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Signalling of indole-3-acetic acid: inhibitory analysis of MAP kinase / phosphatase pathways

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The aim of this work was to study the involvement of MAP kinase / phosphatase pathways in the IAA signal transduction of intensively elongating plant cells by treatment with inhibitors of these pathways and use plants with a disturbed signal transduction cascade.

The results obtained by application of inhibitors such as staurasporine, H8, sodium metavanadate, dideoxyadenosine and activator of membrane-associated protein kinase C – phorbol ester – support the idea of the transduction of IAA-induced signal into the nucleus via the MAP kinase / phosphatase signalling cascade. Hypocotyls of *Arabidopsis thaliana mpk6 knockout* lines showed inhibited signal transduction in comparison to *WT* ones. These data corroborate the idea that MAP kinase modules, at least partially, participate in IAA signal transduction together with the other signalling pathways.

Key words: IAA, inhibitors and activators of signal transduction, wheat coleoptiles, thale cress hypocotyls

INTRODUCTION

The multifunctionality of IAA in the induction of cell division, elongation, morphogenetic and other effects is provided by cross talks in the processes of the perception and transduction of extracellular signal to the nucleus. IAA-dependent changes in cells of different growth phases could be regulated on different levels – during formation of phytohormone–receptor complexes, transduction of signals of these complexes, expression of specific genes and during realization of physiological processes.

Earlier results obtained at Laboratory of Plant Physiology, Institute of Botany [1, 2] have suggested that an essential unit of IAA signalling is the complex of this phytohormone with a receptor protein. Such complexes were registered in different cell compartments such as plasmalemma, tonoplast, cytosol, mitochondria and chloroplasts [3, 4]. However, the way of the signal transduction to the nucleus is not yet clear. One of the possible pathways could be related to the action of the endosomal system by separation of plasmalemmal IAA–protein complexes from the membrane and their transfer to the nucleus [5, 6]. This way is characteristic of plant cells. Extracellular signal transduction through MAP kinases in plant cells is well established [7]. MAP kinase modules are implicated in the processes of IAA and ethylene signalling of cell proliferation, as well as their role is supposed in phytohormone signalling in elongating cells [8, 9] and in the processes of cell differentiation [10].

The aim of this work was to study the involvement of MAP kinase / phosphatase pathways in the IAA signal transduction of intensively elongating plant cells by treatment with inhibitors of these pathways and studying plants with disturbed signal transduction.

OBJECTS AND METHODS

The test objects were intensively elongating wheat (*Triticum aestivum* L. 'Nandu') coleoptiles and thale cress (*Arabidopsis thaliana* Heynh) *mpk6 knock out* mutant hypocotyls. Wheat seedlings were grown in sand at 25 °C in the dark. Four-days-

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old coleoptiles were isolated from the first leaves, decapitated (3 mm, 2x) and kept vertically. Thale cress seedlings were grown aerated for 40 days in the dark on 1/2 Murashige and Skoog medium.

The *mpk6* (At2g43790) T-DNA insertional mutant (SALK_127507) was obtained from SALK collection. The position of T-DNA insertion was identified by PCR using gene-specific and T-DNA left border specific (http://signal.salk.edu) primers. Tandem T-DNA insertion was detected in the fourth exon, approximately 1940 base pairs downstream of the translational start codon ATG. Homozygous for T-DNA insertion plants were selected by PCR using *MPK6* specific primer and Lba1 or *MPK6* specific primers only. *mpk6* plants contain a single T-DNA insertion, as verified by Southern blotting. Reverse transcription PCR (RT-PCR) analysis using *MPK6* specific primers has proved that *MPK6* transcript is absent in *mpk6* plants but detectable in wild type plants. Therefore *mpk6* plants were considered as a *knockout* line.

Pre-treatment by inhibitors *in vivo* was performed by dipping the basal parts of cuttings for 1 h in their solution. Inhibitors and activators used: staurasporine, 2',3'-dideoxyadenosine, phorbol ester–phorbol12-myristate13-acetate (all Sigma–Aldrich, Chemie), H8 {N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide}, Calbiochem), sodium metavanadate ($NaVO_3$, Acros, New Jersey). The concentrations of inhibitors and activators were chosen according to the references [11, 12] and our experimental data and are shown in the results.

In all cases the activity of RNA-polymerase II (RNP-II) in the model RNA synthesis system of isolated nuclei was regarded as the marker of IAA-induced changes in nuclei [13]. The incubating medium consisted of cell nuclei isolated from wheat coleoptile and thale cress seedlings, triphosphates GTP, UTP, CTP and 8-^{14}ATP (51 mCi/mmol, Moravek Biochemicals, California), Tris-HCl (pH 7.8) and, in separate experimental series, additions of plasmalemma and cytosol fractions. After incubation (37 °C) the proteins were precipitated with 3% (final concentration) trichloroacetic acid, filtered and purified on membrane filters of Pragopor tipe (2.5 μ, Czechia). Three variants of the system of isolated nuclei were used:

1. Systems with nuclei isolated from non-treated wheat coleoptiles and thale cress cells (control) and nuclei isolated from plants pre-treated *in vivo* with inhibitors, activators and IAA.

2. Systems with non-treated nuclei supplemented with a plasmalemma fraction treated with inhibitors or activators and enriched *in vitro* by IAA–protein complexes.

3. Systems with non-treated nuclei supplemented with the cytosol fraction isolated from cuttings *in vivo* treated with inhibitors and activators as well as *in vitro* treated with IAA.

The plasmalemma vesicle enriched fraction was obtained by differential centrifugation and purification on sucrose gradient [14, 15]. According to inhibitory and electron microscopy analysis, the fraction localized on 1.13–1.15 g·cm^3 sucrose gradient interphase mostly >90% contains sealed plasmalemma vesicles [15].

Cytosolic proteins were obtained after separation of membrane fractions by precipitation with ammonium sulphate and crude cleaning on a Sephadex PD-10 column. IAA–protein complexes, both in plasmalemma and cytosol preparations, were formed *in vitro* after mixing them (1:1) with the binding medium (Tris-Mes 5 mM, pH 7.2; KCl 50 mM, $MgCl_2$ 3 mM, mercaptoethanol 4 mM). Necessary concentrations of IAA (Merck, USA) were added to the binding medium. After 30 min of incubation (necessary for the formation of IAA–protein complexes), free IAA was removed during a 10-h dialysis. The used dialyzing membrane (Sigma, USA) retained proteins with molecular mass higher than 12 kDa.

Protein content in subcellular fractions was determined according to Bradford [16]. The radioactivity of the preparations was measured with a scintillation counter (Beckman LS 1801, USA).

The experiments were repeated 3–5 times and the statistics of the results was treated to evaluate the mean values, mean square deviations and reability ranges. Only differences significant at $p > 0.95$ were taken into consideration.

RESULTS AND DISCUSSION

1. Changes of the activity of RNP-II in elongating wheat coleoptile cell nuclei under the influence of MAP kinase / phosphatase signal system inhibitors and activators

In a series of experiments where nuclei were isolated after 1 h of wheat coleoptiles pre-treatment with inhibitors or activators and 1 h of incubation with IAA (control without IAA) and a model system for provocating RNP-II activity was created, the obtained results showed that IAA ($1 \cdot 10^{-7}$ M) *in vivo* activated RNP-II up to 42% versus untreated control (Fig. 1–2). Staurasporine at a concentration of $1.6 \cdot 10^{-6}$ M strongly (up to 47%) inhibited activity of RNP-II in IAA-non-treated variants (Fig. 1–3). In the case of IAA treatment staurasporine-dependent RNP-II inhibition was 11.4% (Fig. 1–4). Consequently, staurasporine was less but still associated with the IAA response in the nuclei. Staurasporine inhibits the activity of plasmalemma proteinkinase C by binding as a competitive ligand. In the further experimental series, the fraction of plasmalemma vesicles was isolated and *in vitro* conditions for the formation of IAA–protein complexes were ensured. In such experiments, staurasporine induced ~29% inhibition of RNP-II, versus the variant without inhibitor (Fig. 2–4 versus Fig. 2–2). It is possible that in plasmalemma vesicles IAA–protein complexes were formed, but staurasporine inhibited the transduction of their signal even at a comparatively low (1 μM) concentration. These results suggest a role of proteinkinase C in signal transduction processes of plasmalemmal IAA–protein complexes. This suggestion is also confirmed by data concerning phorbole ester, the activator of proteinkinase C: addition of plasmalemmal fraction treated with phorbole ester to the model RNP-II provocation system activated it up to ~18% (Fig. 2–8 versus Fig. 2–2). Phorbole ester is an activator of cytoplasmic phospholipase A_2 which in active form migrates to the plasmalemma, and plasmalemmal protein kinase C [17, 18]. Therefore our data support the idea that IAA signal via complexes formed in the plasmalemma induces changes in the membrane, which are linked with the functioning of the MAP kinase signal transduction system. Proteinkinase C, localized in plasmalemma, could be one of the links of this system.

The transduction of an external signal via proteinkinase C in HL-60 cells is closely related with the signalling controlled by

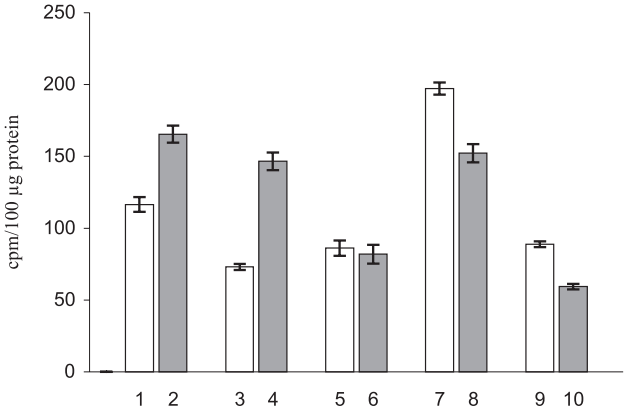

Fig. 1. IAA-dependent activity of RNP-II after pre-treatment (*in vivo*) with: 3, 4 – staurasporine ($1.6 \cdot 10^{-6}$M); 5, 6 – H8 ($2 \cdot 10^{-6}$M); 7, 8 – $NaVO_3$ ($2 \cdot 10^{-4}$M); 9, 10 – dideoxyadenosine ($2 \cdot 10^{-6}$M). Controls: 1 – without inhibitors and IAA; 2 – without inhibitors but pretreated 1 h with IAA ($1 \cdot 10^{-7}$M). Other bright columns – pre-treatment with inhibitor, dark columns – pre-treatment with inhibitor plus IAA (1 h, $1 \cdot 10^{-7}$M)

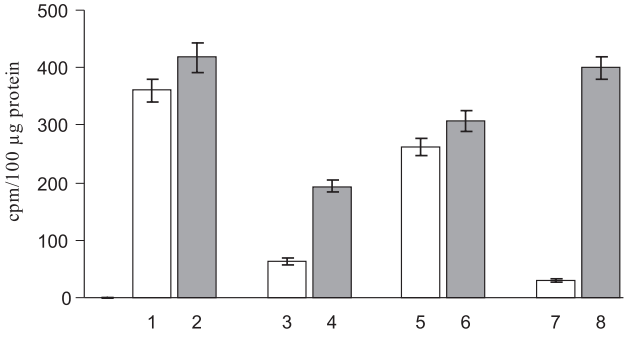

Fig. 3. IAA-dependent activity of RNP-II after pre-treatment of wheat coleoptile: *in vivo* 3, 4 – staurasporine($1 \cdot 10^{-6}$M), 5, 6 – H8 ($1 \cdot 10^{-6}$M) and 7, 8 – dideoxyadenosine ($1 \cdot 10^{-5}$M), separation of cytosolic proteins and addition to the model system of isolated non-treated nuclei. Controls: 1 – without inhibitors and IAA; 2 – without inhibitors, but with IAA ($5 \cdot 10^{-8}$M) *in vitro* treated cytosol protein fraction; bright columns – pre-treatment *in vivo* with inhibitors. Dark columns – pre-treatment *in vivo* with inhibitors and in vitro with IAA ($5 \cdot 10^{-8}$M)

cAMP [19]. Evidently analogous circumstances of signal transduction exist in plants. To test this suggestion, we used H8, an inhibitor of proteinkinase A, and dideoxyadenosine, an inhibitor of AMP cycling. Proteinkinase A and cAMP are related to the cytosolic cell compartment and links just after signal acceptance in the plasmalemma. According to the data obtained, after *in vitro* treatment of the plasmalemma fraction with H8 the activity of RNP-II was slightly inhibited (Fig. 2–5 versus Fig. 2–1), but the influence of IAA was suppressed to 25% (Fig. 2–6 versus Fig. 2–2). After treatment with H8 the summary fraction of cytosolic RNP-II proteins, both IAA-treated and non-treated variants, were inhibited by up to 27% (Fig. 3–5 and 6) versus control (Fig. 3–1 and 3–2).

The influence of dideoxyadenosine was obvious. In the case when wheat coleoptile cuttings were treated with dideoxyadenosine and nuclei were isolated from their cells, the activity of RNP-II in a model system was inhibited by dideoxyadenosine by 24% and 64% in variants without and with IAA, respectively (Fig. 1–9 versus Fig. 1–1 and Fig. 1–10 versus Fig. 1–2). So, according to these data, the cAMP-dependent way is significant for IAA signal transduction to the nucleus.

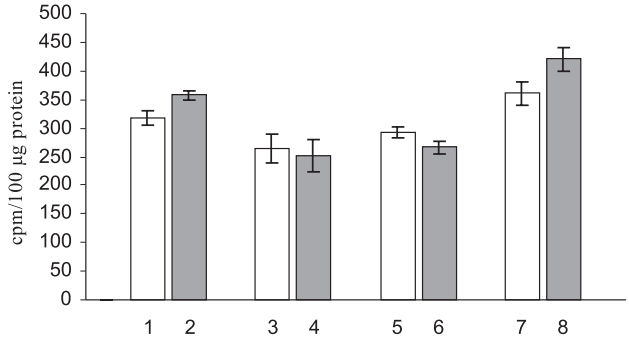

Fig. 2. IAA-dependent activity of RNP-II after pre-treatment of isolated wheat coleoptile cell plasmalemma by: 3, 4 – staurasporine ($1 \cdot 10^{-6}$M); 5, 6 – H8 ($1 \cdot 10^{-6}$M) and 7, 8 – phorbol 12-myristate 13-acetate ($1 \cdot 10^{-6}$M). Controls: 1 – without inhibitors and IAA; 2 – without inhibitors but *in vitro* IAA ($5 \cdot 10^{-8}$M); bright columns – *in vitro* inhibitor; dark columns – inhibitor plus IAA ($5 \cdot 10^{-8}$M)

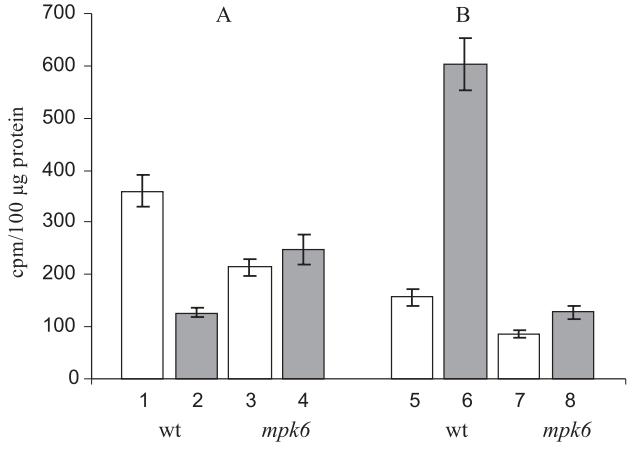

Fig. 4. IAA-dependent RNP-II activity in model system of nuclei isolated from *Arabidopsis thaliana* hypocotyls (A: 1–4) and root (B: 5–8) cells. 1, 2, 5, 6 – wt; 3, 4, 7, 8 – mpk6. Bright columns – without IAA, dark columns – IAA ($1 \cdot 10^{-7}$M 1 h *in vivo*)

Sodium metavanadate, an inhibitor of MAP kinase signal phosphatases, being a negative regulator of these processes induced RNP-II activation in the model system of nuclei treated *in vivo* with this inhibitor (Fig. 1–7 and 1–8).

Thus, the application of inhibitors and of an activator of MAP kinases localized in plasmalemma and cytosol compartments showed that MAP kinase / phosphatase signalling pathways could mediate the transduction of IAA signal. At the same time, the principles of localization of components of MAP kinase signalling pathways in the cytoplasm and nuclei where their substrates could be factors of transcription [20] could be supplemented by the information about the possible links of these signalling pathways localized in the plasmalemma.

2. Influence of IAA on RNP-II activity in nuclei isolated from *Arabidopsis thaliana mpk6 knockout* line cells

A major progress in the growth and development investigations was achieved by employing hormone response mutants. We used the *Arabidopsis mpk6 knockout* line with the non-functioning MPK6 (mitogen activated protein kinase 6).

MPK6 is activated by numerous environmental factors (cold, osmosis, wounding, etc.) [21, 22]. Our test object was etiolated

(40 days old) thale cress seedling hypocotyls and roots which had been treated *in vivo* with IAA. Nuclei isolated from such test objects were used in a model system of RNA synthesis for RNP-II activity provocation. The obtained data show that nuclei from *mpk6* line seedlings inhibited RNP-II activity (Fig. 4). In the nuclei from hypocotyls of the *WT* (Col0) plants, IAA did not activate RNP-II (the reason could be a comparatively high level of endogenous IAA left in the tissues when a seedling is not decapitated), but some RNP-II activation was detectable when IAA-treated *mpk6* hypocotyls were compared with IAA-non-treated ones (Fig. 4–4 versus Fig. 4–3), although the level of RNP-II activity after IAA treatment in *mpk6* cell nuclei was lower than in the *WT* IAA-treated ones. In *WT* roots, IAA activated RNP-II strongly (more than 3 times) (Fig. 4–6 and 4–5). IAA-dependent RNP-II activation to a lower extent was detected in *mpk6* roots (Fig. 4–8 and 4–7). Taken together, these data confirm the report that IAA response mutants are characterized by a lowered MAP kinase signalling way activity [23] and suggest that MAPK signalling could participate in IAA signal transduction. However, when MPK6 is missing, it could be transduced to the nucleus by other pathways.

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INDOLIL-3-ACTO RŪGŠTIES SIGNALO TRANSDUKCIJA: INHIBITORINĖ MAP KINAZIŲ / FOSFATAZIŲ KELIO ANALIZĖ

S a n t r a u k a

Siekiant patikrinti IAR signalo perdavimo galimybę per MAP kinazių sistemą panaudoti MAP kinazinio signalinio kelio inhibitoriai – staurasporinas, H8, dideoksiadenozinas, natrio metavanadatas bei *mpk6* – *Arabidopsis thaliana knock out* mutanto daigų hipokotiliai, turintys sustabdytą MPK6 funkcionavimą. Pagal gautuosius duomenis, MAP kinazinio signalinio kelio pažeidimai ląstelės plazmolemos ir citozolio kompartmentuose yra svarbūs IAR signalo perdavimui į ląstelės branduolį. Manome, kad ši IAR signalo perdavimo sistema funkcionuoja greta kitų, tarp jų ir tiesioginio IAR kompleksų pernešimo į branduolį, sistemų.