Receptor of *trans*-zeatin involved in transcription activation by cytokinin

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Abstract Zeatin-binding protein (67 ± 2 kDa) was isolated from the cytosol of the first leaf of 10-day-old barley plants. The protein fits to all requirements for a zeatin receptor: (i) it binds [^{3}H]*trans*-zeatin reversibly and specifically, (ii) it is recognized by anti-idiotype antibodies from antiserum raised against *trans*-zeatin, (iii) in concert with 10^{-8} M *trans*-zeatin it activates rRNA synthesis in vitro in a transcription elongation system containing chromatin from barley leaves associated with RNA-polymerase I. In the presence of *trans*-zeatin, the protein activates also RNA synthesis directed by RNA-polymerase I and RNA-polymerase II in isolated nuclei from barley leaves.

Key words: *trans*-Zeatin; Zeatin-binding protein; Transcription; Chromatin

1. Introduction

CBPs were isolated from various plant materials, but their function has not been clarified [1]. Previously, we showed that CBP (28–30 kDa) from barley leaf cytosol activated RNA synthesis in the presence of BA in vitro in the transcription elongation system containing RNA-polymerase I associated with chromatin from barley leaves [2]. This protein was isolated by its affinity to the synthetic cytokinin BA, which significantly differs from the natural cytokinin *trans*-zeatin. For instance, CBP isolated from wheat germs by its affinity to BA possessed very low ability to bind zeatin [1]. That was an argument against the receptor function of this protein.

In this context, the goal of the present work was to isolate a protein(s) using its affinity to the *trans*-zeatin, and to study its possible cytokinin-receptor function. Highly sensitive to cytokinin mature barley leaves were used for this work.

2. Materials and methods

Barley plants (*Hordeum vulgare* L. cv. Viner) were cultivated in soil in growth chambers as described [2]. Fully expanded first leaves of 10-day-old plants were used for ZBP isolation.

Z-Sepharose and adenine-Sepharose were prepared by immobilization of *trans*-zeatin or adenine, respectively, to epoxy-activated Sepharose 6B as described in [3].

Ab_z were isolated from antiserum raised against zeatin by chromatography on ZR-Sepharose. Ab_z were immobilized to CNBr-activated Sepharose 4B. $Ab_{a\text{-}i}$ were isolated by immunoaffinity chromatography on Ab_z-Sepharose from antiserum raised against monospecific Ab_z.

All procedures of ZBPs isolation were carried out at 2–4°C. Barley leaves were homogenized in 3–4 volumes of buffer (50 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 250 mM sucrose, 1 mM PMSF, 5 mM 2-mercaptoethanol, and 0.5 M NaCl). The supernatant (160,000 × *g*, 2 h) was passed through a Sephadex G-50 column (buffer: 20 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 0.5 M NaCl, and 5 mM 2-mercaptoethanol) that was followed by hydrophobic chromatography on Phenyl-Sepharose. ZBP was identified at all steps of isolation by its interaction with $Ab_{a\text{-}i}$ in direct ELISA. The fraction containing protein(s) interacting with $Ab_{a\text{-}i}$ was eluted from Phenyl-Sepharose with dist. water. ZBP was purified by affinity chromatography on Z-Sepharose. The protein fraction was applied to affinity matrix in 20 mM Tris-HCl, pH 7.7, with 20 mM NaCl. ZBP was eluted from Z-Sepharose with 1 M NaCl in 50 mM Tris-HCl, pH 7.7, dialyzed against 20 mM Tris-HCl, pH 7.7, and used for analysis. For non-denaturing PAGE of ZBP, the Laemmli procedure [4] was used, but SDS was excluded from the system. The gels were stained as described earlier [2].

Cytokinin-binding properties of isolated proteins were tested by their ability to interact with $Ab_{a\text{-}i}$ from antiserum raised against zeatin as described in [2] and by [^{3}H]*trans*-zeatin-binding assay [5]. [^{3}H]*trans*-zeatin (sp. act. of 95 GBq/mmol) was synthesized in the Institute of Nuclear Biology and Radiochemistry (Czech Republic).

Protein content was determined according to Bradford [6].

The effects of ZBP and cytokinins on the RNA synthesis were studied: (i) in the transcription elongation system containing RNA-polymerase I associated with chromatin from barley leaves [7] and (ii) in isolated nuclei from barley leaves under conditions optimized for RNA-polymerase I or RNA-polymerase II [8]. Chromatin was isolated from 10-day-old barley leaves as described previously [7]. Nuclei were isolated according to [9].

Biochemicals: Sephadex G-50, epoxy-activated Sepharose 6B, CNBr-activated Sepharose 4B, electrophoresis calibration kit were from Pharmacia LKB Biotechnology (Sweden), ovalbumin, *trans*-zeatin, *cis*-zeatin, were from Sigma (USA), antirabbit peroxidase-labelled immunoglobulins were from Gamaleya Inst. of Epidemiology and Microbiology (Russia).

3. Results and discussion

The protein revealed by non-denaturing PAGE as a single band (Fig. 1) with molecular mass of 67 ± 2 kDa was isolated from barley leaf cytosol (160,000 × *g*, 2 h). The protein interacted with $Ab_{a\text{-}i}$ in immunoblotting procedure (data not shown) and in direct ELISA (Fig. 2). Proteins isolated using control adenine-Sepharose column were not recognized by $Ab_{a\text{-}i}$. 67 kDa protein did not interact with preimmune serum and Ab_z used as control antibodies which could not recognize zeatin-binding site. Hence, 67 kDa protein interacted with $Ab_{a\text{-}i}$ very specific, and this enables us to conclude that the protein has a zeatin-binding site. This conclusion was confirmed by [^{3}H]*trans*-zeatin-binding assay (Fig. 3). 67 kDa protein bound [^{3}H]*trans*-zeatin reversibly. 100-fold excess of unlabeled *trans*-zeatin decreased the sample radioactivity to the level of nonspecific label absorption by ovalbumin added as a carrier for protein precipitation with ammonium sulfate.

The functional activity of ZBP was tested in RNA synthesis assay (Fig. 4). In the presence of *trans*-zeatin, the ZBP activated RNA synthesis in vitro in the transcription elongation system

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Abbreviations: BA, 6-benzyladenine; CBP, cytokinin-binding protein; Ab_z, antibodies against *trans*-zeatin; $Ab_{a\text{-}i}$, anti-idiotype antibodies; ZBP, zeatin-binding protein; Z-Sepharose, *trans*-zeatin-Sepharose.

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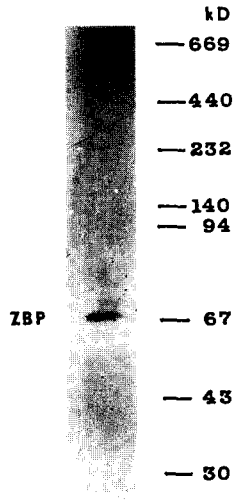

Fig. 1. Non-denaturing PAGE (4–20%) of barley leaf cytosol protein purified on Z-Sepharose. The gel was stained with Coomassie blue R-250. Numbers indicate molecular mass of marker proteins (kDa).

containing RNA-polymerase I associated with chromatin from barley leaves. The ZBP and *trans*-zeatin added separately, had no effect. Transcription activation was strongly dependent on *trans*-zeatin concentration with the maximum response at 10^{-8} M, indicating a high affinity of *trans*-zeatin to the protein.

To check the specificity of ZBP-*trans*-zeatin interaction, the effects of *trans*-zeatin and its analog *cis*-zeatin on transcription regulation were compared. According to X-ray crystallography data [10], a hydrogen bond can be formed between the OH-group of the side chain and N′ of the adenine ring in *cis*-zeatin molecule that should block its interaction with cytokinin-receptor. Such binding is conformationally impossible for *trans*-zeatin. Therefore, *cis*-zeatin could be considered as the best control substance to study the specificity of *trans*-zeatin-receptor interaction. The negative results obtained with *cis*-zeatin (Fig. 4) showed that the ZBP specifically recognized *trans*-zeatin resulting in active cytokininreceptor complex formation.

The ZBP in concert with *trans*-zeatin activated also RNA synthesis in nuclei isolated from barley leaves in the conditions

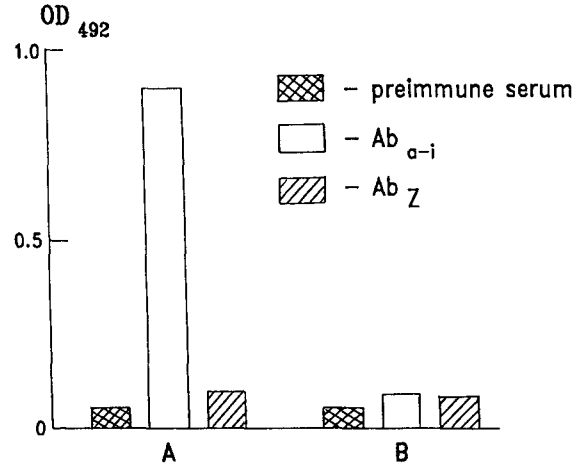

Fig. 2. Interaction of Ab_{a-i} from antiserum raised against zeatin with barley leaf cytosol proteins purified with Z-Sepharose (A) and adenine-Sepharose (B). Data were obtained in direct ELISA.

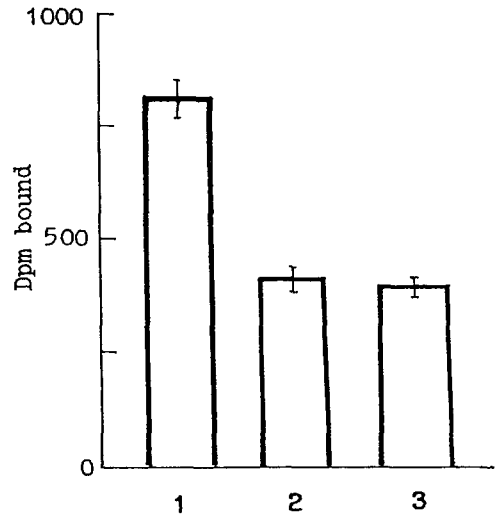

Fig. 3. [^{3}H]*trans*-Zeatin binding to 67 kDa protein isolated from barley leaf cytosol. Incubation medium (200 μl) contained 50 mM Tris-HCl (pH 7.9), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, ZBP (4 μg), [^{3}H]*trans*-zeatin 5.6×10^{-9} M. Ovalbumin 30 μg was added as a carrier for protein precipitation by ammonium sulfate. For the competition assay 2.5×10^{-7} M cold *trans*-zeatin was used. The test system contained: (1) ZBP, ovalbumin, [^{3}H]*trans*-zeatin; (2) ZBP, ovalbumin, [^{3}H]*trans*-zeatin, unlabeled *trans*-zeatin; (3) ovalbumin, [^{3}H]*trans*-zeatin. Bars represent standard error of means (n = 3).

optimized for RNA-polymerase I as well as RNA-polymerase II (Table 1). The experiments with alfa-amanitin confirmed that the ZBP-*trans*-zeatin-complex activated RNA synthesis directed by both RNA-polymerases (Table 1).

As it was shown in our preliminary experiments, RNA synthesis in isolated nuclei as well as in the system containing RNA-polymerase I associated with chromatin is a result of transcription elongation.

To our knowledge, the data presented are the first conclusive evidence for ZBP, which fits to all requirements for zeatin receptor: the protein reversibly and specifically binds *trans*-zeatin, resulting in complex formation that activated in vitro

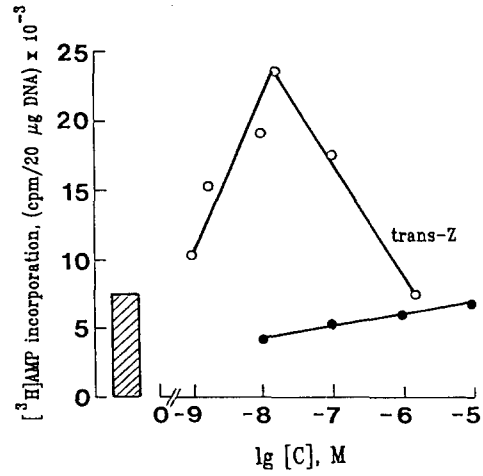

Fig. 4. The effect of *trans*-zeatin and *cis*-zeatin on ZBP-mediated regulation of RNA synthesis in vitro in the system containing chromatin-bound RNA polymerase I from barley leaves. The shaded column represents control levels of RNA synthesis.

Table 1
Effect of the ZBP and *trans*-zeatin (10^{-7} M) on RNA synthesis in isolated nuclei from barley leaves under conditions optimized for RNA-polymerase I and RNA-polymerase II

ZBP	*trans*-Zeatin	α-Amanitin (4 μg/ml)	[α-^{33}P]AMP incorporation into RNA, cpm per 20 μg chromatin DNA	
			RNA-polymerase I	RNA-polymerase II
−	−	−	5,350 ± 210 (100)	9,720 ± 350 (100)
+	−	−	5,060 ± 170 (95)	9,460 ± 310 (97)
−	+	−	5,230 ± 230 (98)	11,060 ± 440 (114)
+	+	−	17,400 ± 640 (325)	36,300 ± 1,020 (373)
+	+	+	15,930 ± 580 (298)	3,120 ± 110 (32)
−	−	+	5,030 ± 180 (94)	3,600 ± 320 (37)

Results are mean ± S.E.M. (n = 3), each individual assay being performed in triplicate. There are percent of control within parentheses.

transcription elongation by RNA-polymerases I and II. These in vitro obtained results correspond to in vivo cytokinin-induced activation of RNA synthesis in leaf cells. Hence, we suggest that the *trans*-zeatin receptor isolated from barley leaves should be a transfactor mediating cytokinin-dependent activation of transcription elongation in leaf cells. In the context of this conclusion it is necessary to emphasize that, according to the latest data, stimulation of transcription elongation is important for regulation of gene expression in animal cells [11].

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