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Receptor-Like Cytokinin-Binding Protein(s) from Barley Leaves

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Abstract. Purified fractions of soluble proteins from barley leaves have been shown to contain specific binding sites for *trans*-zeatin, a natural cytokinin. Such binding is very strong in vitro in concentrated solutions of some salts (ammonium sulfate or potassium phosphate) with optimum at pH 7–8 and temperature within the range 0–20°C. The cytokinin-binding sites have high affinity for zeatin ($K_d \sim 1.5 \cdot 10^{-8}$ M) and low capacity corresponding to 1–1.5 pmol zeatin per milligram of initial soluble protein. Cytokinin binding is reversible; it is due to protein (or proteins) with molecular weight 40–45 kDa. This protein(s) does not bind ^{3}H-adenine and ^{3}H-abscisic acid. The ability of various compounds to displace ^{3}H-zeatin from its high-affinity binding sites is in strict accordance with their biological cytokinin activities. Other phytohormones as well as fusicoccin do not displace ^{3}H-zeatin from its binding sites. Specific zeatin binding is sensitive to heat, alkali, and pronase, but not to RNase treatment. The 150- to 200-fold purification of cytokinin-binding proteins was achieved by a combination of ammonium sulfate precipitation and Ultrogel AcA-54- and DEAE-cellulose chromatography. The zeatin-binding protein(s) from barley leaves is suggested to take part in cytokinin action in vivo.

The primary recognition of hormones at the molecular level suggests the existence of cellular macromolecules ("receptors") capable of binding particular hormones and evoking some active responses from the cells (Kulaeva 1973, Kende and Gardner 1976, Dodds and Hall 1980, Stoddart and Venis 1980). In recent years, many studies have appeared dealing with isolation of proteins that bind phytohormones (Stoddart and Venis 1980), and cytokinins in particular (Kulaeva 1982). At present, cytokinin-binding proteins (CBPs) from wheat germ have been isolated and well characterized (Fox and Erion 1975, Moore 1979, Keim et al. 1981, Polya and Davies 1983). However, some properties of

CBPs from wheat germ argue against their specific cytokinin receptor function. These CBPs have much lower affinity for zeatin than for synthetic cytokinins such as N^6-benzylaminopurine (BAP) or kinetin. Moreover, these CBPs are abundant in embryos (as much as 2 mg/g fresh weight), but absent in cytokinin-sensitive vegetative parts of plants (Polya and Davis 1978, Keim et al. 1981). This has caused investigators to search for proteins that bind natural cytokinins (zeatin), using plant material highly sensitive to cytokinins, i.e., obviously containing cytokinin receptors.

Isolated barley leaves were shown earlier to display high sensitivity to cytokinins: BAP delays leaf senescence and activates RNA as well as protein syntheses in leaf cells (Kulaeva 1973, 1981, 1982, 1985). These results indicate the presence of active cytokinin receptor in barley leaves. We have investigated the nature and main characteristics of cytokinin-binding sites in the cytoplasmic fraction from barley leaves. ^{3}H-*trans*-zeatin of high specific activity was used to detect cytokinin-binding moieties. The ability of a protein fraction from barley leaves to bind ^{3}H-zeatin specifically was shown recently (Romanov et al. 1986 a,b).

Materials and Methods

Plant Material

Barley plants (*Hordeum vulgare* L. cv. Viner) were grown in a soil in growth chambers. The external factors were programmed for humidity, 70%; length of light period, 16 h/day; light intensity, ~50 W/m^2; day temperature, 22–23°C; and night temperature, 18°C. For each experiment usually 200 g of 8- to 10-day-old first leaves was used.

Extraction and Purification of Soluble Proteins

Leaf homogenization and all subsequent operations, unless otherwise indicated, were performed strictly at 2–4°C. The extraction buffer (Buffer A) was composed of 50 mM KCl, 2 mM 1,4-dithiothreitol, 5 mM EDTA, 2.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.7, at 2–4°C, 0.1% (w/w) L-ascorbic acid, and 0.2 mM phenylmethylsulfonyl fluoride. The fragmented leaves were homogenized in an MPW 302 blender (Poland) at 16,000 rpm for two 3-min periods. Buffer A: plant material ratio was 1.4–1.5 (wt/wt). Before homogenization, 5–10% (w/w) of Polyclar AT (Serva) was added to leaf pieces. The slurry was filtered through two layers of unbleached calico and centrifuged at 23,000 *g* for 40 min. The supernatant was brought to 90% (0°C) ammonium sulfate saturation by rapid addition of granular salt (pH was maintained at ~7.5 by addition of conc. NH_4OH) and kept at −10°C for several hours. The mixture was then centrifuged at 23,000 *g* for 40 min, and the pellet was redissolved in 30–50 ml of Buffer A (undissolved particles were removed by centrifugation) and applied to a G-50 Sephadex column (3.5 × 40 cm) equilibrated in the same buffer. The column was eluted with Buffer A at a flow rate of ~100 ml/h. The pooled

protein fractions eluting in the column void volume were utilized in subsequent experiments.

Binding Activity Assay

^{3}H-*trans*-zeatin (6-(4-hydroxy-3-methyl-2-butenylamino) [8-^{3}H]purine, specific activity 95 GBq/mmol) was received from the Institute of Nuclear Biology and Radiochemistry (ČSSR). ^{3}H-zeatin was obtained from *trans*-zeatin (Sigma) by catalyzed tritium exchange (Černý et al. 1978). Labeled zeatin was purified using reverse-phase high-performance liquid chromatography. The final product contained no less than 90% ^{3}H-*trans*-zeatin with tritium mainly in the C8-position of adenine residue. ^{3}H-zeatin was additionally purified just before use by thin-layer chromatography (TLC) on Silufol UV-254 (Kavalier). The purity of radiolabeled zeatin was checked by TLC with water-saturated *n*-butanol as solvent, paper chromatography with isopropanol:H_2O:conc. HCl (130:37:33) as solvent, and gel chromatography on LH-20 Sephadex column with 35% ethanol as eluent. In all cases, not less than 98% of the total radioactivity moved like zeatin. The purified ^{3}H-zeatin was stored in redistilled ethanol at -10°C for several months without appreciable decomposition.

The loss of tritium in aqueous solutions (pH 6–8, 2–4°C) was negligible: no less than 95% radioactivity remained within ^{3}H-zeatin even after prolonged (2–3 weeks) incubation.

Some other labeled ligands were also used in binding experiments: ^{3}H-dihydrozeatin (Amersham), specific activity 1.37 GBq/μmol; [8-^{3}H]adenine (Amersham), specific activity 740 GBq/mmol; D,L-*cis*, *trans*-[G-^{3}H]abscisic acid (Amersham), specific activity 814 GBq/mmol; and benzyl[8-^{14}C]adenine (Amersham), specific activity 2.0 GBq/mmol. The purity of all labeled compounds used was checked by TLC. Immediately before use ^{3}H-zeatin was redissolved in Buffer A (~6 pmol for each assay) and mixed with soluble proteins at 2–4°C. After incubation, 4–9 volumes of cold 100% saturated ammonium sulfate in water (pH 7.5) was added, followed by centrifugation at 23,000 *g* for 20 min. The supernatant was discarded and the pellet was rewashed with 2–3 ml of cold 90% saturated ammonium sulfate (the washing procedure may be omitted). All remaining liquid was thoroughly removed from the tube walls and the pellets were solubilized in 1% (w/w) sodium dodecyl sulfate in water and counted.

In some cases, all binding procedures, including incubation of protein with ^{3}H-zeatin, were carried out at concentrated salt solutions. The binding of other labeled ligands to proteins was accomplished in the same way.

Dialysis was performed in Plexiglas cells (TechniLab, Fisher Scientific Co.) separated into two equal chambers by dialysis membrane (Serva). An aliquot (1–3.5 ml) of protein-containing solution was placed on one side of the cell and the opposite side was filled with the same solution, but without protein. Both sides contained equal concentrations of ^{3}H-zeatin. The cells were continuously shaken at 2–4°C; 0.2-ml aliquots were then taken from each chamber for radioactivity counting in the course of hormone translocation (dialysis kinetics) or after the equilibrium was attained (equilibrium dialysis).

Column Chromatography

All fractionation procedures were carried out at 2–4°C using the LKB chromatographic system. Gel filtration was performed using Ultrogel AcA-54 (LKB). The exclusion volume of the column (2.6 × 100 cm) was determined with dextran blue 2000 (Pharmacia). For column calibration, standard reference proteins (Pharmacia) were employed. The column was equilibrated in a low-salt buffer, and 100–150 mg protein in 10 ml of the same buffer was applied to the column. Proteins were eluted with constant flow rate (40–50 ml/h) and monitoring eluent at 278 nm. Fractions of 5–7 ml were collected and analyzed for protein content and hormone binding.

Ion-exchange chromatography was performed using DEAE-cellulose column (0.8 × 2 cm). DEAE-cellulose (Whatman DE-52) was prewashed with 1 M $(NH_4)_2SO_4$ (pH 8) containing 10 mM EDTA. The column was then equilibrated in a low-salt buffer. The diluted protein solution in the same buffer was applied to the column. The column was subsequently eluted with the initial buffer (25 ml/h) followed by a linear gradient (0–0.25 M) of ammonium sulfate in the same buffer. Fractions of 1–5 ml were collected and analyzed as above.

Analytical Techniques

All salts used were of analytical grade. Concentrated salt solutions in 50 mM Tris-HCl and 1 mM EDTA were purified with Whatman GF/C filters. Solutions were then cooled to 2–4°C and pH was adjusted to 7.2–7.4 by an appropriate acid or base (e.g., potassium phosphate solution with KOH and phosphoric acid). For protein precipitation, zinc salt or polyethylene glycol was also used. The precipitation by zinc salt was performed using 10% $ZnSO_4$ together with 0.3 M $Ba(OH)_2$, the final $ZnSO_4$ concentration being 1% (pH 8.0–8.1) (Roe 1955). Protein precipitation with polyethylene glycol was performed using PEG 6000 (Merck), the final PEG concentration being about 12% (w/v). Minicolumns (PD-10, LKB) filled with Sephadex G-50 fine were used for protein desalting as well as buffer change. Protein was measured by the method of Bradford (1976) using ovalbumin (Sigma) as a standard.

Radioactivity was counted on a 6892 spectrometer (Tracer Analytic) at a counting efficiency of about 35%.

Results

Extensive purification of the initial crude protein extract from barley leaves is necessary for further binding to be detected. The combination of ammonium sulfate precipitation and gel filtration provides rapid and practically complete purification of proteins from low molecular weight contaminants, including inhibitors of specific ^{3}H-zeatin binding (mainly endogenous cytokinins and their derivatives). The purified protein fraction has an absorption spectrum characteristic of protein with an A_{280} of ~1.2 at a protein concentration of 1 mg ml^{-1}.

Purified proteins from barley leaves were tested for their ability to bind ^{3}H-

zeatin specifically. The nonspecific binding in the presence of a 600-fold excess of unlabeled zeatin was subtracted (Romanov et al. 1986a). Different independent procedures were used for assaying specific zeatin binding. Among them, protein precipitation through 80–90% saturated ammonium sulfate always indicated the presence of high-affinity zeatin-binding sites. Protein precipitation by 40% saturated ammonium sulfate demonstrated the absence of an appreciable difference between total and nonspecific ^{3}H-zeatin binding, although not less than 80% of the total protein was pelleted in this solution. These pelletable proteins (fractions 0–40) were unable to bind zeatin specifically even at higher salt concentrations (Fig. 1) (Romanov et al. 1986a). On the contrary, proteins precipitating between 40 and 90% (or 80%) saturated ammonium sulfate (fractions 40–90) exhibited distinct ability to bind specifically ^{3}H-zeatin, which was 4- to 6-fold increased as compared with the initial protein solution. This binding versus protein concentration was linear (Fig. 1). Dialysis was used to verify the validity of results obtained by ammonium precipitation assays. Equilibrium dialysis in the presence of 80% ammonium sulfate saturation demonstrated a significant ^{3}H-zeatin translocation from pure solution to the protein-containing one. The specific binding activity determined by equilibrium dialysis was close to that determined in parallel by precipitation assay (Table 1). In low-salt buffers (i.e., in the absence of concentrated ammonium sulfate), hormone did not accumulate in the protein-containing chamber. Moreover, in the course of dialysis in low-salt solution, a progressive loss of zeatin-binding activity was shown using the ammonium precipitation test: 16-h and 38-h dialysis resulted in no more than 50% and 20–25% of the initial activity, respectively. This lability of CBPs makes the equilibrium dialysis method in low-salt solution unsuitable for quantitative characterization of zeatin-binding sites.

Using a dialysis cell makes it possible to study the influence of some salts and other compounds on the zeatin-binding activity of fraction 40–90. As shown, fraction 40–90 exhibited zeatin-binding capacity not only in the presence of concentrated ammonium sulfate solution (80% saturation), but also in the presence of concentrated potassium phosphate (80% saturation)—quite a different salt—although in the last case the binding was less intensive. But in the presence of other salts, which also quantitatively precipitate proteins–sodium citrate (80% saturation) and a mixture of $ZnSO_4$ and $Ba(OH)_2$, protein precipitates failed to bind ^{3}H-zeatin. The same was observed in the presence of polyethylene glycol (12%, w/v). The specific effect of different salts on the ^{3}H-zeatin binding by fraction 40–90 is clearly shown in Fig. 2, where the redistribution kinetics of ^{3}H-zeatin between buffer- and protein-containing chambers of the dialysis cell are demonstrated.

Among different commercial proteins (ovalbumin, RNase, pronase, concanavalin A, bovine serum albumin), only BSA was able to bind ^{3}H-zeatin specifically, although its affinity for hormone was of an order of magnitude less than that of crude protein fraction 40–90. This property of albumin was not unexpected because its ability for binding different low molecular ligands, including phytohormones, has been established (Murphy 1979).

The influence of pH and temperature on ^{3}H-zeatin binding is demonstrated in Figs. 3 and 4. In both cases, only specific binding was sensitive to changes in

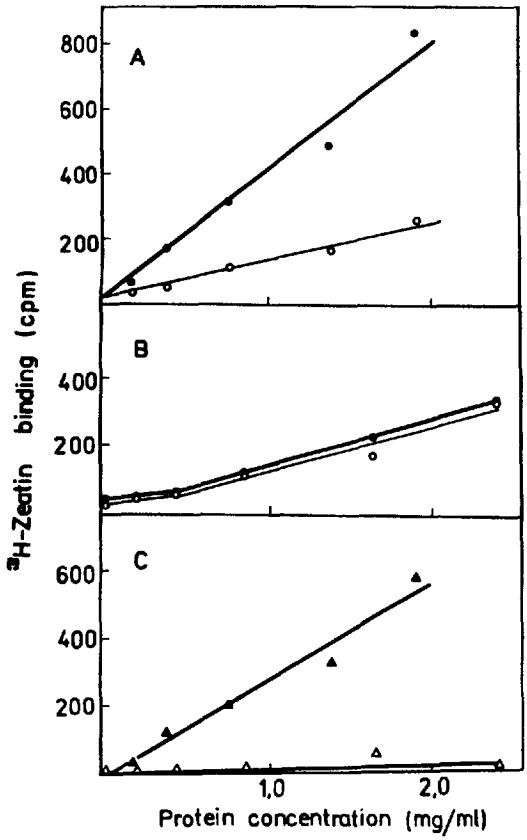

Fig. 1. ^{3}H-zeatin binding relative to protein concentration of fraction 40–90 (precipitated by between 40 and 90% saturated ammonium sulfate) (A) and fraction 0–40 (precipitated by 40% saturated ammonium sulfate) (B). Total (●) and nonspecific (○) binding is plotted. Specific ^{3}H-zeatin binding (C) by fraction 40–90 (▲) and fraction 0–40 (△) represents the difference between values of corresponding total and nonspecific binding.

environment whereas nonspecific binding was mainly invariable. The maximal binding was observed at pH 7–8 (Fig. 3) and 0–20°C (Fig. 4).

The incubation of protein solution at 70°C for only 5 min results in nearly complete and irreversible loss of binding activity, as does the short alkali treatment (0.1–0.2 M NaOH). Heating and alkali treatment obviously cause irreversible denaturation of zeatin-binding sites, which thus appear to be proteinaceous. Incubation of protein solution with ribonuclease has a negligible effect on zeatin-binding activity whereas pronase largely abolishes zeatin binding (Table 2). All this proves that ^{3}H-zeatin-binding sites are proteins or at least contain some protein components.

The change of other salt concentrations has almost no effect on zeatin-binding activity. Neither KCl up to 1 M nor $CaCl_2$ up to 10 mM has an effect. The specific zeatin binding is quantitatively retained in the presence of a small amount of ethanol (up to 7.5% v/v); 6 M urea completely blocked the specific binding of ^{3}H-zeatin.

The apparent dissociation constant (K_d) for ^{3}H-zeatin–CBP interaction was determined by the Scatchard method (Fig. 5). The biphasic curve obtained is

Table 1. Comparison of different methods for determination of ^{3}H-zeatin specific binding to barley leaf CBPs.

Methods	^{3}H-zeatin binding (dpm ± SE)		
	Total	Nonspecific	Specific
Equilibrium dialysis	534 ± 23	265 ± 16	269
Ammonium sulfate precipitation	853 ± 36	610 ± 18	243

Equilibrium dialysis was carried out in a Plexiglas cell divided in half by membrane. One side of the cell contained the protein fraction 40–90 (protein conc. 1.7 mg ml^{-1}) in 80% saturated ammonium sulfate, and the opposite side contained the same volume of 80% saturated ammonium sulfate without any protein. Equal amounts of ^{3}H-zeatin were added to both sides (final conc. ~70 Bq ml^{-1}). Nonspecific binding in the presence of 10^3-fold excess of unlabeled zeatin was also determined. The cell was placed on a shaker at 2–4°C and after 38-h aliquots were taken from each side for radioactivity counting. Precipitation assay was performed by a routine procedure (see Materials and Methods) with samples containing the same concentrations of components as used for the equilibrium dialysis method.

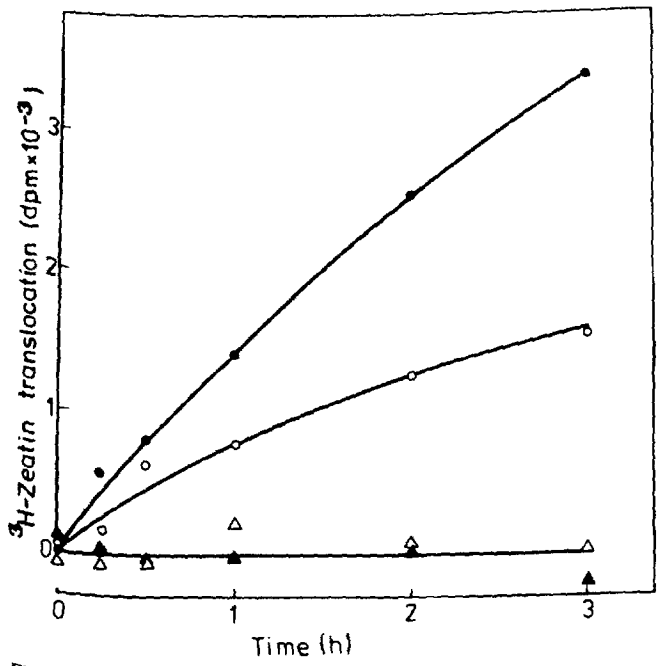

Fig. 2. Kinetics of ^{3}H-zeatin migration from buffer- to protein-containing chambers of the dialysis cell. Both sides of the dialysis cell contained the same salt solution, the protein of the fraction 40–90 being present only on one side. Proteins were desalted by passing through a PD-10 column equilibrated in 10 mM Tris-HCl, pH 7.4, and then mixed with appropriate salt solution. The final protein and ^{3}H-zeatin concentrations in each series were 2 mg ml^{-1} and 1.5 kBq ml^{-1}, respectively. For other details, see Materials and Methods and the legend to the Table 1. The following salts were used: 80% saturated ammonium sulfate, pH 7.4 (●); 80% saturated potassium phosphate, pH 7.4 (○); 80% saturated sodium citrate, pH 7.2 (△); and the mixture of 1% $ZnSO_4$ and 0.06 M $Ba(OH)_2$, pH 8.0 (▲).

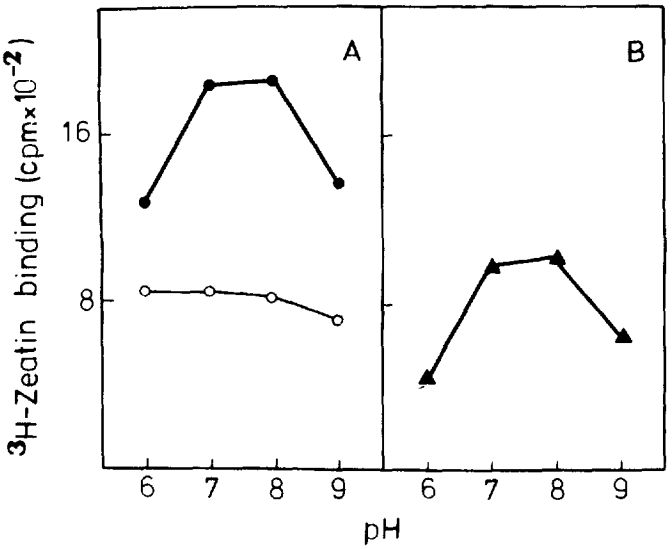

Fig. 3. Dependence of ^{3}H-zeatin binding on pH. Equal amounts of protein (fraction 40–90) were transferred into test tubes (about 0.5 mg protein per tube) and mixed with ^{3}H-zeatin. Equal volumes of concentrated ammonium sulfate solutions were then added to the mixture; pH's of ammonium sulfate solutions were previously adjusted by Tris to values from 6 to 9. The samples were then incubated in the cold for 20 min and treated by a routine procedure. (A) Total (●) and nonspecific (○) binding. The mean values ± SE are presented. Specific binding (B) represents the difference between mean values of corresponding total and nonspecific binding.

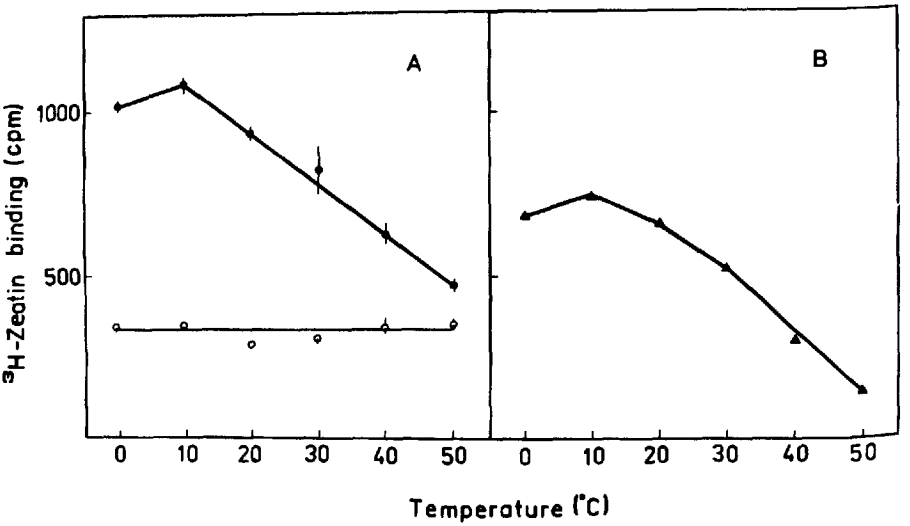

Fig. 4. Effect of temperature on ^{3}H-zeatin binding. ^{3}H-zeatin was added to purified proteins (fraction 40–90) and aliquots were incubated in parallel for 1.75 h at temperatures indicated. (A) Total (●) and nonspecific (○) binding. The mean values ± SE are presented. When no error is given, the SE is smaller than the symbol used. Specific binding (B) is determined as described in Fig. 2.

Table 2. Effect of enzyme treatment on specific zeatin binding.

	Protein concentration after treatment	^{3}H-zeatin specific binding	
	mg ml^{-1}	cpm ± SE	%
None	4.54	237 ± 4	100
RNase	5.10	213 ± 52	90
Pronase	1.74	44 ± 13	19

Pronase (Calbiochem) was preincubated at 37°C for 1 h, and pancreatic RNase (Reanal) was heated at 80°C for 20 min; 0.1 ml of each enzyme solution at concentration 10 mg ml^{-1} was added to 0.5 ml of purified barley leaf protein. Nonspecific ^{3}H-zeatin binding was determined in the presence of 600-fold excess of unlabeled zeatin. The incubation buffer was devoid of phenylmethylsulfonyl fluoride. After the incubation (38°C, 30 min) protein concentrations were checked for and adjusted to nearly equal by ovalbumin addition followed by ammonium sulfate precipitation as described (see Materials and Methods).

characteristic of high-affinity binding sites in the presence of multiple low-affinity sites. Points of high-affinity binding obtained by the Rosenthal method (Rosenthal 1967) give a straight line in a Scatchard graph (Fig. 5). Linearity is indicative of a single class of noninteracting binding sites with a K_d equal to $1.5 \cdot 10^{-8}$ M and a capacity of ~6 pmol/mg protein of fraction 40–90. Taking into account that fraction 40–90 comprises only 15–20% of total soluble protein, the specific binding site content is no more than 1–1.5 pmol/mg solubilized protein from barley leaves.

K_d characteristic of high-affinity interaction (Fig. 5) and temperature suppression of ^{3}H-zeatin binding (Fig. 4) argue against the partial metabolization of labeled hormone and subsequent isotope incorporation into macromolecules. More direct evidence also proves nonartifactual label association with protein: (1) reversibility of binding since the bound hormone can be released from binding sites by gel chromatography or ethanol extraction; (2) no appreciable ^{3}H-zeatin decomposition or modification as judged by TLC of extracted hormone after prolonged incubation with purified barley leaf proteins; and (3) the rapidity of specific ^{3}H-zeatin binding, which appears no longer than after 1 min of incubation with proteins in the ammonium precipitation assay.

The dynamics of ^{3}H-zeatin displacement from binding sites in the presence of various concentrations of unlabeled zeatin, kinetin, and adenine are shown in Fig. 6. Both unlabeled cytokinins actively displace ^{3}H-zeatin in an apparently competitive fashion and with almost similar effectiveness. Hence, zeatin and kinetin have the same affinity for CBP from barley leaves. The ^{3}H-zeatin binding is diminished to a plateau corresponding to nonsaturable binding when the ^{3}H-zeatin/unlabeled cytokinin molar ratio is ~500 or more (Table 3). As these cytokinins are adenine derivatives, unmodified adenine was also tested. Although it displaces ^{3}H-zeatin from specific binding sites, too, the effect of adenine becomes significant only at concentrations more than 2 orders of magnitude higher than that of "classic" cytokinins (Fig. 6).

The binding specificity of CBPs from barley leaves was tested by comparing the ability of various structurally disparate compounds with or without cytokinin activity to displace bound ^{3}H-zeatin. Table 2 shows a strong correlation between cytokinin activity and the affinity for CBP among the compounds

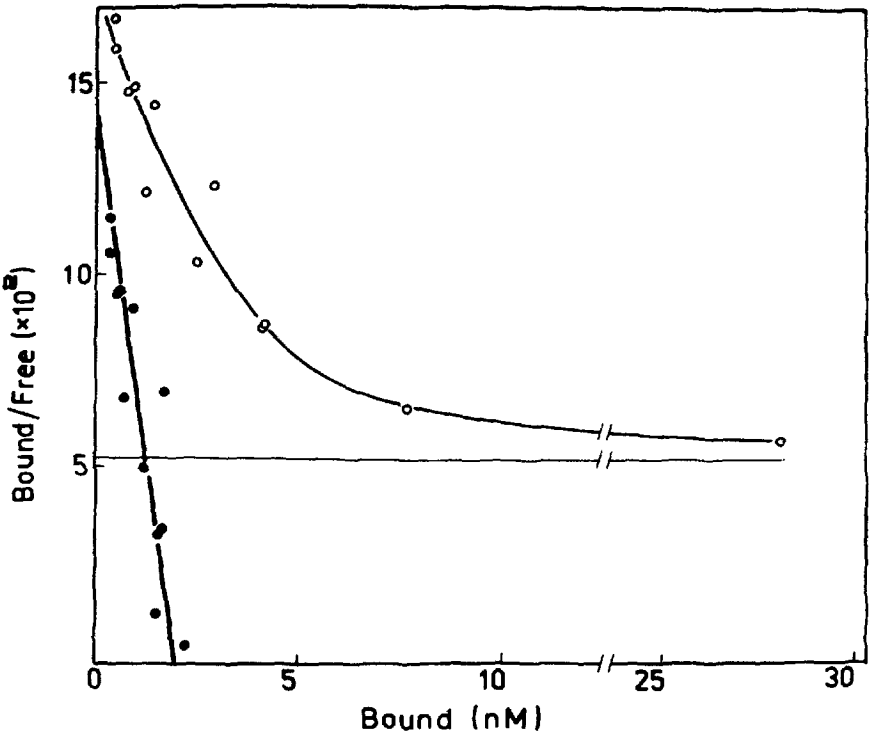

Fig. 5. The Scatchard plot of concentration-dependent ^{3}H-zeatin binding by protein fraction 40–90 (O). A number of test tubes were prepared, all containing the same amount (6 pmol) of ^{3}H-zeatin and various amounts (0–36.5 nmol) of unlabeled zeatin; 0.6 ml of protein-containing solution (protein conc. 3.2 mg ml^{-1}) was added to each tube followed by ~9 volumes of saturated ammonium sulfate, the final volume being 6 ml. After incubation for 60 min at 2–4°C, proteins were pelleted by centrifugation, and the supernatants were thoroughly removed. To ensure conditions close to equilibrium, the precipitate washing was omitted and the initial pellets were directly used for radioactivity counting. The total concentration of the bound ligand is given on the abscissa, and the ratio of bound to free ligands, taken as a ratio between bound and free radioactivities, is plotted on the ordinate. The level of nonspecific ^{3}H-zeatin binding (parallels the abscissa) corresponds to 6000-fold excess of unlabeled zeatin relative to the labeled one. The line of high-affinity binding (●) is plotted according to the method of Rosenthal (1967).

tested. Widely used very potent cytokinins such as zeatin, dihydrozeatin, kinetin, isopentenyladenine, and BAP displace ^{3}H-zeatin most effectively. The compounds of moderate and low cytokinin activities such as BAP riboside, 4-benzylamino-6-methyl pyrimidine, and adenine (Kulaeva 1973) are much weaker inhibitors of specific zeatin binding. Both anticytokinins used have prominent activity to displace bound ^{3}H-zeatin. This means that anticytokinin activity of 4-amino-2-methylamino-2-methyl pyrrolo-[2,3-*d*]pyrimidine derivatives may be due to their ability to compete with zeatin for receptor binding. It should be also taken into account that in some biotests these compounds exhibit not anticytokinin, but true cytokinin, activity (Skoog and Ghani 1981, Kuroedov et al. 1983). No other phytohormones—namely, indole-3-acetic acid and 2,4-D (auxins), gibberellic acid (GA_3), abscisic acid, and fusicoccin—

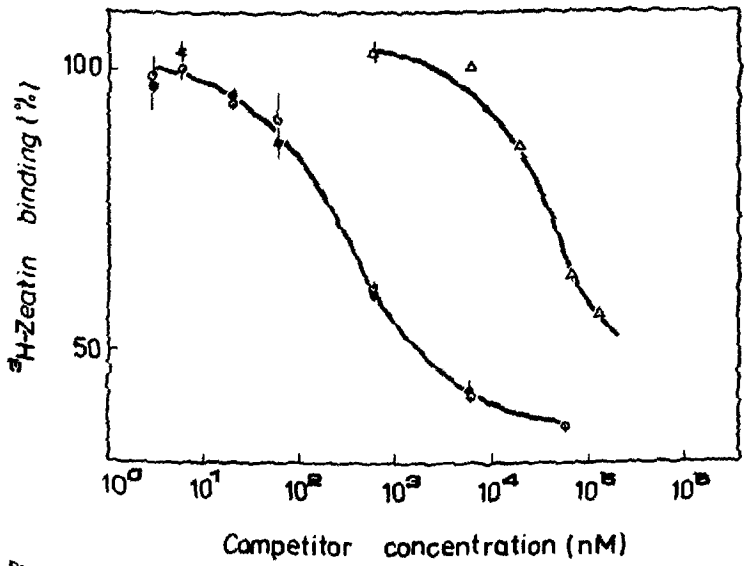

Fig. 6. The displacement of bound ^{3}H-zeatin by unlabeled zeatin (○), kinetin (●), and adenine (△). The concentration of ^{3}H-zeatin was 10 nM. Protein fraction 40–90 was used. The mean values ± SE are presented.

compete with ^{3}H-zeatin for specific binding sites. The effect of some nonhormonal compounds on ^{3}H-zeatin binding to CBPs from barley leaves was also examined. L-tryptophan and tryptamine are ineffective in displacing zeatin from CBPs; the herbicide atrazine (6-isopropylamino-2-chloro-4-ethylamino-*sym*-triazine) possesses a weak inhibitory activity close to that of adenine (Table 2). Thus, CBPs from barley leaves stand in marked contrast to CBPs from wheat germ (Polya and Bowman 1979, Moore 1979, Keim et al. 1981) in regard to their specificity of ligand binding.

For further CBP characterization and partial purification, the proteins of fraction 40–90 were chromatographed on Ultrogel AcA-54. Proteins pelleted in ammonium sulfate were redissolved in Buffer A and immediately applied to an Ultrogel column (100 × 2.6 cm) equilibrated in the same buffer. The eluent was monitored at 278 nm; ^{3}H-zeatin-binding activity in all fractions was determined (Fig. 7). Two main radioactivity peaks were observed. The first peak coincided with the eluting zone of the bulk of proteins and was obviously due to nonspecific binding of ^{3}H-zeatin. The second, greatest peak did not correspond with the profile of the UV-absorbing material and coincided with the eluting zone of the reference protein ovalbumin (molecular weight, 43 kDa). A 600-fold excess of unlabeled zeatin eliminated the second binding peak, confirming that this peak represented specific binding of ^{3}H-zeatin.

Another independent approach, namely, a direct binding experiment, was employed to evaluate cytokinin-binding specificity. Protein fractions from an AcA-54 column were tested for binding of various labeled ligands. The binding with equal amounts of proteins from each fraction occurred strictly under identical conditions (2–4°C, 80% saturated ammonium sulfate, pH 6). The existence of a single binding peak for ^{3}H-zeatin in the elution zone characteristic

Table 3. The displacement of bound ^{3}H-zeatin by various compounds.

Compound	Displacement (%) at molar ratio of 600	Displacement (%) at molar ratio of 6,000	Experimental series no.
Strong cytokinins			
trans-zeatin	100	100	1, 2, 3
N^6-benzylaminopurine	96.1	94.0	1
Kinetin	101.3	90.0	1
Isopentenyladenine	101.0	99.4	1
N-(α-phenylethylamino)purine	109.6	102.6	2
Dihydrozeatin	99.1	—	3
Moderate and weak cytokinins			
N^6-benzylaminopurine riboside	61.3	72.5	1
N^6-benzylaminopurine riboside	62.9	66.4	3
4-Benzylamino-6-methylpyrimidine	44.0	80.4	2
Adenine	20.5	40.8	1
Pyrrolo[2,3-d]pyrimidine derivatives			
4-Cyclopentylamino-2-methylpyrrolo[2,3-*d*]pyrimidine	51.6	81.5	1
4-Cyclopropylmethylamino-2-methylpyrrolo[2,3-*d*]pyrimidine	58.4	102.7	2
Other phytohormones			
Indole-3-acetic acid	0	0	1
2,4-D	—	3.4	2
Gibberellic acid	0	0	1
Abscisic acid (Serva)	0	0	1
Abscisic acid (Sigma)	0	0	2
Fusicoccin	0	0	1
Other compounds			
Atrazine	17.8	44.1	2
L-tryptophan	—	0	2
Tryptamine	—	0	2

^{3}H-zeatin binding was determined at 600- and 6000-fold molar excess of unlabeled compounds. For each ratio, the inhibition of binding by unlabeled zeatin was taken as 100%. Each value is the average of two independent assays; deviations as a rule do not exceed 2–3%. N^6-benzylaminopurine and its riboside, tryptamine-HCl, indole-3-acetic acid, and abscisic acid (± *cis-trans*) were from Sigma; gibberellic acid, abscisin II, and atrazine (2-ethylamino-4-chlor-6-isopropylamino-1,3,5-triazine) were from Serva; *trans*-zeatin and dihydrozeatin were from Calbiochem; kinetin was from Loba Chemie and Roth; L-tryptophan and adenine were from Reanal; 2,4-D (2,4-dichlorophenoxyacetic acid), phenylurea, *N*-(α-phenylethylamino)purine and 4-benzylamino-6-methylpyrimidine were from the USSR. Isopentenyladenine and anticytokinins were kind gifts from Prof. F. Skoog. Fusicoccin was a kind gift from Prof. G. S. Muromtsev. Thidiazuron [*N,N'*-dimethyl-*N*-(5-ethylsulfonyl-1,3,4-thiadiazolyl-2)urea] was a gift from Prof. Yu. A. Baskakov. Each experimental series represents the assays carried out in parallel.

for proteins with an M_r of 40–45 kDa was confirmed (Fig. 8). This peak indicated specific binding because it disappeared in the presence of excessive unlabeled zeatin (Fig. 8A). The similar binding profile with the single peak in the ovalbumin elution zone was obtained with ^{3}H-dihydrozeatin instead of ^{3}H-zeatin or with potassium phosphate instead of ammonium sulfate (not shown). The binding profile of ^{14}C-benzyladenine of low specific activity was inexpressive and similar to that of nonspecific ^{3}H-zeatin binding. This result together with very strong nonspecific adsorption of ^{14}C-BAP on proteins under routine

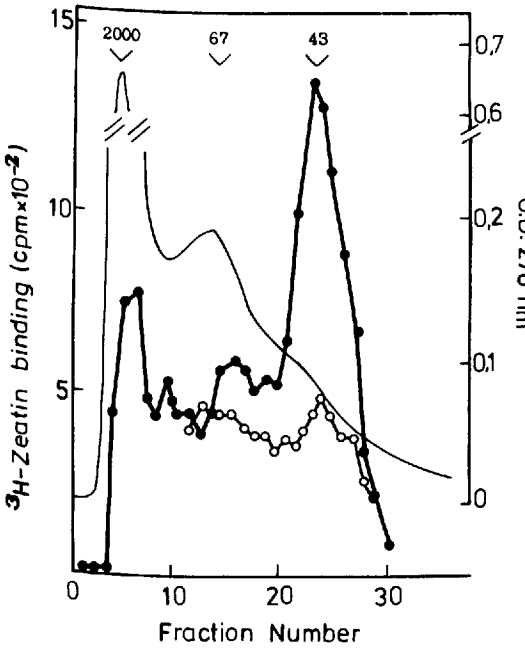

Fig. 7. Chromatography of ^{3}H-zeatin-binding material on Ultrogel AcA-54. Protein fraction 40–90 (~100 mg protein) in 10 ml of Buffer A was applied to a column (530 ml) of Ultrogel AcA-54 and eluted with the same buffer at a flow rate 40 ml h^{-1}. Fractions (5 ml) were collected and 0.5-ml aliquots of each fraction were immediately tested for total (●) and nonspecific (○) ^{3}H-zeatin binding. The solid line represents the O.D. 278 nm protein tracing monitored using LKB Uvicord S II. M_r standards were (at the top): dextran blue, 2000 kDa; BSA, 67 kDa; and ovalbumin, 43 kDa.

binding conditions (Fig. 8B) led us to consider ^{14}C-BAP as useless in revealing CBPs in plant material. The binding profiles of ^{3}H-adenine, a cytokinin-like nonhormonal compound, as well as another phytohormone, ^{3}H-abscisic acid (both of very high specific activity), had nothing in common with the ^{3}H-zeatin-binding profile (Fig. 8). Hence the result of direct binding gave strong evidence of high selectivity of cytokinin binding by barley CBPs with an M_r of 40–45 kDa.

The gel chromatography on Ultrogel AcA-54 has presented an opportunity not only to characterize CBPs, but also their 4- to 6-fold purification. Further CBP purification was achieved using DEAE-cellulose ion-exchange chromatography (Fig. 9). CBP-containing fractions from an AcA-54 column (fractions 22–27, see Fig. 7) were pooled and applied to a DEAE-cellulose column in a low-salt buffer. The bulk of applied proteins was not retarded by the resin and eluted with the initial buffer. This flow-through fraction lacked ^{3}H-zeatin-binding activity (Fig. 9). Another protein fraction eluting from DEAE-cellulose with buffer containing ammonium sulfate (up to 0.1–0.2 M) was enriched by zeatin-binding sites. This procedure provided an additional 5- to 7-fold CBP purification. On the whole, a series of purification steps described above (salting out by ammonium sulfate, gel chromatography on Ultrogel AcA-54, and DEAE-cellulose chromatography) produced an overall 150- to 200-fold purification of CBPs from crude initial leaf extract.

Discussion

Among isolated and well-characterized CBPs, there are soluble cytoplasmic ones (Moore 1979, Chen et al. 1980, Erion and Fox 1981, Klämbt 1981, Reddy

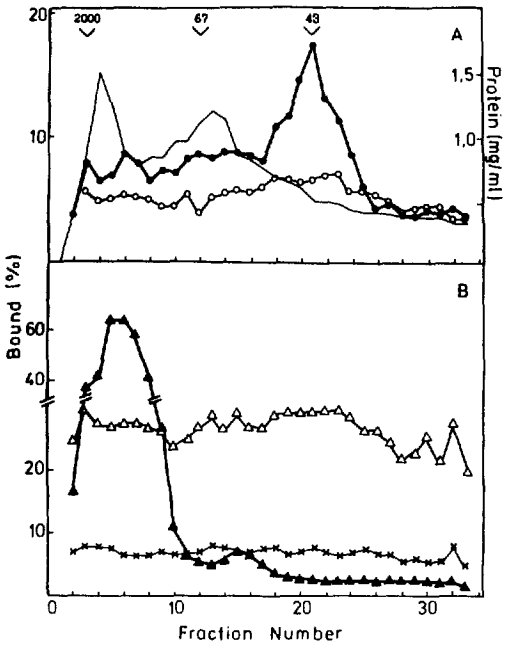

Fig. 8. Comparison of binding of different labeled ligands to protein fraction 40–90 chromatographed on Ultrogel AcA-54. Conditions of gel filtration chromatography were as above (see the legend to Fig. 7), except that protein content was determined in each fraction (A, solid line). Each fraction was then made up to 80% saturated ammonium sulfate by adding dry salt (final pH was ~6) and stored in the cold. Binding activities were assayed with equal amounts (0.25 mg) of protein from each fraction. Ligands were (A) ^{3}H-zeatin, total (●) and nonspecific (○) binding; and (B) ^{3}H-adenine (▲), ^{14}C-benzyladenine (△) and ^{3}H-abscisic acid (×).

et al. 1983), while others seem to be associated with ribosomal (Fox and Erion 1975, Yoshida and Takegami 1977, Chung et al. 1979, Namaguchi et al. 1985), mitochondrial (Keim et al. 1981), and apparently microsomal (Sussman and Kende 1978) fractions of plant homogenate. Barley leaf CBPs described here belong to soluble proteins since they were readily extracted with aqueous solutions and not pelleted by ultracentrifugation even at 160,000 *g*. However, these proteins strongly differ from other known soluble CBPs isolated from the germs or cultivated cells (for a review, see Venis 1985). The most important distinction is their high affinity to zeatin, a natural cytokinin. Furthermore these proteins are distinguished by their molecular mass (cf. Venis 1985) and ligand specificity of binding (cf. Polya and Bowman 1979, Keim et al. 1981, Chen et al. 1980, Moore 1979). An unusual feature of these isolated proteins is their ability to bind cytokinin in vitro in concentrated salt solutions, and in 80–90% saturated ammonium sulfate solutions (3.1–3.5 M) in particular. This property appears not to be unique for phytohormone-binding proteins because some recently described soluble auxin-binding proteins are active only in a high-salt medium (Venis 1984).

Although an opinion was expressed that high concentrations of ammonium sulfate could somehow provoke an artifactual phytohormone binding (Venis 1984), we cannot share this view with respect to our data. Firstly, not only in the presence of ammonium sulfate, but of a quite different salt (potassium phosphate), too, the similar binding of cytokinins by the proteins occurred. This binding can be reproduced by different methods, including equilibrium dialysis. The hormone obviously was not simply trapped in the pellet because similar protein pellets produced by sodium citrate, zinc salt, or polyethylene glycol were not capable of zeatin-specific binding. Secondly, cytokinin binding

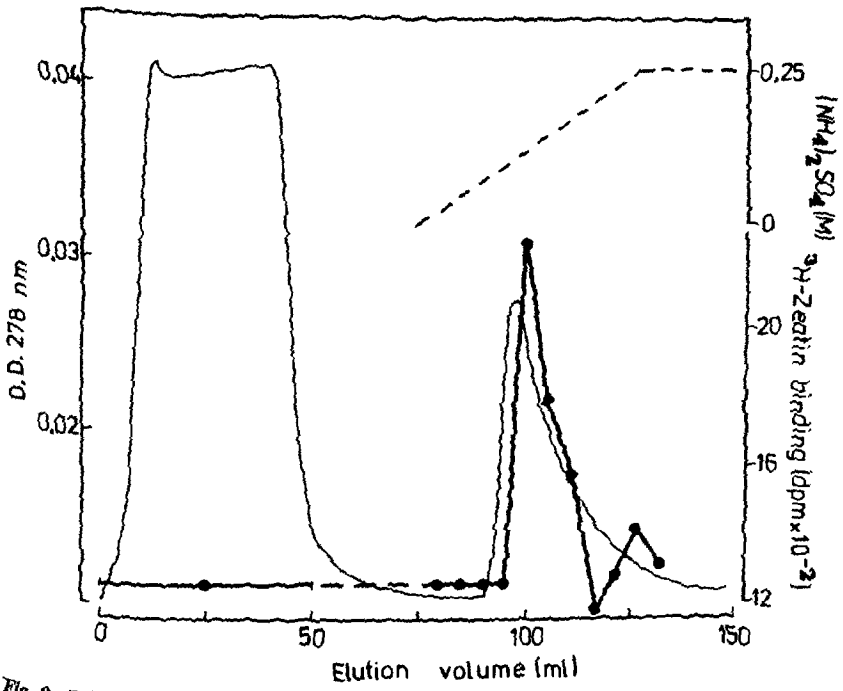

Fig. 9. DEAE-cellulose chromatography of partially purified CBPs. Pooled active protein fractions (*NN* 22–27, Fig. 7) from Ultrogel AcA-54 were applied to the DEAE-cellulose column (0.8 × 2 cm) in Buffer A. The column was eluted (25 ml h^{-1}) with initial buffer followed by a linear gradient (0–0.25 M) of ammonium sulfate in Buffer A. Fractions of 5 ml were collected and assayed for ^{3}H-zeatin binding (●) with ovalbumin (0.5 mg) as a carrier. Optical density (278 nm) is shown by the solid line and ammonium sulfate gradient by the broken line.

is very specific with respect to proteins. Among a great number of soluble proteins in the barley leaves, only a single protein (or a set of similar proteins) with molecular mass of 40–45 kDa was able to bind the cytokinins specifically. Almost all commercially available proteins tested (except albumin, with very low activity) lacked this ability. Thirdly, CBPs from barley leaves have demonstrated a clear ligand specifity of binding. Zeatin exhibited high affinity to cytokinin-binding proteins (apparent $K_d \approx 1.5 \cdot 10^{-8}$ M). Other active cytokinins, for example, BAP, IPA, and kinetin, showed the same high affinity to these proteins. The phytohormones of other types as well as other organic ligands revealed no significant affinity to CBPs. The summarized results of all either direct or competitive binding experiments have demonstrated a clear positive correlation between physiological activity of cytokinins and their affinity to CBPs.

The described CBPs are present in barley leaves that are highly sensitive to cytokinins (Kulaeva 1973, 1982). It appears reasonable to assume these CBPs to be important for the mechanism of cytokinin action. The activity of CBPs under high ionic strength in vitro can be explained, on the one hand, by stabilization of protein conformation necessary for cytokinin binding, with high concentrations of the definite salts. The similar conformation can result in vivo either from interaction of CBPs with specific regulatory micromolecules or

macromolecules or from some cooperative interaction possibly with participation of membranes. On the other hand, it is not excluded that inside a plant cell, too, the cytokinin-binding proteins can be located in some high-salt compartments. Some preliminary experiments have shown that such CBP activity could be revealed in vitro beginning from rather moderate salt concentrations, i.e., 1–1.5 M, of potassium phosphate or ammonium sulfate.

In conclusion, it should be emphasized that CBPs found and partially purified by us have no analogues among other recently described cytokinin-binding proteins (Venis 1985) and possess some properties of receptor proteins, namely, (1) the reversibility of binding and its resistance to medium changes; (2) high affinity to zeatin, a natural cytokinin; (3) clear positive correlation of the affinity to various cytokinins with their bioassay activities; (4) no interaction with other phytohormones; and (5) the low concentration of binding sites detected and their occurrence in a plant tissue (barley leaves) highly sensitive to cytokinins.

It cannot be excluded also that these CBPs can play a very important role as regulators of the concentration of free active cytokinins in plant leaves. Further investigation of receptor-like CBPs and elucidation of the mode of their action on cellular metabolism are needed to clarify the functional role of these proteins.

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