A new family of cytokinin receptors from Cereales

O.N. Kulaeva[a,*], T.K. Zagranichnaya[b], F.A. Brovko[b], N.N. Karavaiko[a], S.Yu. Selivankina[a], Ya.V. Zemlyachenko[a], M. Hall[c], V.M. Lipkin[b], Kh.M. Boziev[b]

[a] *Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya 35, Moscow 127276, Russia*
[b] *Pushchino Branch of Ovchinnikov-Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Pushchino, Moscow region 142292, Russia*
[c] *Institute of Biological Sciences, The University of Wales, Aberystwyth, Dyfed SY23 3DA, UK*

Received 12 January 1998

Abstract The highly specific recognition of a natural cytokinin, *trans*-zeatin, by cytokinin-binding protein (CBP) of 67 kDa from barley leaves was detected with an assay developed on the basis of cytokinin competition in ELISA with anti-idiotype antibodies (raised against antibodies to zeatin) for complex formation with CBP. Monoclonal antibodies (mAbs) raised against 70 kDa CBP from etiolated maize seedlings cross-reacted with barley 67 kDa CBP and prevented barley CBP and *trans*-zeatin induced activation of transcription elongation directed by RNA polymerase I associated with barley chromatin. One mAb (Z-6) had an agonistic effect. Maize CBP replaced barley CBP in activation of RNA synthesis with cytokinin in the barley transcription system. Hence, a new family of cytokinin receptors with common functions and immunodeterminants including maize and barley CBPs was found.

Key words: Cytokinin-binding protein; Anti-idiotype antibody; Monoclonal antibody; Chromatin; RNA polymerase I

1. Introduction

Phytohormones, cytokinins in particular, play an important role in the regulation of plant growth, morphogenesis, and senescence [1]. Attempts to isolate a cytokinin receptor from plants were not successful for a long time. Cytokinin-binding proteins (CBPs) have been isolated from various plant materials, but their functions have not been clarified [2]. A CBP (67 kDa) was isolated in our previous work from the cytosol of barley leaves which, in concert with the natural cytokinin *trans*-zeatin, activated RNA synthesis in the transcriptional elongation system containing chromatin from barley leaves associated with RNA polymerase I. In the presence of *trans*-zeatin, the protein also activated RNA synthesis directed by RNA polymerase I and RNA polymerase II in isolated nuclei from barley leaves [3]. These data together with results on the reversible zeatin binding moiety of the 67 kDa protein provided definitive proof to consider this protein a cytokinin receptor in barley leaf cells responsible for cytokinin activation of transcription elongation directed by both RNA polymerase I and RNA polymerase II. However, the specificity of the 67 kDa CBP interaction with natural and synthetic cytokinins was not studied though this information is absolutely necessary to confirm that the protein is a real cytokinin receptor. For this reason, the first aim of the present work was to study the interaction of cytokinins, their analogs and other phytohormones with the barley 67 kDa CBP. A very sensitive assay was developed based on substance competition with Ab_{a-i}, raised against Ab_z for formation of the complex with 67 kDa CBP in ELISA. The second aim of this work was to study the function of another cytosolic CBP (70 kDa) isolated from etiolated 5-day-old maize seedlings [4] and compare the properties of CBPs from mature barley leaves and growing maize seedlings to elucidate whether these proteins belong to the same receptor family with common functions and immunodeterminants or whether the two CBPs are different in their properties.

2. Materials and methods

Barley plants (*Hordeum vulgare* L., cv. Viener) were growth in soil culture in a growth chamber as described earlier [5]. Fully expanded first leaves of 10-day-old plants were used for CBP isolation. Maize (*Zea mais* L., cv. Elbrus) seeds were germinated on moist filter paper in darkness at 27°C for 5 days [4]. Coleoptiles with primary leaf inside were excised and used for CBP isolation.

Z-Sepharose was prepared by *trans*-zeatin immobilization to Epoxy-activated Sepharose 6B [6]. Ado-T and Zr-T were synthesized as described earlier [4].

Ab_{a-i} were raised against monospecific Ab_z and purified from rabbit antiserum by immunoaffinity chromatography on Ab_z-Sepharose [7].

67 kDa CBP was isolated from barley leaf cytosol (160 000 × *g*, 2 h) by an earlier described procedure including affinity chromatography on Z-Sepharose [3]. CBP was identified at all steps of purification by Ab_{a-i} in direct ELISA.

70 kDa CBP from maize coleoptiles with primary leaf inside was isolated by a previously developed procedure [4], involving affinity chromatography on Ado- and Zr-T columns. CBP was detected during purification by the [^{3}H]dihydrozeatin binding test.

Interaction of cytokinins with 67 kDa CBP was tested by the ELISA technique based on their competition with Ab_{a-i} in the formation of complex with the protein immobilized on a polystyrene microtiter plate.

mAbs were raised against maize 70 kDa CBP as described in [8] according to a routine technique [9,10]. mAbs were isolated from ascites liquid by ammonium sulfate precipitation followed by chromatography on MonoQ HR (FPLC).

For SDS-PAGE of CBPs, the Laemmli procedure was used [11]. 67 kDa CBP was revealed after SDS-PAGE by the Western blot technique [12], using mAbs raised against maize 70 kDa CBP.

Protein content was determined according to Bradford [13].

The effects of CBP and mAbs on RNA synthesis were studied in the transcriptional elongation system containing RNA polymerase I associated with chromatin isolated from barley leaves as described in [14].

*Corresponding author. Fax: (7) (95) 482-1685.
E-mail: vladimir@ad.plantphys.msk.ru

Abbreviations: Ab_{a-i}, anti-idiotype antibodies; Ab_z, antibodies against *trans*-zeatin; Ado-T, adenosine-Toyopearl; BA, 6-benzyladenine; CBP, cytokinin-binding protein; mAb, monoclonal antibody; ZBP, zeatin-binding protein; Z-Sepharose, *trans*-zeatin Sepharose; Zr-T, zeatin riboside-Toyopearl

PII S0014-5793(98)00101-X

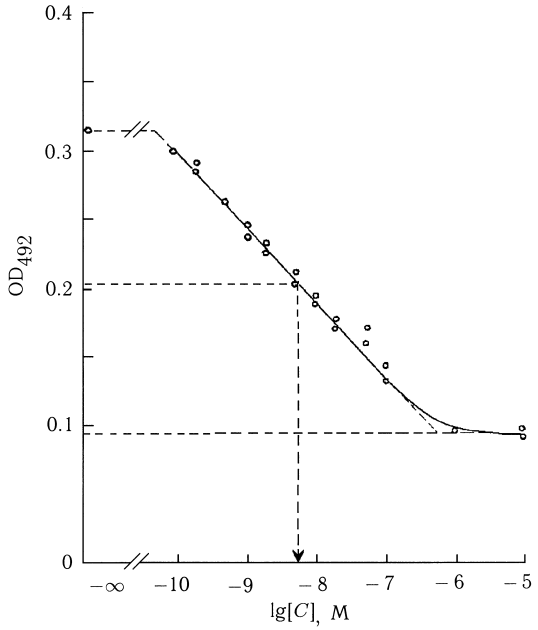

Fig. 1. Decrease of Ab_{a-i} (raised against Ab_z) interaction with barley cytokinin-binding protein (67 kDa) caused by *trans*-zeatin. Data were obtained by competitive ELISA. The concentration of *trans*-zeatin resulting in 50% inhibition of Ab_{a-i} interaction with CBP is marked by an arrow.

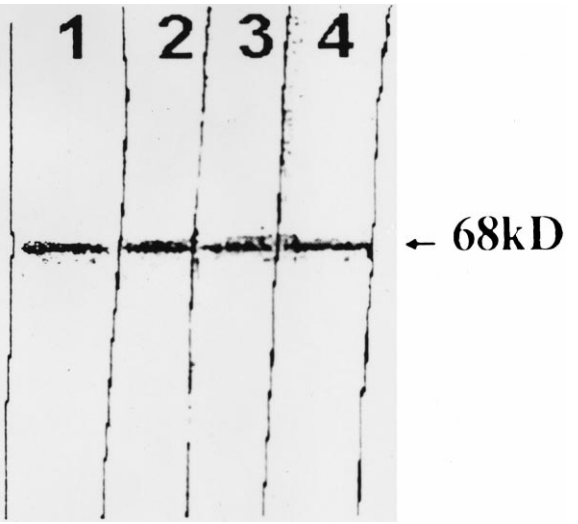

Fig. 2. Western blot of the barley leaf cytokinin receptor (67 kDa) using monoclonal antibodies raised against maize CBP 70 kDa. The position corresponding to BSA 68 kDa, used as a marker, is shown. Z-1, Z-5, Z-6 and Z-8 are different monoclonal antibodies raised against CBP (70 kDa).

2.1. Chemicals

Sephadex G-50 and G-25, Epoxy-activated Sepharose 6B, CNBr-activated Sepharose 4B, Sephacryl S-200, DEAE-Sepharose, electrophoresis calibration kit were from Pharmacia LKB Biotechnology (Sweden). Tris, Tween 20, adenosine, *o*-phenylenediamine, sodium periodate, ovalbumin, *trans*-zeatin, *cis*-zeatin, dihydrozeatin, *trans*-zeatin-riboside, 6-benzyladenine, gibberellin A_3, abscisic acid, indole-3-acetic acid, adenine were from Sigma (USA), Toyopearl HW-60 and Toyopearl HW-65 were from Tosoh (Japan), *trans*-zeatin-*O*-glucoside was kindly provided by Dr. J. Corse (Western Regional Center, USDA Agricultural Research Service, Albany, NY, USA), anti-rabbit peroxidase-labelled immunoglobulins were from the Gamaleya Institute of Epidemiology and Microbiology (Russia). RPMI 1640 medium, selective media HAT and HT, embryonic calf serum, 96-well microtiter plates for ELISA, and 24- and 96-well Linbro plates were from Flow Laboratories (UK). PEG-3000 and dimethyl sulfoxide were from Merck (Germany).

3. Results and discussion

A highly sensitive assay was developed to study cytokinin affinity for CBP. The assay is based on the competition between cytokinins and Ab_{a-i} (raised against Ab_z) for the formation of complex with 67 kDa CBP. CBP (67 kDa) isolated from barley leaves was immobilized on a polystyrene microtiter plate, and cytokinin competition with Ab_{a-i} for complex formation with the protein was estimated by ELISA (Fig. 1). *Trans*-zeatin, a natural cytokinin, competed with Ab_{a-i} in a dose-dependent manner. The concentration of cytokinin resulting in 50% inhibition of Ab_{a-i} interaction with CBP was very low (5×10^{-9} M) and close to that expected from the studies of RNA synthesis activation by *trans*-zeatin-receptor complex [3]. The results demonstrated a high affinity and specificity of *trans*-zeatin interaction with 67 kDa CBP from barley leaves (Table 1). Any changes in the *trans*-zeatin molecule led to a decrease in its affinity for receptor protein. For instance, dihydrozeatin (saturation in side chain) had 30-fold lower affinity for the protein than *trans*-zeatin. *O*-Glucosylation of *trans*-zeatin reduced its affinity for the receptor approximately 100 times, which could be expected from the absence of physiological activity of hormone glucosides.

Cis-zeatin had 200-fold lower affinity for the receptor than *trans*-zeatin. The results confirmed the hypothesis, based on X-ray crystallography data, that, in the *cis*-zeatin molecule, a hydrogen bond can be formed between the OH group of the side chain and N′ of the adenine ring [15]. Such bonding should block *cis*-zeatin interaction with a cytokinin receptor in plant cells. This bonding is conformationally impossible for *trans*-zeatin. Dramatic differences in *cis*- and *trans*-zeatin af-

Table 1
The effect of cytokinins, their analogs and other phytohormones on the interection between Ab_{a-i} (raised against Ab_z) and barley CBP 67 kDa as revealed by ELISA

Compound	Amount of compound resulting in 50% inhibition of Ab_{a-i} interaction with CBP of 67 kDa (pmol)
Trans-zeatin	0.7 ± 0.2
Dihydrozeatin	22.5 ± 3.2
N^6-Benzyladenine	34.5 ± 8.5
Trans-zeatin-*O*-glucoside	60.5 ± 13.8
Cis-zeatin	140 ± 2.2
Adenine	no effect
Gibberellic acid, abscisic acid, indole-3-acetic aid	no effect

Table 2
Effect of maize CBP (70 kDa) and *trans*-zeatin on RNA synthesis in the transcription elongation system, containing chromatin and RNA polymerase I from barley leaves

CBP (1.2 μg)	*Trans*-zeatin concentration (M)	[α-^{33}P]AMP incorporation into RNA (cpm/50 μg DNA)	%
−	−	19 059 ± 262	100
+	−	20 965 ± 944	110
−	10^{-6}	18 849 ± 324	99
+	10^{-8}	38 066 ± 1934	200
+	10^{-7}	41 577 ± 1977	218
+	10^{-6}	57 740 ± 1728	302

finity for 67 kDa CBP found in our experiments confirmed this theoretical prediction.

The approach used in the experiment permitted us to detect a lower affinity of synthetic cytokinin BA for the receptor protein in comparison with *trans*-zeatin, unlike bioassays, which demonstrated a higher activity of BA due to its stability in plant cells [16]. Adenine, an analog of cytokinins lacking their physiological activity, as well as other tested phytohormones (auxin, gibberellic and abscisic acids) had no affinity for the cytokinin-binding site of the CBP, thus confirming its cytokinin-receptor properties.

Therefore, the high specificity of cytokinin interaction with its receptor from barley leaves was established.

It is necessary to emphasize that the developed assay of cytokinin activity based on the cytokinin-receptor interaction is very useful for the study of cytokinin structure/activity relationships and has several advantages in comparison with bioassays, because it does not depend on substance uptake by the cell and their metabolization.

The data on cytokinin-receptor interaction obtained in this assay correlate well with effects of substances in complex with 67 kDa CBP on RNA synthesis in vitro in transcription test systems (data not shown).

A further goal of the work was to elucidate the function of 70 kDa CBP isolated from etiolated 5-day-old maize seedlings and to compare the properties of this protein and the cytokinin receptor 67 kDa from mature barley leaves.

In our previous work we showed that barley 67 kDa CBP mediated cytokinin-dependent activation of RNA synthesis in vitro in elongation of transcription system containing RNA polymerase I associated with chromatin from mature barley leaves [3]. To elucidate maize 70 kDa CBP functions, an attempt was made to substitute barley CBP in the transcription system by maize CBP. The results (Table 2) demonstrated that, in the presence of *trans*-zeatin, maize 70 kDa CBP activated RNA synthesis in the heterologous transcription system. 70 kDa CBP alone and *trans*-zeatin alone were not active. The activation of RNA synthesis by 70 kDa CBP was revealed at a very low concentration of *trans*-zeatin (10^{-8} M) in the reaction medium, showing a high affinity of the cytokinin for the protein.

From these data the conclusion can be drawn that maize 70 kDa CBP could replace the barley receptor of cytokinins in the hormone-dependent activation of RNA synthesis. Therefore, both proteins have similar functions and thus belong to the same receptor family.

A set of mAbs raised against maize 70 kDa CBP recognized the cytokinin receptor from barley leaves as revealed by Western blot analysis (Fig. 2) and by ELISA techniques (data not shown), demonstrating the presence of common immunodeterminants in the proteins under investigation.

These mAbs, being added to the transcription test system from barley, prevented RNA synthesis activation caused by barley 67 kDa CBP in the presence of *trans*-zeatin (Table 3). Hence, the mAbs recognized the barley receptor and inhibited its functional activity. One of the mAbs (Z-6) was found to increase transcription activation induced by *trans*-zeatin and barley cytokinin receptor (67 kDa). Such agonistic effect is known for Abs raised against the peptide corresponding to the auxin-binding site of the auxin-binding protein with properties of the auxin receptor [17]. In this context, mAb Z-6 is of special interest for further CBP studies. mAb Z-6 had practically no effect on barley CBP induced activation of RNA synthesis.

To summarize all the data presented, it is possible to conclude that the 67 kDa CBP from mature barley leaves and the 70 kDa CBP from growing etiolated maize seedlings have common functional and immunological properties and belong to the family of cytokinin receptors mediating the cytokinin-dependent regulation of transcription.

A set of mAbs, raised against maize receptor of cytokinin, permits the epitope analysis of members of this new receptor family.

Acknowledgements: We are very grateful to Dr. J. Corse for the gift of zeatin-*O*-glucoside. This work was partially supported by the Russian

Table 3
Effect of monoclonal antibodies (mAbs) raised against maize CBP of 70 kDa on transcription activation by barley CBP of 67 kDa in concert with *trans*-zeatin in the system containing RNA polymerase I associated with chromatin from barley leaves

CBP	*Trans*-zeatin	mAb	[^{3}H]AMP incorporation into RNA (cpm/50 μg DNA)	%
−	−	−	10 275 ± 780	100
+	+	−	17 515 ± 944	170
+	+	Z-1	7 661 ± 548	75
+	+	Z-15	12 215 ± 641	119
+	+	Z-8	11 483 ± 211	112
−	−	−	9 010 ± 833	100
+	+	−	15 479 ± 922	172
+	+	Z-6	25 038 ± 1987	278
		Z-7	13 523 ± 689	150

Foundation for Fundamental Research (Grants 96-04-50705, 96-04-00128 G and 96-15-98030) and by INTAS Grant 93-678 EXT.

References

[1] Kulaeva, O.N., Karavaiko, N.N., Selivankina, S.Yu., Moshkov, I.E., Novikova, G.V., Zemlyachenko, Ya.V., Shipilova, S.V. and Orudgev, E.M. (1996) Plant Growth Regul. 18, 29–37.
[2] Brinegar, C. (1994) in: Cytokinins: Chemistry, Activity and Function (Mok, D. and Mok, M., Eds.), pp. 217–232, CRC Press, Boca Raton, FL.
[3] Kulaeva, O.N., Karavaiko, N.N., Selivankina, S.Yu., Zemlyachenko, Ya.V. and Shipilova, S.V. (1995) FEBS Lett. 366, 26–28.
[4] Brovko, F.A., Zagranichnaya, T.K., Boziev, Kh.M., Lipkin, V.M., Karavaiko, N.N., Selivankina, S.Yu. and Kulaeva, O.N. (1996) Russ. J. Plant Physiol. 43, 467–473.
[5] Kulaeva, O.N., Karavaiko, N.N., Moshkov, I.E., Selivankina, S.Yu. and Novikova, G.V. (1990) FEBS Lett. 261, 410–412.
[6] Momotani, E. and Tsuji, H. (1992) Plant Cell Physiol. 33, 407–412.
[7] Karavaiko, N.N., Zemlyachenko, Ya.V., Selivankina, S.Yu. and Kulaeva, O.N. (1995) Russ. J. Plant Physiol. 42, 481–487.
[8] Zagranichnaya, T.K., Brovko, F.A., Shepelyakovskaya, A.O., Boziev, Kh.M. and Lipkin, V.M. (1997) Russ. J. Plant Physiol. 44, 1–5.
[9] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
[10] Fazekas de St. Groth, S. (1985) in: Handbook of Monoclonal Antibodies. Applications in Biology and Medicine (Ferrone, S. and Dierich, M.P., Eds.), pp. 9–10, Noyes, Park Ridge, IL.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Selivankina, S.Yu., Romanko, E.G., Ovcharov, A.K. and Kharchenko, V.I. (1982) Sov. Plant Physiol. 29, 274–281.
[15] Chen, C.-M., Jin, G., Andersen, B.R. and Ertl, J.R. (1993) Aust. J. Plant Physiol. 20, 609–619.
[16] Zemlyachenko, Ya.V., Karavaiko, N.N., Maslova, G.G. and Kulaeva, O.N. (1997) Dokl. Akad. Nauk. 353, 261–263.
[17] Venis, M.A., Napier, R.M., Barbier Brygoo, H., Maurel, C., Perrot-Rechenmann, C. and Guern, J. (1992) Proc. Natl. Acad. Sci. USA 89, 7208–7212.