders of magnitude with small inter- and intraassay variation.

The specificity of antibodies was determined by cross-reactivity studies, and the results are shown in Table 2. The compounds were tested for antibody binding over a range from 0.01 up to 5,000 pmol/assay. Data for cytokinins and related compounds producing molar cross-reactivities lower than 0.01% are not shown, namely, no cross-reactivity was found for adenine, adenosine, adenosine 5′-monophosphate, inosine, *N,N′*-diphenylurea, zeatin 7-glucoside, dihydrozeatin 7-glucoside, dihydrozeatin 9-glucoside, and *O*-glucosides of zeatin and dihydrozeatin even when tested in amounts up to 5,000 pmol/assay. Other natural isoprenoid cytokinins such as zeatin, zeatin riboside, zeatin 9-glucoside, zeatin riboside 5′-monophosphate, *cis*-zeatin, *cis*-zeatin riboside, dihydrozeatin, and dihydrozeatin riboside showed at most only slight cross-reactivity. In addition to the riboside, the antibodies cross-reacted strongly with respective free bases, riboside 5′-monophosphates, and N^9-glucosides. The slopes of the log/logit transformation of all N^9-substituted derivatives were similar to the standard curves of [9*R*]BAP and (mOH)[9*R*]BAP, respectively (data not shown).

Surprisingly, there was a very low level of competition by N^6-benzyladenine, N^6-(*ortho*-hydroxybenzyl)adenine, and their N^9-glycosides for antibodies raised against *meta*-derivative. Thus, the position of the hydroxyl group on the benzyl ring is a crucial factor for antibody recognition. As expected, the anti-[9*R*]BAP antibodies were reactive with isopentenyladenine, kinetin, and their N^9-glycosides because of the apolar side chain of the original antigen. Interestingly, this antibody also bound appreciably benzyladenine 3-glucoside and N^6-(*meta*-hydroxybenzyl)adenine and its N^9-substituted derivatives. In consequence, by replacing of [9*R*]BAP with (mOH)[9*R*]BAP tracer, the assay is also suitable for [9*R*]BAP analysis.

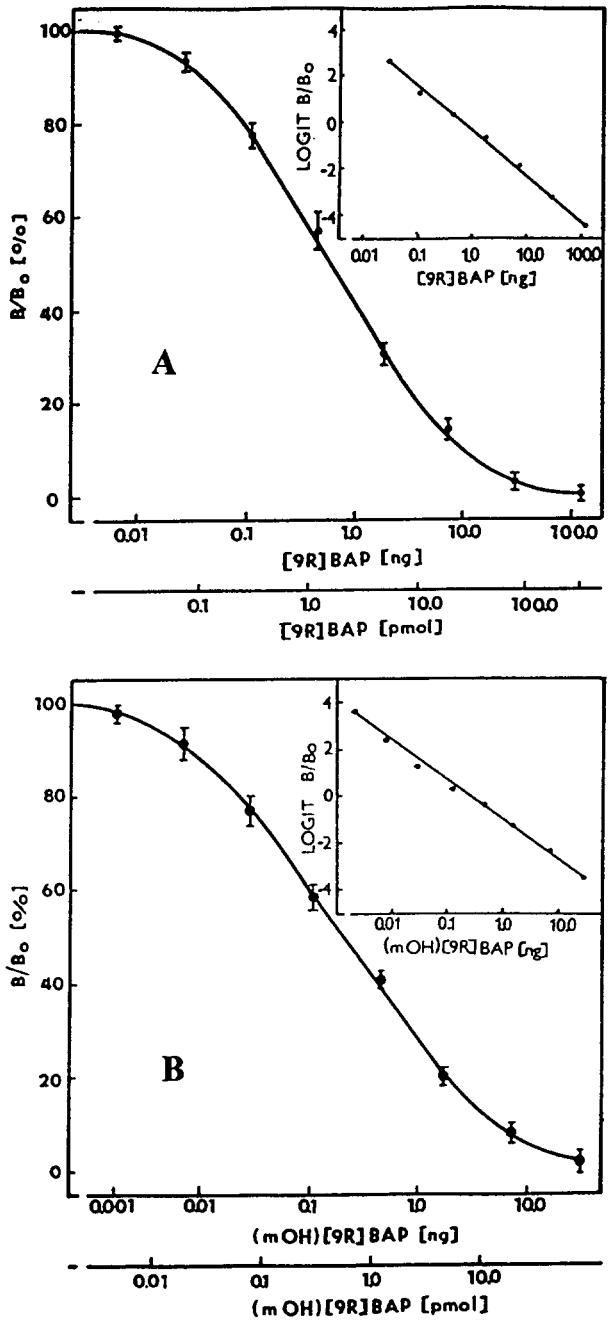

Fig. 1. Typical standard curves obtained for [9*R*]BAP and (mOH)[9*R*]BAP ELISAs and linearized logit/log plot of the same data **(inset). Bars** indicate standard deviation of duplicates (n = 20); B and B_0 represent binding of alkaline phosphatase tracer in the presence and absence of [9*R*]BAP and (mOH)[9*R*]BAP, respectively.

Validation of the ELISAs

Validation of assay performance at different purification steps was carried out to assess the reliability of the ELISAs. Details for one of the sampled (*P.* × *Robusta*) are shown in Fig. 2. When serially diluted crude extracts were analyzed by ELISA, parallel curves were always obtained (Fig. 2), suggesting that these samples did not contain substances interfering with the assays. When activity was detected in HPLC fractions that were assayed at more than one dilution, the dilution curves were also parallel to the standard curves (Fig. 2). Similarly, the recoveries of internal standards added to the crude and HPLC-purified extracts were found to produce satisfactory parallel lines (Fig. 2). Accurate quantification of the cytokinins in plant extracts was performed by ELISA of HPLC-purified extracts in conjunction with recoveries of internal radiolabeled standards. Recoveries of 83% for [2-^{3}H](mOH)[9*R*]BAP and 76% for [2-^{3}H][9*R*]BAP were obtained, whereas the recoveries of corresponding free bases were 68 and 63%, respectively. Tritium-labeled cytokinins used in this study proved also to have a useful application in locating the HPLC fractions containing immunoreactive cytokinins. Thus, any possible spread of the immunoactivity into neighboring fractions could be detected and accounted for, based on these recovery markers.

Immunodetection and Quantification of Cytokinins in Plant Extracts

The broad specificity of the antibodies for N^9-substituents of aromatic cytokinin allowed these forms to be quantified together with those for which the assays were developed. This was achieved by separating all cross-reactive compounds using two different HPLC systems on the Separon SGX C_{18} column (Fig. 3). This column is unique in that it separates cytokinins in methanolic gradient (system A) according to their apparent hydrophobicity; but when separated in system B (acetonitrile-TEAA buffer as a solvent), the N^9-glucosides eluted first followed by the free bases, and the ribosides were retained most strongly among their corresponding N^9-substituted derivatives (Fig. 3*B*). Furthermore, there was a good separation of aromatic cytokinins from isoprenoid ones (see Strnad et al. 1990, Jones et al. 1996). However, the batch-to-batch variability in the stationary phase was quite high.

Assay of HPLC-purified extract of *P.* × *Robusta* leaves with the ELISA for (mOH)[9*R*]BAP detected cross-reactive compounds coeluting with those of authentic and labeled (mOH)[9*R*]BAP and its free base (Fig. 4). The values obtained from two HPLC systems of the three duplicate estimates were 20.8 + 3.4 and 7.2 ± 0.9 pmol · g^{-1} FW, respectively. ELISA using anti-[9*R*]BAP antibodies revealed peaks corresponding to N^6-benzyladenosine (0.68 ± 0.12 pmol · g^{-1} FW) and N^6-benzyladenine (0.31 ± 0.09 pmol · g^{-1} FW). In addition, the anti-[9*R*]BAP antibodies cross-reacted with a compound that in the methanolic gradient had a retention time of 13.8 min (Fig. 4). It was deduced to be

Table 2. Molar cross-reactivities of various cytokinins with N^6-benzyladenosine and N^6-(*meta*-hydroxybenzyl)adenosine antibodies. Data presented are expressed as the percentage ratio of molar concentration of [9R]BAP or (mOH)[9R]BAP and competitor giving 50% binding.

	Cross-reactivity (%)	
	Anti-[9R]BAP	Anti-(mOH)[9R]BAP
N^6-Benzyladenosine	100	0.07
N^6-Benzyladenine	72.2	0.05
N^6-Benzyladenine 3-glucoside	23.3	<0.01
N^6-Benzyladenine 7-glucoside	0.1	<0.01
N^6-Benzyladenine 9-glucoside	79.5	0.05
N^6-Benzyladenosine 5′-monophosphate	86	0.08
N^6-(*meta*-Hydroxybenzyl)adenosine	6.8	100
N^6-(*meta*-Hydroxybenzyl)adenine	2.8	43.4
N^6-(*meta*-Hydroxybenzyl)adenine 9-glucoside	4.7	68.7
N^6-(*ortho*-Hydroxybenzyl)adenosine	0.04	0.06
N^6-(*ortho*-Hydroxybenzyl)adenine	<0.01	0.03
N^6-(*ortho*-Hydroxybenzyl)adenine 9-glucoside	<0.01	0.04
Zeatin riboside	0.07	<0.01
Zeatin	0.05	<0.01
Zeatin 9-glucoside	0.06	<0.01
Zeatin riboside 5′-monophosphate	0.04	<0.01
cis-Zeatin riboside	0.09	<0.01
cis-Zeatin	0.06	<0.01
Dihydrozeatin riboside	0.09	<0.01
Dihydrozeatin	0.04	<0.01
Dihydrozeatin riboside 5′-monophosphate	0.03	<0.01
Isopentenyladenine	2.33	<0.01
Isopentenyladenine 9-glucoside	1.22	<0.01
Isopentenyladenosine 5′-monophosphate	2.12	<0.01
Kinetin riboside	2.53	<0.01
Kinetin	1.72	<0.01

(mOH)[9*R*]BAP because the dame fraction gave a high level of activity in the (mOH)[9*R*]BAP assay. Identity of the peak was confirmed by coelution of authentic radioactive and immunoactive compound on Microsorb C_{18} (Rainin) column and by mass spectrometry (Strnad et al. 1997). Furthermore, the amount of (mOH)[9*R*]BAP found after correction for cross-reactivity (6.8% in [9*R*]BAP assay) was approximately the same as that determined in the appropriate ELISA.

Fig. 5 shows the immunohistograms of the extracts from transformed and untransformed *Solanum* shoots analyzed by HPLC-ELISAs. Control potato shoots cultivated in vitro contained amounts of [9*R*]BAP and (mOH)[9*R*]BAP cytokinins too low to detect by this method (detection limit 0.2 pmol · g^{-1} FW), whereas a 4-week-old teratoma shoot culture showed considerably higher levels of (mOH)[9*R*]BAP and benzyladenine 9-glucoside (Fig. 5, *B* and *D*). The peak that eluted before benzyladenine 9-glucoside is almost certainly due to the immunoactivity of (mOH)[9*R*]BAP in the [9*R*]BAP assay as described above, e.g. it coeluted at the retention time of an authentic standard (labeled and unlabeled), and the tissue content (19.8 ± 1.7 pmol · g^{-1}FW) calculated from its cross-reactivity was similar to that obtained in the (mOH)[9*R*]BAP ELISA (see Fig. 5).

Discussion

The ELISAs for N^6-benzyladenosine and N^6-(*meta*-hydroxybenzyl)adenosine described here have a slightly higher sensitivity than the immunoassays for isoprenoid cytokinins described previously (Hansen et al. 1984, Barthe and Stewart 1985, Cahill et al. 1986, Eberle et al. 1986). Cross-reactivity data revealed that as for other cytokinin antibodies (Weiler 1980, Badenoch-Jones et al. 1984, Turnbull and Hanke 1985) there is a marked selectivity for features of the N^6-side chain, e.g. the presence of a polar group and its position on the side chain but lack of specificity for N^9-substituents (Strnad et al. 1992a). The cross-reactivities of amino acid conjugates are unknown, but they may cross-react and thus presumably could be measured by ELISAs (Badenoch-Jones et al. 1987b). The binding of benzyladenine 7-glucoside to anti-[9*R*]BAP antibodies, as expected, was low (see Badenoch-Jones et al. 1984), but the 3-glucoside was highly immunoactive. Unfortunately, *meta*-hydroxybenzyladenine 3-glucoside was not available for testing in the corresponding assay. In general, other workers have not determined the cross-reactivity of their antibodies with 3-glucosides, but the antiserum against isopentenyladenosine developed by Weiler and Spanier (1981) was

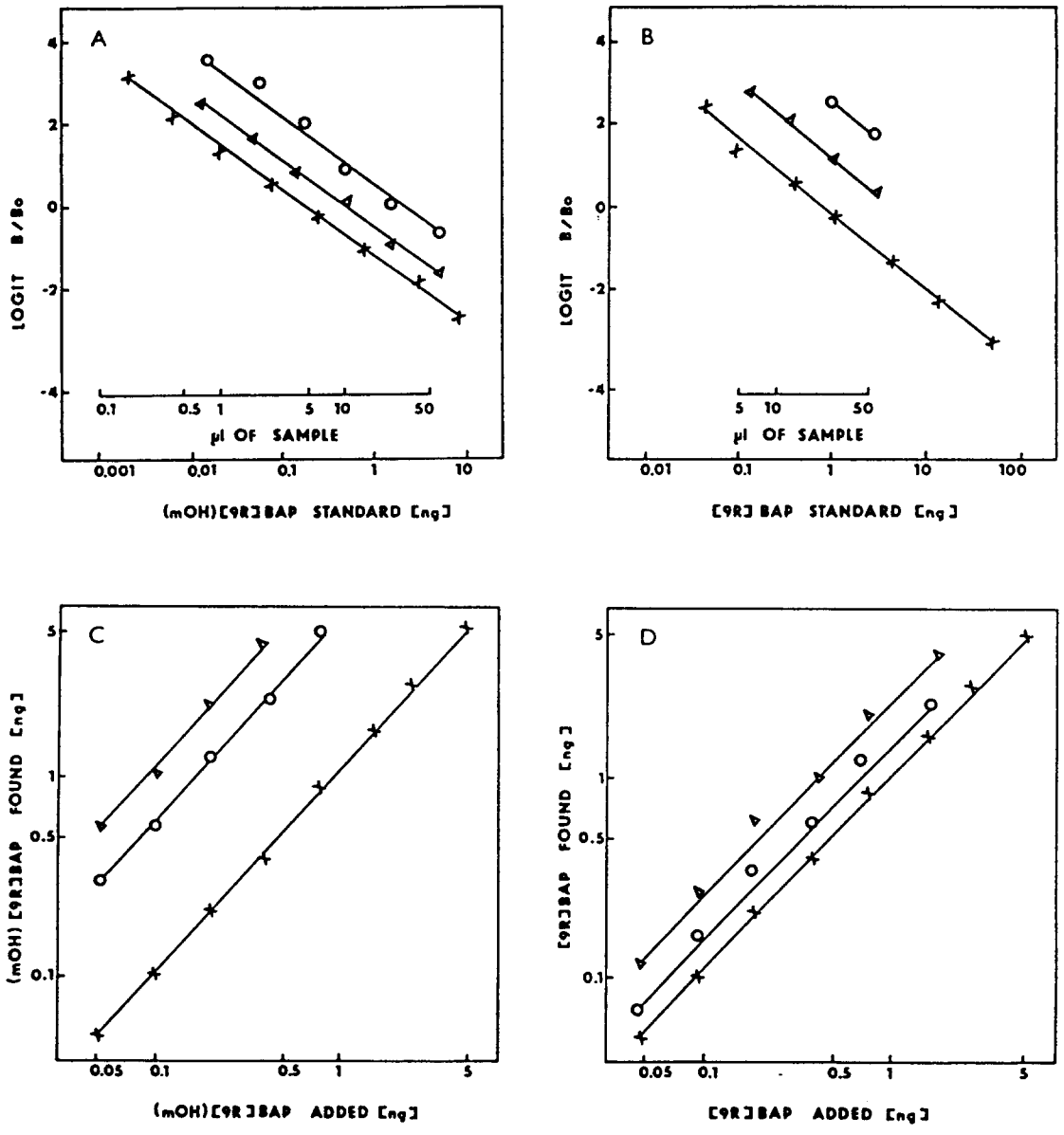

Fig. 2. Validation of the ELISA data for [9*R*]BAP (**right**) and (mOH)[9*R*]BAP (**left**). **A** and **B**, logit transformation of ELISA standard curves (×) and dilution curves of crude extract (○) and HPLC fractions containing corresponding cytokinin (▽).**C** and **D**, internal standardization using different amounts of unlabeled standard added to a fixed amount (50 µL) of crude (○) or HPLC-purified (▽) extract and comparison with the calibration line (×). Logit $B/B_0 = \ln[(B/B_o)/(100 - B/B_0)]$.

reported to show high cross-reactivity with benzyladenine 3-glucoside. The antibodies raised against hydroxylated benzyladenines are highly specific for the N^6-substituent (Strnad et al. 1992b), less so in the case of antibodies to [9*R*]BAP. As with the antibodies against isopentenyladenosine (Ernst et al. 1983a, Sotta et al. 1987) the antibodies against [9*R*]BAP showed moderate cross-reactivity against cytokinins bearing an apolar N^6-side chain such as kinetin, isopentenyladenine, and their N^9-substituted derivatives. The reasons for the high cross-reactivity of *meta*-hydroxybenzyladenines are not yet clear. However, the same degree of cross-reactivity toward (mOH)BAP-type cytokinins was obtained with anti-[9*R*]BAP antibodies raised against 5′-hemisuccinyl and 2′,3′-acetyl [9*R*]BAP derivatives (Siglerová and Strnad 1996, unpublished). The question arises as to whether the benzyl ring of [9*R*]BAP in the antigen might not have been specifically hydroxylated in the *meta*-position during immunization.

To demonstrate the applicability of the assays for the analysis of [9*R*]BAP and (mOH)[9*R*]BAP, young poplar leaves (*P.* × *canadensis* Moech., cv. *Robusta*) and trans-

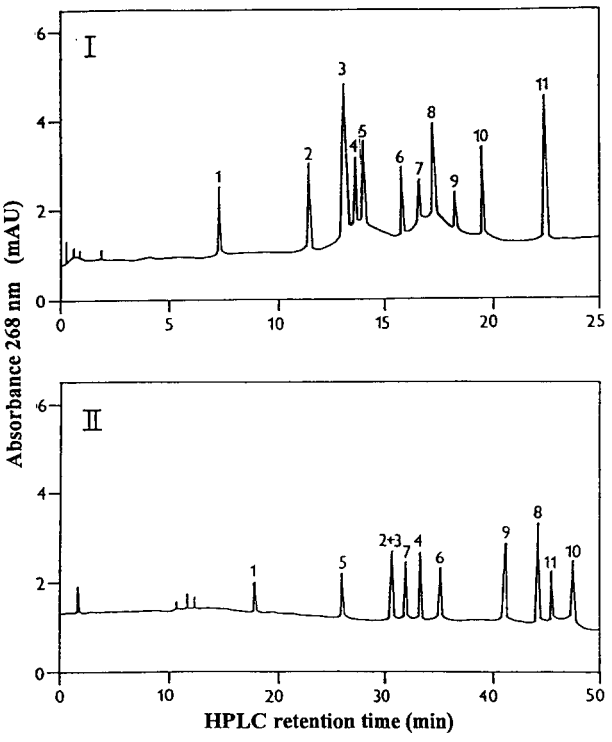

Fig. 3. Reversed phase HPLC separation of aromatic cytokinin standards. Column: 250 × 4.0 mm inner diameter, 7 μm Separon SGX C_{18}; detector at 268 nm. System A: flow rate, 1.0 mL/min, gradient between methanol-TEAA buffer (40 mM, pH 3.35) was 0 min 26%, 15 min 44%, 25 min 56%. System B: gradient between acetonitrile-TEAA buffer (40 mM, pH 3.35) was 0 min 5%, 10 min 7%, 30 min 10%, 40 min 15%, 50 min 14%. Peak numbers: **1,** N^6-(*meta*-hydroxybenzyl)adenine 9-glucoside; **2,** N^6-(*ortho*-hydroxybenzyl)adenine 9-glucoside; **3,** N^6-(*meta*-hydroxybenzyl)adenosine; **4,** N^6-benzyladenine 9-glucoside; **5,** N^6-(*meta*-hydroxybenzyl)adenine; **6,** N^6-(*para*-hydroxybenzyl)adenosine; **7,** N^6-(*para*-hydroxybenzyl)adenine; **8,** N^6-(*ortho*-hydroxybenzyl)adenosine; **9,** N^6-(*ortho*-hydroxybenzyl)adenine; **10,** N^6-benzyladenosine; **11,** N^6-benzyladenine.

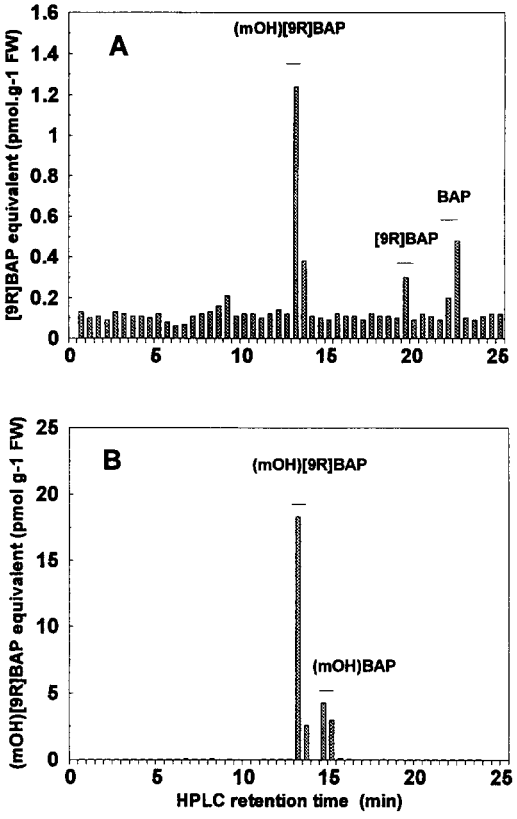

Fig. 4. Immunodetection of aromatic cytokinins in HPLC-fractionated extracts from young *P.* × *canadensis* Moench., cv. *Robusta* leaves by ELISAs for N^6-benzyladenosine **(A)** and N^6-(*meta*-hydroxybenzyl)adenosine **(B)**. HPLC conditions for separation of aromatic cytokinins in methanolic gradient are as in Fig. 3a. Retention times of 2-^{3}H-labeled standards are indicated by **horizontal bars.**

formed *Solanum* plants known to contain aromatic cytokinins (Horgan et al. 1973, Strnad et al. 1992b, Nandi et al. 1989a) were analyzed. Interference in the assay by other compounds in the extracts, as indicated by nonparallelism of the standard curve and sample dilution curves, proved not to be a problem for any sample, even when crude extracts were analyzed by ELISAs. In addition, spiking with authentic standards for internal standardization of either crude extracts or HPLC fractions containing immunoactive substances produced parallel lines, as generally reported for cytokinin immunoassays (Weiler 1980, Badenoch-Jones et al. 1984, Eberle et al. 1986). Moreover, HPLC immunohistograms showed single peaks of immunoreactivity cochromatographing with the corresponding labeled and unlabeled standards. In spite of this evidence supporting the validity of the HPLC-ELISA, the identification of individual compounds in plant extracts by the method should be regarded as tentative for the following three reasons. First, the large number of different cytokinins in extracts makes it difficult to resolve them all unambiguously, especially by collecting fractions. Second, retention times in HPLC are subjected to slight variation due to impurities in the extract, fluctuating temperature, etc. Third, in some cases the levels of individual cytokinins can be extremely high, leading to appreciable immunoreactivity even in ELISAs in which such compounds show low cross-reactivity. It has been suggested that the immunoassays could be used in conjunction with appropriate systems for cytokinin separation (MacDonald et al. 1981, Badenoch-Jones et al. 1984). Clearly, it is important to separate all cytokinin metabolites even when cross-reactivities are lower than 0.1%. For the samples examined by [9*R*]BAP ELISA in the present study, a rechromatographing the putative *meta*-hydroxybenzyladenine-like cytokinins confirmed this (see Fig. 4). Furthermore, we have already detected very high N^6-(*ortho*-hydroxybenzyl)adenosine levels (as much as 1.0

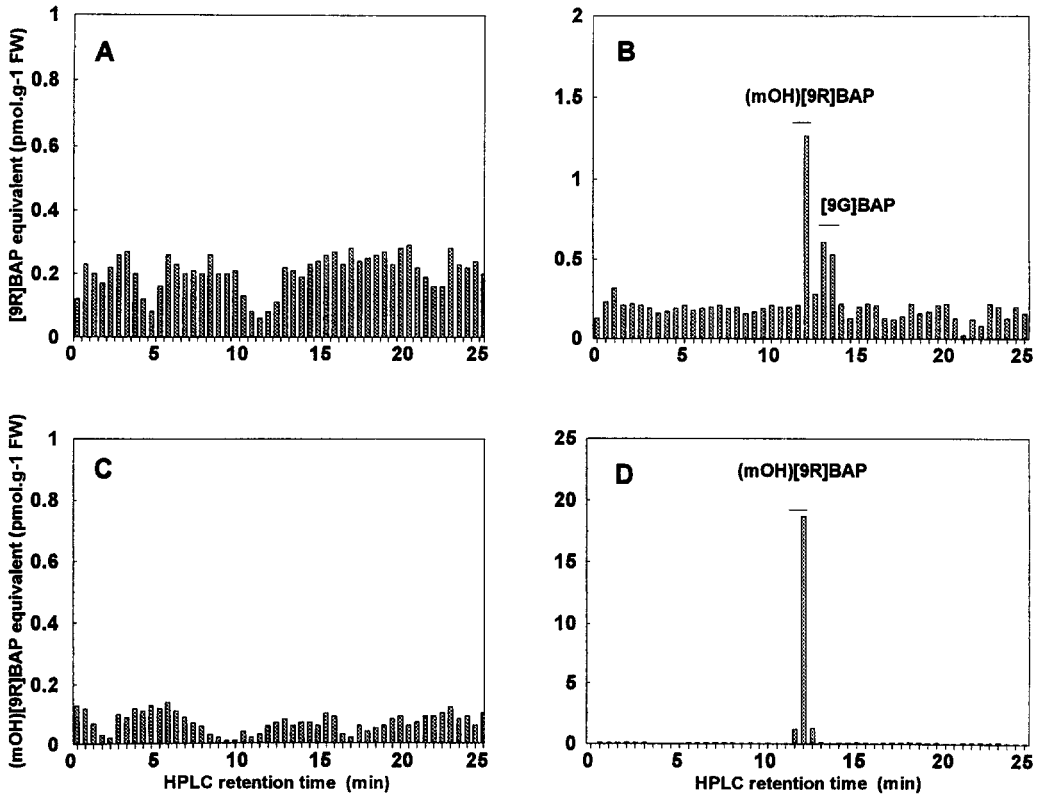

Fig. 5. Immunodetection of aromatic cytokinins in HPLC-fractionated extracts in untransformed (**A** and **C**) and transformed (**B** and **D**) and potato shoots by T-DNA gene 4 (*ipt*) by ELISAs for N^6-benzyladenosine (**A** and **B**) and N^6-(*meta*-hydroxybenzyl)adenosine (**C** and **D**). HPLC conditions for separation of aromatic cytokinins in methanolic gradient are as in Fig. 3a. Retention times of authentic and 2-^{3}H-labeled standards are indicated by **horizontal bars.**

nmol · g^{-1} FW) in HPLC-purified extracts either by appropriate assay (Strnad et al. 1992b) or by ELISAs for zeatin riboside, dihydrozeatin riboside, isopentenyladenosine, N^6-benzyladenosine, and N^6-(*meta*-hydroxybenzyl)adenosine (data not shown). Immunoassays developed in the present study proved to have useful application in locating and estimating the aromatic cytokinin-like substances in HPLC fractions. One method for achieving this in the presence of a high UV background was based on cochromatography of immunoactive substances with authentic radiolabeled standards and had already been developed for estimation of isoprenoid cytokinins (Badenoch-Jones et al. 1987a, Hocart et al. 1988). The addition of 2-^{3}H-labeled cytokinins of the highest activity available (approximately 1.0 TBq · $mmol^{-1}$) to the extracts facilitated detection of the immunoactive fractions, giving better resolution of compounds that elute close together, e.g., N^6-benzyladenine and isopentenyladenine, as well as a measure of the percentage recovery of each cytokinin after purification. Chromatographic procedures used do not generally resolve these cytokinins, and thus the immunoactivity attributed to isopentenyladenine and related derivatives could in some cases turn out to be due to the presence of N^6-benzyladenine-like substances (Nandi et al. 1989b). HPLC-ELISAs of extracts of teratoma shoot culture derived from transformed *Solanum* leaf discs by T-DNA gene 4 (*ipt*) indicated that the high endogenous levels of N^6-benzyladenine-type cytokinins found in crown galls (Nandi et al. 1989a) and teratoma shoot culture (present study) are probably induced by elevated levels of isoprenoid cytokinins (see Ondřej et al. 1990) rather than synthesized by enzymes encoded in T-DNA. T-DNA gene 4 involved in the production of isoprenoid cytokinins may well interfere with wild-type biosynthetic pathways of aromatic cytokinins. Furthermore, it has been postulated that cytokinins induce their own synthesis when present at higher than threshold concentration (Meins and Hensen 1985). The effects of exogenous cytokinins on the accumulation of endogenous zeatin and zeatin riboside which would support this hypothesis have been reported (Mok et al. 1982, Vaňková et al. 1987). Thus, the data obtained in the present study indicate that an increase in isoprenoid cytokinin levels due to transgenesis by T-DNA gene 4 can induce an increase in the level of aromatic cytokinins in plant cells. The identity of

the aromatic cytokinins of potato transformants is under investigation.

The presence of putative N^6-(*meta*-hydroxybenzyl)adenine cytokinins in *P.* × *Robusta* leaves was confirmed by gas chromatography-mass spectrometry (Strnad et al. 1997), but the identification of endogenous N^6-benzyladenine in this tissue is still in progress. The unambiguous identification of aromatic cytokinins and their detection at appreciable levels in mature poplar leaves, fruits of *Zantedeschia aethiopica* (Chaves das Neves and Pais 1980), an old anise cell culture (Ernst et al. 1983b), and crown galls (Nandi et al. 1989b) clearly showed that these cytokinins are present in cells that have ceased cell division. Because of this they have been regarded as senescence-retarding factors (Horgan et al. 1975). The relatively low level of N^6-benzyladenosine and its hydroxylated derivatives in growing *P.* × *Robusta* leaves is at least consistent with this idea.

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