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A Modified Enzyme Immunoassay Method for Determination of cAMP in Plant Cells

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1. Introduction

A substantial experimental material shown that an adenylate cyclase signaling system is active in plants (Yavorskaya and Kalinin, 1984; Cooke et al. 1994; Newton et al., 1999; Moutinho et al., 2001; Richards et al., 2002). Convincing data have been obtained, which are related to the main components of the scrutinized system: cAMP, adenylate cyclase, phosphodiesterase, cAMP-binding proteins (Brown et al., 1980; Polya and Bowman, 1981; Phedenko et al., 1983; Yavorskaya and Kalinin, 1984; Tarchevsky, 2001), nucleotide-gated channels (Martinez-Atienza et al., 2007). The concentration of the endogenous cAMP is an indicator of the functional activity for this signaling. Several methods are known to be used to the aim of its determination, of which Gilman's method was the most popular (Gilman, 1970). This method is based on the competitive replacement of the unlabelled nucleotide, which is present in the complex containing the cAMP-binding protein in the test sample, with 8-^{3}H-cAMP. The method of radioimmune dilution (Rosenberg et al., 1982) represents another approach to the analysis. It is based on the primary binding together the standard antigen, which is present in excess and is generally labeled with a radioactive iodine isotope, with antibodies, which are deficient. On the next stage, the unlabeled antigen is added to the generated complex, which competitively supplants the radioactive label. On the basis of a decrease in the radioactivity observed, is make conclusion about the amount of the bound antigen. More rarely the following complex and labour-consuming methods are applied: mass spectrometry and high-performance gas-liquid chromatography (Newton et al., 1980), and also the bioluminescent method allowing to monitor the dynamics of cAMP in the living cell (Nicolaev and Lohse, 2006). Application of the all mentioned methods, in general, requires multi-step purification with the use of different ion-exchange resins, high voltage electrophoresis that can result in partial loss of the cyclic nucleotide. Therefore some authors, having detected cAMP with the use of enumerated methods, note that the results achieved are obviously underestimated (Yavorskaya and Kalinin, 1984). In case of application of Gilman's method and the method of radioimmune dilution there appears the hazard of radioactive pollution of the environmental. Furthermore, and this quite important, it is necessary have to possess expensive equipment in order to detect of the levels radioactivity. Mass-spectrometry and gas-liquid chromatography belong to the set of precise

methods, but their application presumes i) employment of specially trained operators as well as ii) the process of long-term and multi-step preparing of the sample.

On account of above considerations, the objective of the present research presumed modification of the immunoenzyme method (EIA), which is widely applied in analysis of other substances, in order to determine the level of cAMP in plants.

One of the critical stages in the analysis conducted with the employment of EIA is proper preparing of the plant sample. The main requirement to the sample presumes efficient removal of the admixtures under the condition of maximal retaining of the antigen to be determined in the sample. This is conditioned by the fact that any contamination can cause the process of nonspecific binding the antibodies and hence distortion of the result. Therefore, previously to verify purity of the sample prepared, we applied two independent methods: NMR and capillary electrophoresis. The concentration of cAMP in plant samples (plants of potato *in vitro*) was determined by capillary electrophoresis. We also used this method to compare the value of concentration to the value obtained by EIA (Lomovatskaya et al., 2011).

It has been determined in the comparative experiments conducted earlier (Lomovatskaya et al., 2011) that the sensitivity of modified method is about 5 pM, which is eight to ten times more precise than that obtained using the acetylated version of EIA from Sigma–Aldrich and two times better than that from GE Healthcare. Furthermore, the EIAs from Sigma–Aldrich and GE Healthcare require the use of compounds, which possess lachrymator and corrosive effects; the prices of the reagents used for our modified method are some three times cheaper than those using the method from Sigma–Aldrich. Therefore, modified EIA, compared with that from commercial suppliers, is more sensitive, safer and more economical. The results considered suggest that the application of modified EIA may be useful for further investigations of the cAMP content, and accordingly, properties and functios.

Our modification of the method EIA has given us the possibility to determine the level of cAMP in various plant objects under the influence of stressors of abiotic and biotic natures. The following model systems have been employed: the suspension culture of arabidopsis cells + soft and rough heat shock, the potato plants *in vitro* + exopolysaccharides of bacterial initiators of ring rot pathogen *Clavibacter michiganensis* subsp. *sepedonicus*, and also red beet root-crops (*Betula vulgaris)* + fungus *Botritis cinerea.*

2. Experimental procedures

2.1 The plant material

The cell culture of Arabidopsis thaliana (ecotype Columbia) was growing in 250-mL flasks containing the medium comprised by mineral salts prepared according to Murashige and Skoog (1962), 3% sucrose, 0.5 mg/l thiamin–HCl and 0.1 mg/l 2,4-D. The flasks were placed on a shaker (80 r.p.m.) and incubated in darkness at 26º C. In our experiments, 8-day-old cultures in a logarithmic phase of growth were used. In order to create stress conditions, the suspension (10 ml) was held in conic flasks, under permanent wobbling and in bath-marie at 37°C or 50°C for 2 min. During this time period, an assigned temperature level in the medium of suspension growth was. After that, the samples were additionally incubated during 1, 3, 5 or 15 minutes. The suspension, which did not undergo the effect of thermal

shock, was the check sample. After the exposition, the suspension was quickly filtered through fine-meshed fosta nylon and immobilized with the use of liquid nitrogen.

2-week-old potato plants were used. *In vitro* the plants were grown in test-tubes in a growth chamber (16 h light, 6 klux, 20°C/8 h darkness, 15°C) on a liquid salt Murashige and Skoog medium (Murashige and Skoog, 1962). The following chemicals were added: 20 g/L sucrose (Reakhim, Russia), 1.0 mg/L thiamine, 0.5 mg/L pyridoxine, 0.1 mg/L indole-3-butyric acid and 0.02 mg/L ferulic acid (Sigma, USA). To create stress conditions, solution of exopolysaccharides extracted from *Clavibacter michiganensis* subsp. *sepedonicus* bacterium causing potato tubers ring rot was added to potato plants growing medium in final concentration 0.1% and left for 1 min, then the plants were frozen in liquid nitrogen. Vacuoles from red beet root *Betula vulgaris* parenchyma cells were obtained through preparation method (Salyaev et al., 1981). This method allows to obtain fractions of "heavy" and "light" vacuoles, the names being due to the difference in floating density. Red beet root infecting by fungus *Botritis cinerea* was used as a stress factor. Root tissue, which was not infected by fungus, was used for the investigation.

2.2 Preparing samples for analysis of cAMP

Homogenization of plant tissues (stems and roots of potato plants *in vitro*, 10 grams) and suspension cells was conducted with the application of a homogenizer in some isolation medium of the following composition (Fluka, Sweden): 3 ml of 50 mM tris-HCl, pH 7.2, 0.1 mM theophylline (inhibitor of phosphodiesterase), 1 mM dithyothreitol (protector of SH-groups), 0.5 mg/mL polyvinylpyrrolidon (sorbing phenols). To the end of additional binding of phenols (Karimova et al., 1993) we used ione-exchange resin Dowex–50 ("Sigma", USA), which had the weight ratio (i.e. the *resin* to *plant* weight ratio) of 1:5. Crude homogenate was filtered through fine-meshed fosta nylon, this process being followed by centrifugation of the filtrate during 40 minutes at 20 000 g. The level of cAMP was determined in the supernatant.

There were also used vacuole fractions placed in isolation medium (1:1 by volume), where these organelles were destroyed under hypotonic shock. The level of cAMP was determined in the acquired vacuolar sap.

2.3 Supernatant cleaning from admixtures of other nucleotides

1 ml of sample was put into the column with neutral aluminum oxide, the thickness of its layer being 1 sm^3, which was balanced with the buffer. Elution was conducted with the use of the same buffer, and analysis of the substance's spectrum was carried out with the aid of a chromatograph ("Uvicord", Sweden) at the wavelength of 276 nm. The set of standard test specimens included adenosine, 5'-AMP, cAMP, cGMP, cTMP, cCMP ("Sigma", USA) in concentration of 100 pM. In this case, cAMP eluded earlier than other compounds and was discovered already in the second ml, what coordinates with the information, which can be found in the literature (White and Zenser, 1971).

Next, the eluate (of total volume 8 ml) was evaporated on a rotary evaporator under vacuum. Its dry residue was washed in 1 ml 100% dimethylsulfoxide («Reachim», Russia) to remove salts, and then it was centrifuged during 5 min at 20000g. Dimethylsulfoxide was removed and the residue was washed with the use of acetone («ECOS-1», Russia). The specimen was air-dried at room temperature and analyzed to determine the concentration of cAMP by EIA.

2.4 Analysis by EIA to determine the concentration of cAMP in the samples

To the end of antigen immobilization we prepared a mixture, which contained 1 mL of the tested sample dissolved in 1 ml of PBS (i.e. 20 mM of phosphate buffer plus 0.1 M NaCl, pH 7.0), 0.5 mg of the dry plant sample plus 0.08 mL 25% glutaraldehyde plus 1 mg/mL BSA (Sigma), 0.1 mL of which was added into each well of the polystyrene multiwell plate. The plate was incubated during 15 hours at 37°C, and the wells were rinsed three times with the solution of PTBS (20 mM of phosphate buffer plus 0.1 M NaCl plus 0.3% Tween 100, pH 7.0). To avoid non-specific binding of the antibodies with the solid carrier 0.1 mL of horse serum (Allergen, Russia), diluted 1:10 PBS was added to each well, and the plate was left for 1 h at 37° C and next was rinsed three times with PTBS. After that, a solution prepared of primary rabbit antibodies was added to cAMP («Sigma», USA) (60 mkg per ml in PBS plus 0.1% BSA) and incubated during 2 h at 37° C. Next, the samples were flushed in PTBS again and treated with horse serum. After that, secondary goat's antibodies anti rabbit labeled with the use of peroxidase («Sigma», USA) on 0.1 M carbonate-bicarbonate buffer, pH 8.3, were added in each well and incubated during 1 h at room temperature, whereupon these were rinsed three times with PTBS.

In order to remove the detergent from the wells, we conducted its repeated washing with the use of 0.1 M phosphate-citrate buffer, pH 5.3. In case of peroxidase reaction, 0.1 mL 0.15% orthophenylendiamine (Sigma, USA) diluted in the 0.1 M phosphate-citrate buffer, pH 5.3, and 3 µL 3% H_2O_2 was added into each well. Color developed during 20 min. The reaction was stopped by adding 0.1 mL/well 4 N H_2SO_4. The measurements of the intensity of light absorption by the solution were made with the use of spectrophotometer C-101-46 (BioRad, Germany) at the wavelength of 490 nm. The negative control was represented by the buffer of isolation without plant samples. The concentration of cAMP in the sample was determined with the help of the calibration plot built for cAMP ("Sigma", USA), (Fig.1). This calibration plot was represented as positive control.

For the purpose of additional verification of the specificity of binding the antibodies with cAMP, adenosine (0.1 µM concentration), 5'-AMP, and other cyclical nucleotides, such as cGMP, cUMP, cTMP, cCMP, were tested as the anti-genes.
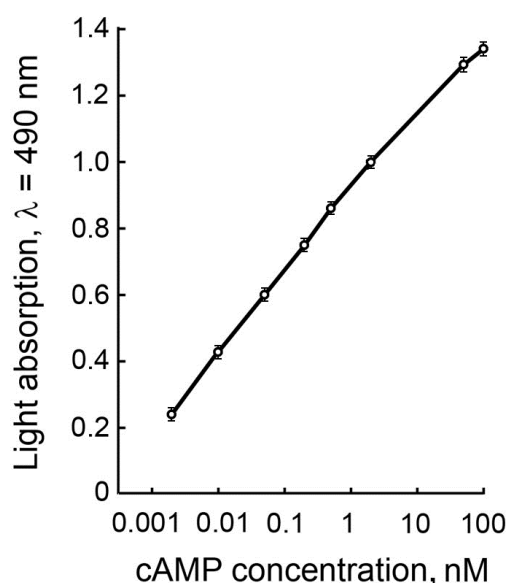

Fig. 1. The calibration plot built for cAMP. Concentrations of cAMP are represented in the logarithmic form.

2.5 Analysis of the data

Eight (EIA) replicates were carried out in experiments, which were repeated two times. Results are expressed as means ± standard errors.

2.6 Results and discussion

2.6.1 Analysis by EIA

Verification of specificity of the process of binding the antibodies, which were harvested against cAMP, has given evidence that such junctions as adenosine, 5'-AMP and other cyclic nucleotides (cGMP, cUMP, cTMP, cCMP) did not react with antibodies. The results turned out to be at the level characteristic of the control sample without any plant material. When employing the radio-immune method with the application of polyclonal antibodies against cAMP, Roef et al. (1996) have obtained similar data related to the specificity of binding cAMP.

Therefore, despite the fact of obvious simplicity of preparing samples and trivial scheme of analysis, the proposed method possesses rather high rates of sensitivity and specificy.

In this connection, it was of interest for us to apply this modification of EIA for revealing variations of cAMP concentrations, if these variations occur in plant cells in response to the abiotic stress, particularly, in case of thermal influences on the cells of arabidopsis.

2.6.2 Results obtained with the use of a modified version of EIA

Our investigations have given evidence that all the kinds of stress had a substantial effect upon the variation of cAMP concentrations in the scrutinized plant objects (Tables 1, 2, 3), what reflects the variations of activities of adenylate cyclase and, obviously, phosphodiesterase as the main components of the adenylate cyclase signaling system.

Temperature and incubation time	cAMP concentration			
	Suspension cells	% to control	Growth medium	% to control
26°C	1,87±0,11	100	3,6±0,25	100
37°C 1 min	32,8±2,40	1754	8,9±0,7	247
37°C 3 min	17,2±1,30	916	8,3±0,8	230
37°C 5 min	17,4±1,30	914	3,6±0,2	100
37°C 15 min	2,7±0,19	144	0,7±0,06	20
50°C 1 min	53,7±4,80	2871	11,5±0,9	321
50°C 3 min	5,5±0,44	294	7,1±0,4	198
50°C 5 min	2,2±0,18	118	5,4±0,3	151
50°C 15 min	13,1±0,90	700	0,7±0,05	20

Table 1. Changes of cAMP concentration, nMol/g raw weight, in Arabidopsis suspension cell culture (*Arabidopsis taliana*) (temperature) stressor and in the environment of their growth under the influence of the short-term abiotic.

The results presented in Table 1 allow to conclude that in Arabidopsis suspension cells both mild and severe heat stresses induce the highest cAMP level already 1 minute after the impact. At 37° C this provokes activation of heat shock protection system, in particular, induction of HSP synthesis (Rikhvanov et al., 2007). Nevertheless, sharp increase of cAMP level at 50° C already 1 minute after, and then its sharp drop, indicated of signal alarm response, due to which there apparently takes place qualitative change-over of intracellular metabolic paths. In Arabidopsis suspension cells this leads, as previously shown, to the development of programmable cell death (apoptosis).

So, both abiotic and biotic stresses cause early activation of adenylate cyclase signal system in different plant species. According to literary data, differences in the induction of early signal pathways are of importance for further adaptive processes. Due to this intensity of the acting stress factor may produce a differentiating effect on the activity of various components of this or that signal system. As Tables 2 and 3 show, biotic stresses also significantly affect activity of adenylate cyclase signal system, despite the fact that in case of *in vitro* potato plants exometabolites of potato ring rot bacterial pathogen act as a stressor, whereas in case of red beet root the stressor is a cultivar of fungus gray rot pathogen.

cAMP concentration			
Resistant cultivar		Susceptible cultivar	
Control	+EPS*, 1 min	Control	+EPS*, 1 min
17,4±0,7	111±4,3	12,8±0,5	0,47±0,02

* EPS – exopolysaccharides of the potato ring rot pathogen *Clavibacter michiganensis* subsp. *sepedonicus*.

Table 2. Changes of cAMP concentration, nMol/g raw weight, in stems of potato plants *in vitro (Solanum tuberosum)* under the influence of the biotic stressor (exopolysaccharides of the potato ring rot pathogen).

cAMP concentration			
Vacuole fractions			
"light"		"heavy"	
control	+*Botritis cinerea*	control	+*Botritis cinerea*
12±0,5	160±7,0	63±3,0	800±37,0

Table 3. Changes of cAMP concentration, nMol/mg protein, in vacuoles isolated from red beet root-crops (*Betula vulgaris)* under the influence of the biotic stressor (infecting with the root-crops by fungus *Botritis cinerea*).

3. Conclusion

The data obtained allow us to assume that variation of the level of intracellular cAMP determines the adaptive and restorative capabilities of plant cells in many respects. We are sure that the proposed modification of EIA may be successfully applied in investigations these or other physiological-biochemical processes in plants. It should be noted that the advantage of this method as compared to analogous method of Sigma-Aldrich and GE Healthcare companies is its hogher sensitivity and relatively lower cost of reagents.

So, for the purpose of determination of cAMP from plant samples we can propose the following scheme (Fig. 2).
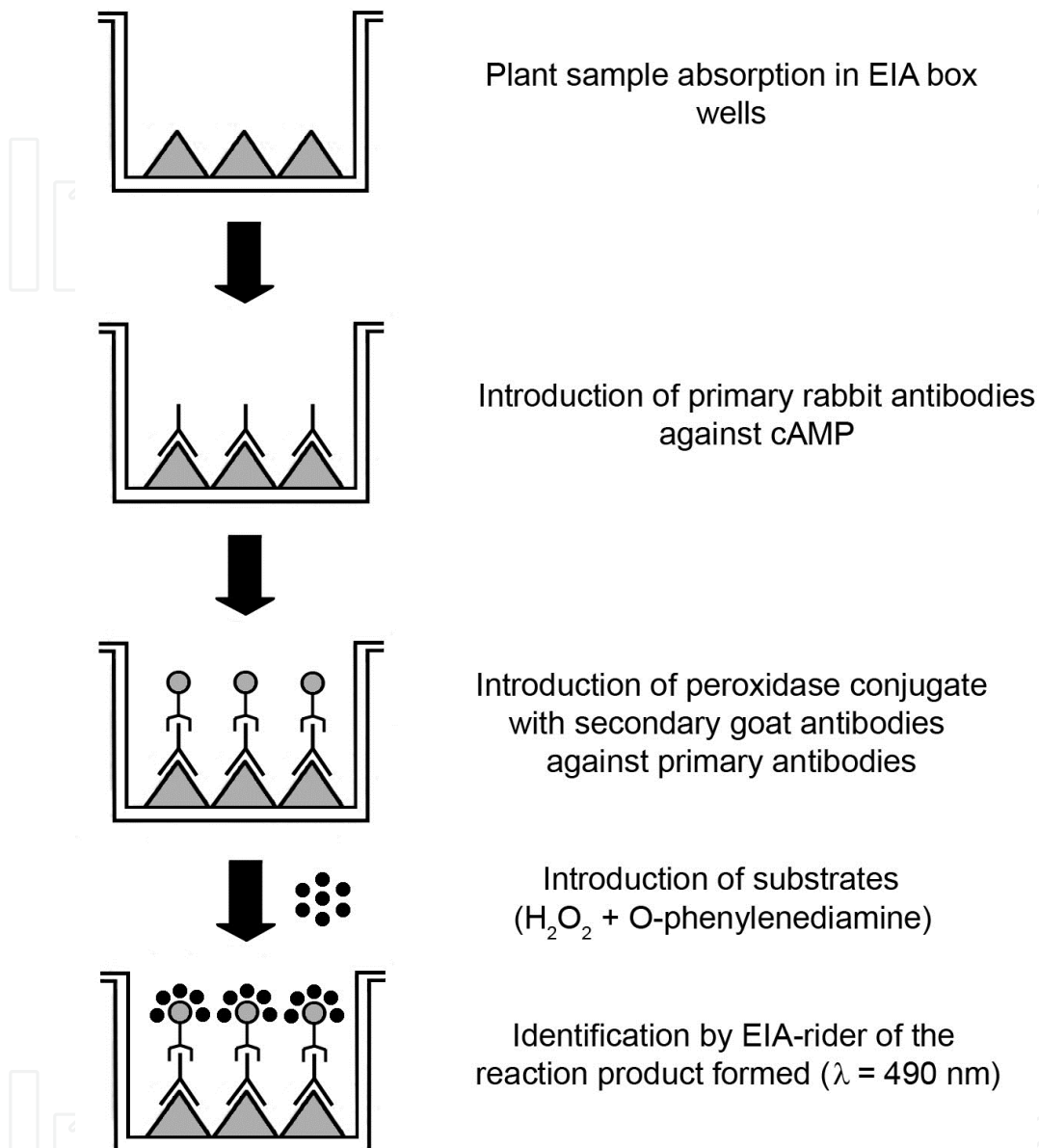

Plant sample absorption in EIA box wells

Introduction of primary rabbit antibodies against cAMP

Introduction of peroxidase conjugate with secondary goat antibodies against primary antibodies

Introduction of substrates (H_2O_2 + O-phenylenediamine)

Identification by EIA-rider of the reaction product formed (λ = 490 nm)

Fig. 2. Major stages of EIA method to determine cAMP concentration in plant sample.

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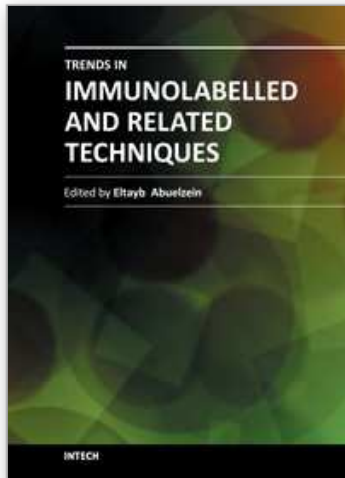
Trends in Immunolabelled and Related Techniques
Edited by Dr. Eltayb Abuelzein

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