ABSTRACTS
POSTER PRESENTATIONS

SESSION 1:
Mechanisms of cytokinin signaling and action

EXPRESSION OF GENES PARTICIPATING IN THE CONTROL OF PLANT CELL DIVISION IN TUMOR-PRODUCING RADISH (*RAPHANUS SATIVUS* VAR. RADICULA PERS.) INBRED LINES

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Tumors in higher plants are suitable model for studying mechanisms of systemic control plant cell division and differentiation. Probably, tumor formation is under control of numerous genes. On spontaneous and pathogen-induced tumors in different plant species it was shown that the key role in tumor formation plays alteration of cytokinins/auxins ratio. In the other hand, tumor formation is accompanied by change of expression levels and patterns next of genes that participate in cell cycle control, homeobox-containing genes controlling meristem cell specifity and genes of primary response to auxins and cytokinins.

Spontaneous tumors on inbred lines from radish (*Raphanus sativus* var. Radicula Pers.) genetic collection are used for study the mechanisms of tumor growth in higher plants. Two types of tumors were described for radish lines: undifferentiated tumors which form on the crop-roots of several lines during flowering and tumors on ovaries which undergo redifferentiation and form ectopic shoot meristems. Besides that, cultivation of young aseptic plants from many lines of radish on the mediums with cytokinins leads to formation of tumors in the lower part of hypocotyl. These tumors are close resemble to tumors in the crop-roots in their anatomy and are capable to hormone-independent growth for a long time. It was shown that lines which are tumor-producing *in vivo* have increased level of free and bound forms of cytokinins and decreased level of IAA. Explants of tumorous lines demonstrate high sensitivity to exogenous auxins and cytokinins *in vitro*.

We have used RT-PCR to analyze expression of some cell cycle genes (*CycD3*), meristem-specific genes (*STM, KNAT1, WUS)* and primary response genes to auxins (*IAA1*) and cytokinins (*ARR5*) during tumor formation in radish lines. PCR-products which were obtained during PCR of radish the DNA and cDNA with specific primers to *CycD3, STM, KNAT1, WUS IAA1* and *ARR5* genes demonstrated high level of homology with the corresponding genes of *Arabidopsis thaliana*. Expression of named genes was studied on different tissues of lines producing tumors in the crop-roots, lines forming ovary tumors and non-tumorous lines in several stages of development; besides that we have analysed their expression during formation cytokinins-induced tumors on aseptic plants. The expression of *CycD3* and *KNAT1* genes was increased during the development of spontaneous tumors on the crop-roots and cytokinins-induced tumors. Formation of ovary tumors was accompanied by increased expression levels of *CycD3, STM* and *WUS* genes. The treatment of radish plants by cytokinins induces the incease of *ARR5* gene expression level. We have shown that the expression of *ARR5* is induced early and stronger in tumorous lines. In line which has maximal rate of tumor formation, we observed high level of *ARR5* expression without any cytokinins treatment. On the contrary, increased level of *IAA1* expression we observed in non-tumorous lines. So that, *CycD3, STM, KNAT1, WUS* and *ARR5* genes may participate in the control of tumor development on radish lines.

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PARTICIPATION OF PIP_2-PHOSPHOLIPASE D IN CYTOKININ SIGNALING

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Cytokinins are class of plant hormones that play central role in many aspects of plant growth and development including apical dominance, formation and activity of shoot meristems, flower and fruit development, leaf senescence, source-sink relations, photosynthesis, seed germination, control of cell division, pigment production and plant defense responses. They also appear to mediate a number of light-regulated processes, such as deetiolation and chloroplast differentiation. For cytokinin signaling, most clear evidence was obtained on the involvement of Phospholipase D (PLD). This evidence is based on a dose-dependent inhibition of cytokinin-induced pigment accumulation in *Amaranthus caudatus* L. by low concentrations of primary alcohols, known specific inhibitors of phosphaditic acid formation by PLD. Secondary alcohols, which do not interfere with PLD action, lack such inhibitory effect. Also, primary alcohols partially prevented cytokinin-responsive *ARR5* gene promoter activity and reduced an accumulation of *ARR5* gene transcripts in *Arabidopsis thaliana*.

Phospholipase D (PLD) constitutes a major plant phospholipase family in plants involved in many cellular processes such as signal transduction, membrane remodeling, and lipid degradation. Stimulation of PLD also has been shown in plants in response to variety stress and hormone treatments.

The effect of cytokinin (BA) on the phosphatidylbutanol (PtdBut) accumulation was tested in tissues of *Amaranthus caudatus*. Detached shoots of *Amaranthus* were placed in flasks containing [^{33}P]orthophosphate and incubated for 14 h at 25 ˚C. BA was added at different time-points, and lipids were extracted and analyzed. PLD activity was measured according to phosphatidylbutanol accumulation upon 1-butanol addition. Cytokinin application during 5-30 min caused increase in phosphotidylbutanol accumulation.

In order to reveal the type of PLD activated upon cytokinin action, we used different modifiers of signaling cascades: EGTA that binds calcium, verapamil that inhibits plasma membrane calcium channels and neomycin that binds PIP_2 and inhibits PLD activity. The modifiers were applied together with 5 μM BA. As the result, EGTA, verapamil and neomycin blocked cytokinin-induced PtdBut formation in the following way: neomycin > verapamil > EGTA. Therefore, among the inhibitors tested, the neomycin was the most potent in reducing PtdBut levels. These results indicate that PLD activated by cytokinin can be PIP_2-dependent.

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FUNCTIONAL HETEROLOGOUS EXPRESSION OF *ARABIDOPSIS THALIANA* CYTOKININ OXIDASE/DEHYDROGENASE FAMILY

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Cytokinins are plant hormones able to promote cell division, therefore contributing to the regulation of a variety of developmental processes including apical dominance, flower and fruit development, leaf senescence, and seed germination.
The degradation metabolism of cytokinins is an important process that controls the levels of this hormone active forms and their distribution in plant tissues. The enzyme catalyzing the cleavage of N^6-side chain of free cytokinin bases and their ribosides is a flavoprotein classified as a dehydrogenase, but able to work also in an oxidase mode (CKX, EC 1.5.99.12). In *Arabidopsis thaliana*, seven distinct CKX-encoding genes were identified. Amino acid sequence comparison revealed that individual CKX proteins from *Arabidopsis* share conserved regions of high homology (e.g. FAD-binding domain), but their sequences outside of these domains display strong divergence. Therefore these isoenzymes differ in their catalytic properties, their subcellular localization and their expression domains.
Functional expression of several recombinant AtCKX proteins has been obtained successfully in the *Pichia pastoris* system, with protein secretion to the medium. In the case of AtCKX1 and AtCKX3, N-terminal sequence-specific vacuolar sorting signal (ssVSS) was identified that had to be deleted to achieve secretion of active proteins from the yeast cells.
In order to facilitate purification, recombinant proteins were fused with His-tag domain. The fusion on the C-terminal end of the proteins, however, proved to be useless in affinity purification, suggesting that polyhistidines are buried inside the protein structure and cannot bind to the metal-chelating resin. Proteins with N-terminal His-tag domain are retained on Ni-NTA columns but the binding strength differs significantly for each protein.
In neutral conditions the dehydrogenase activity of AtCKX enzymes is the highest with N^6-(2-isopentenyl) adenine (iP), whereas in slightly acidic pH most enzymes prefer iP9-glucoside. Oxidase activity is the highest with iP9-glucoside in neutral conditions. Aromatic cytokinins and their ribosides are relatively good substrates only for AtCKX1 and AtCKX3 in slightly acidic conditions with 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0) as the electron acceptor.

DIMETHYLSULFOXIDE SELECTIVELY STRENGTHENS THE CYTOKININ ACTION IN ARABIDOPSIS

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Dimethylsulfoxide (DMSO) is one of the widespread solvents used for pharmacological screening, in biology in particular. Also DMSO can affect biological membranes and can be employed for membrane permeabilization. We have used DMSO previously to study the influence of different growth substances/inhibitors on the expression of *ARR5::GUS* construct in transgenic Arabidopsis (Romanov et al., FEBS Letters, 2002). The construct represents the fusion of the promoter from the cytokinin primary response gene *ARR5* with the reporter gene *GUS* (Brandstatter & Kieber, Plant Cell, 1998). We remarked that in control series some concentrations of plain DMSO influenced somehow cytokinin effects. For example, DMSO at concentration 2-7% enhanced cytokinin action by 2-3-fold. Therefore we have checked the influence of different DMSO concentrations on the induction of *ARR5* promoter by cytokinins, using different transgenic clones of Arabidopsis. For experiments, we have used 3-4-day-old seedlings of double Arabidopsis mutants expressing only one of three cytokinin receptors (Riefler et al., Plant Cell, 2006): *ahk2/ahk3*, *ahk2/ahk4* and *ahk3/ahk4* expressing sole receptors CRE1/AHK4, AHK3 or AHK2, respectively. These mutants were additionally transformed with *ARR5::GUS* construct (Riefler et al., unpublished). Quantitative GUS assays have shown that double mutants differed in their response to DMSO. The mutant *ahk2/ahk4* (expressing AHK3) seemed to be most responsive (5-fold increase at DMSO concentration 4.2%), the mutant *ahk3/ahk4* (expressing AHK2) less responsive (2-fold increase at the same DMSO concentration) and the mutant *ahk2/ahk3* (expressing CRE1/AHK4) almost non-responsive. Direct binding assays have shown no any positive influence of DMSO on the affinity of receptors to cytokinins.

In order to study this phenomenon in more detail, we employed the histochemical staining of Arabidopsis on GUS activity. Seedlings were incubated on DMSO solution (4%) or DMSO together with cytokinin (BA, 5 μM) for 5 h. Seedlings grown on water or BA solutions served as controls. Our preliminary data showed difference in the pattern of GUS activity between mutants. For example, in all experimental and control series with *ahk2/ahk3* mutant only the extensive coloring of root (especially root tip) was obvious, but not of hypocotyls or cotyledons. The clone *ahk2/ahk4* showed, on the contrary, marked coloring in hypocotyl and cotyledons. This coloring became more intensive after DMSO treatment.

These results give evidence that the positive influence of DMSO on cytokinin action is rather selective depending on the type of expressed cytokinin receptor. The different responsiveness of mutants can be, at least partly, explained by the different subcellular localization of the receptors.

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TWO MAIZE CYTOKININ RECEPTORS, ZmHK1 AND ZmHK2, HAVE DIFFERENT LIGAND-BINDING PROPERTIES

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Cytokinins are "classical" plant hormones that regulate various processes of plant growth and development. The molecular mechanism of cytokinin signaling is now under extensive studies. At present, it is commonly assumed that cytokinin signal is transduced via two-component system, with hybrid sensor histidine kinases as receptors. Three cytokinin receptors were characterized from maize, ZmHK1, 2 and 3a (Yonekura-Sakakibara et al., Plant Physiol., 2004). The ZmHKs were expressed in the Δ*rcsC* and *cps::LacZ* mutant background of *E. coli*. ZmHK1 and ZmHK2 were able to complement the function of RcsC in a cytokinin-dependent manner. Hence these receptors obviously retained their functional state upon expression in bacteria.

Using transformed bacterial cells, we investigated cytokinin-binding properties of ZmHKs by a direct radioligand method (Romanov et al., 2005). Highly labeled ^{3}H-*trans*-zeatin served as a ligand. Both receptors bound *trans*-zeatin with high affinity: dissociation constants (Kd) for ZmHK1 and ZmHK2 corresponded to approx. 80 and 1 nM, respectively. Receptors had different preference for some cytokinins. Among numerous substances tested ZmHK2 displayed high affinity to only *trans*-zeatin. By contrast, ZmHK1 tightly bound also isopentenyladenine (iP, Kd 5 nM) and benzyladenine (BA, Kd 12 nM). Functional test on the *cps::LacZ* construct activation by different cytokinins showed reasonable correlation with binding data.

The influence of media conditions on cytokinin binding to receptors was also investigated. We found that mono- and divalent cations (K^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}, Mn^{2+}) at physiological concentrations had no marked effect. The receptors differed in pH-dependence of ligand binding. The binding activity of ZmHK2 was almost not affected by pH change (from 5 to 9). On the contrary, ZmHK1 was shown to be strong pH-dependent. We supposed that difference in pH-dependence of hormone-binding properties might be due to different subcellular localization of receptors. Our first results revealed some difference in cytokinin binding sites between plasma- and inner (ER) membranes. Such difference might indicate that ZmHK1 is mainly located on inner membranes, whereas ZmHK2 on plasma membrane.

Thus, maize cytokinin receptors ZmHK1 and ZmHK2 are quite different in their ligand-binding properties. This makes clear parallel between maize cytokinin receptors and corresponding receptors from Arabidopsis, AHK3 and CRE1/AHK4. The revealed differences in ligand-binding properties might be connected with different pattern of *in planta* expression and/or different intracellular localization of cytokinin receptors.

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THE PARTICIPATION OF CYTOKININS IN THE GENERAL ADAPTATION SYNDROME IN WINTER WHEAT

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Cytokinins are a class of plant growth regulators that plays a key role in different processes of development such as control of apical dominance in the shoot, shoot morphogenesis in cell and tissue culture, root growth, chloroplast development, leaf senescence. It is known that phytohormones take a part in the regulation of plant stress too. This is supposedly triggered by hormonal changes such as increased levels of ABA and decreased concentrations of cytokinins. Although a role for ABA in mediating many physiological responses to environmental stress is now well-established, evidence has been presented for the existence of five signal transduction pathways that regulate drought- and cold-inducible genes, only two are dependent on ABA action. Furthermore, recent studies had been shown that some stress-related genes might express under high cytokinin concentrations. Therefore the aim of our research was the investigation of the possible participation of cytokinins in realization of stress responses.

We studied the effects of high temperature (38-40°C) on the balance of two cytokinin forms (zeatin and it riboside) in leaves, transpiration rate and root meristematic activity of eight-day-old plants of four winter wheat cultivars - Khar'kovskaya 81, Khar'kovskaya 96, Donetskaya 46 and Polukarlik 3 – after 1-, 2-, 5-, 10-, 15-, 30-, 45-, 60- and 90-minutes exposition.

Under the optimum temperature (22-24°C) the content of zeatin and zeatin riboside was not changed dramatically in the leaves of both cultivar, but we observed some oscillation of them. Probably it is caused by the day periodic activity of plants, because our experiment started at 12 a.m. High temperature had the positive effect on the zeatin concentration in leaves tissues of all cultivars, although the time reaction was markedly different. Then zeatin content was decreased rapidly. At the same time zeatin riboside dynamics had another character – it was decreased after the start of stress and increased in the second half of hour. Perhaps the pool of zeatin riboside is the reserve for zeatin in leaves but this reserve is not enough for the maintenance of concentration of physiological active forma of cytokinins.

Transpiration rate had been changed two times during the experiment. At the beginning of experiment transpiration rate had been decreased and the first maximum of evaporation coincided with the peak of cytokinin content in the leaves, but the second one was differed from it one.

Meristematic activity had been decreased under high temperature conditions in roots of all cultivars. But the long-stemmed cultivars had prepotent and faster reduction of meristematic index than semi-dwarf one. At the same time we observed the increase of relative duration of prophase and telophase when the anaphase and metaphase one were reduced substantially.

So it is possible that at the first minutes of high temperature stress the synthesis and transport of active forms of cytokinins were not inhibited and they supported cell division in root meristem. However the cells were remarkable by their competence towards cytokinins and ABA that might result in the stomatal guard cells aperture.

PARTICIPATION PA IN TRANSPORT OF IONS AND BAP SIGNALING

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Phosphatidic acid (PA) takes special place among signal lipids because it is a key link in the membrane lipid metabolism, on the one hand, and it performs functions of lipid signal molecule, on the other hand. Amount of PA in plant cells elevates transiently under the influence of pathogens, ROS, elicitors, ABA and ethylene. Changes in PA level influence physical properties of membranes and their ability to form vesicles. Some data confirms the ability of PA to transport Ca^{2+} ions through membranes of muscular and nervous cells.

We investigated action of PA with different composition (dioleoyl, dipalmitoyl, from egg yolk) on membrane transport of Ca^{2+}, Mg^{2+} and H^+ using plasma and endomembranes vesicles from maize (Zea mays L.) roots and coleoptiles. Also, of this investigation was to study the influence of BAP on the level of PA.

The 4-day-old etiolated maize seedlings were used. The vesicle preparations were obtained by differential centrifugation and subsequent separation in the PEG/DEX aqueous polymer two-phase system (for isolation of plasma membranes) or in the sucrose density gradient (for isolation of endomembrane fraction). The transport of ions were assayed using Ca-sensitive fluorescent probe Indo-1 (loaded into the membrane vesicles) and Ca^{2+}(Mg^{2+})-sensitive probe chlortetracycline (added to the incubation medium). The proton gradient on the tonoplast vesicles was created by activation of vacuolar H^+-ATPase. The reaction was initiated by the addition of ATP. The increasing of H^+ concentration inside vesicles was registered by the potential density probe acridine orange. The preparation of total pure lipids extracts was isolated on method Bligh and Dyer. The fraction of phospholipids was divided TLC a method Vaskovsky V.E.

Three types of PA were used in experiments; PA was allocated from an egg yolk; PA containing two residues of palmitic acid and PA containing two residues of oleic acid. The greatest effect was observed with PA consisted of two residues of oleic fatty acid. PA including in structure two residues of palmitic fatty acid had the least ionophore properties. The ability of PAs to transport calcium ions across membranes is higher at alkaline conditions than at acidic conditions. We had showed that the ionophore properties of PA are not specific. They are capable to function as membranous transmitting agent not only for Ca^{2+}, but also for Mg^{2+}.

The movement of protons along pH gradient was facilitated by PA as well as FCCP, a well-known protonophore. The highest rate of proton membrane gradient dissipation of tonoplast vesicles caused PA, which consists of two residues of oleic acid. PA with two residues of palmitic acid had the least ionophore activities.

Than we analyzed the influence BAP on change level of PA in cells. We show that the action of BAP on coleoptiles or roots induced the increase of quantity PA and change of fatty-acid composition PA. The received results testify that is probable the action BAP activated PLD. We assume, that PA produced by PLD in plant cells.

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MOLECULAR CHARACTERIZATION OF NOVEL CYTOKININ RECEPTORS IN MAIZE

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Cytokinin (CK) plays an important role for plant growth and development such as cell division, regulation of organogenesis, leaf senescence, chloroplast development and nutrient signaling. We have already identified three genes encoding CK receptors (*ZmHK1*, *ZmHK2* and *ZmHK3*) from maize. Our analyses revealed that the ZmHKs differ in ligand preference, and that the orthologs from maize and *Arabidopsis* show different ligand specificity (Plant Physiol., 2004, 134: 1654-1661).

Here we have isolated three novel genes encoding CK receptors (*ZmHK1a2*, *ZmHK1b1* and *ZmHK1b2*) from maize. *ZmHK1* shows over 90% identity with *ZmHK1a2* and about 60% with *ZmHK1b1* and *ZmHK1b2* at amino acid level. Orthologs of the *ZmHK*s were also found in rice genome, suggesting that they commonly function in monocots. Heterologous expression of each of the ZmHKs in *Escherichia coli* mutant having the Δ*RcsC* and *cps::lacZ* genetic background conferred CK-inducibility of the *lacZ* expression on the bacteria. As is the case with ZmHK1, ZmHK1a2 could respond to *cis*-zeatin at similar extent with *trans*-zeatin in the *Escherichia coli* mutant [Δ*RcsC, cps::lacZ*] assay. These data support our hypothesis that *cis*-type CK is physiologically active in some plant species including maize. ZmHK1b-type receptors showed different ligand preference from those of ZmHK1a-type (ZmHK1 and ZmHK1a2). Another assay with *Saccharomyces cerevisiae sln1* mutant showed essentially same results.

Based on the multiple sequence alignment of CK receptors from maize, rice and *Arabidopsis*, we introduced site-directed mutagenesis into *ZmHK1* and *ZmHK1b2*. The substitutions of specific amino acid residues alter the ligand preference of each receptor. Single amino acid substitution in *ZmHK1* confers constitutive activity to ZmHK1.

Western blot analyses using the antibody against ZmHK1/ZmHK1a2 suggest that subcellular localization of ZmHK1/ZmHK1a2 may be different from those of ZmHK1b-type.