O7-1: Cytokinin induced photomorphogenesis in dark-grown *Arabidopsis*: a proteome analysis

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Depending on light access plants use two distinct developmental programs. Dark-grown plants invest stored nutrients on stem elongation (skotomorphogenesis) while under light conditions development of the photosynthetic apparatus is supported (photomorphogenesis). Cytokinins (CKs) can partially replace effect of light in a transition from skoto- to photomorphogenesis. We investigated, at the proteome level, the transition induced in *Arabidopsis* dark-grown seedlings by increased levels of endogenous CKs achieved using a pOp-ipt/LhGR system. Total seedling proteins were separated by two-dimensional electrophoresis. Image analysis revealed spots that differed significantly between controls and seedlings with increased CK levels. Candidate proteins were identified by both MALDI-MS and LC-MS/MS. Chloroplast and nuclear proteins represent two major groups in the candidates. A number of them might be involved in chloroplast maturation induced by increased levels of endogenous CKs in the dark grown seedlings. The relevance of the candidate proteins in the CK induced transition from skoto- to photomorphogenesis will be determined in subsequent experiments. (Supported by grants MSM143100008, MSM0021622415, FRVŠ 804/2005, IAA600380507 and AVOZ50040507.)

O7-2: Extraction and purification of cytokinins for their determination using HPLC-MS

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Increasing use of mass spectrometry for determination of cytokinins has raised special requirements for extraction and purification of this class of plant hormones.We have compared three different extraction solvents (a) Bieleski's mixture (methanol, chloroform/formic acid/water, 12/5/1/2, v/v/v/v), (b) modified Bieleski's mixture (methanol/formic acid/water, 15/1/4, v/v/v) and (c) 80% methanol and three different purification procedures (a) mixed-mode solid-phase extraction, (b) DEAE Sephadex–RP-C18 chromatography and (c) liquid-liquid partition. Modified Bieleski's solvent, which prevented dephosphorylation of ZRMP, gave high yields of cytokinins and is easy to handle, appeared to be the most suitable for extraction of cytokinins. Purification of cytokinins using MM-SPE as compared to DEAE Sephadex-RP-C18 method provided slightly lower yields of cytokinin N-glucosides, very similar yields of storage cytokinin O-glucosides and higher yields of physiologically active cytokinin ribosides and bases. Samples purified by the former method contained lower levels of contaminants that may interfere with HPLC/MS analysis and provided higher relative recoveries of internal standards. This method was found simpler, more operational and faster allowing more complex plant hormone analysis.

O7-3: New technologies for cytokinin and auxin analyses

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The problem associated with the isolation, identification and analysis of cytokinins and auxins are considerable, although in general they are similar to those encountered with other endogenous plant substances, namely the purification and identification of submicrogram quantities of compounds from large amounts of interfering material, and their accurate and precise measurement. Liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS) in particular have recently become more important for both qualitative and quantitative analyses of all phytohormones. Unfortunately, the deuterated standards are not usually available for this analytical technology and have to be still prepared. Furthermore, specific purification is usually necessary prior to LC-MS. Immunoaffinity chromatography based on generic polyclonal and monoclonal antibodies have been recently introduced in this field giving us a possibility to get samples of almost 100% purity which contain unusual amount of very different cytokinin and auxin metabolites. In conclusion, there are several excellent procedures based on combined immunoaffinity chromatography-elektrospray single- and triplequadrupole/Q-Tof mass spectrometric analyses of cytokinins and auxins, which we hope, will be further improved.

P7-1: Analyses of cytokinins in coconut (*Cocos nucifera* L.) water using capillary electrophoresis

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Two capillary electrophoresis (CE) methods were developed for the separation of cytokinins (CKs) in young coconut water (CW). The first CE method was used to separate both isoprenoid and aromatic CKs, including Z, ZOG, DZ, DZOG, mT, iP and BA; while the second CE method was used to separate the six positional isomers of hydroxylated aromatic CKs (topolin and topolin riboside), including oT, mT, oTR, mTR, and pTR. The advantages of the CE methods are short analysis time (< 20 min), low solvent and sample consumption, and separation could be achieved by a simple silica capillary without the use of expensive chromatographic columns. Both CE methods had good reproducibility and were successfully applied to screen for the presence of CKs in CW extract purified using C_{18} and mixed-mode SPE columns. Two putative isoprenoid CKs (ZOG, DZOG) and one putative aromatic CK (oT) were identified and quantified from CW. In addition, the presence of CKs in CW was further confirmed by independent HPLC and LCMS experiments. The efficacy of CW as an excellent growth supplement could now be explained, at least in part, that it is driven by both isoprenoid and hydroxylated aromatic CKs in regulating growth and morphological development in tissue culture.

P7-2: Extraction of plant growth regulating compounds for analysis in LS-MS/MS: different procedures compared

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Relatively few papers describe the use of the sensitive combination of liquid chromatography and electrospray tandem mass spectrometry (LC-MS/MS) for determination of plant growth regulating compounds. In a study of branch hierarchy and crown structure in a conifer tree, *Abies nordmanniana*, two different published procedures for extraction were tested using LC-MS/MS for identification and quantification of plant hormones including cytokinins, gibberellins and auxin. The first procedure was discarded due to several limitations: Deuterium labelling of the internal standard for IAA was partly or entirely lost, separation of phosphorylated and non phosphorylated cytokinins was incomplete, and both ABA and gibberellins were lost in the procedure. The second procedure recovered all compounds of interest.

P7-3: Immunomagnetic separation method for isolation of new cytokinin derivatives from various biological materials

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Dynabeads M-450 Epoxy are uniform, superparamagnetic, polystyrene beads which are designed as a matrix for immunomagnetic separation. Antibodies may be directly coupled to the magnetic beads and this system could be used for the isolation of various substances from samples of different biological origin. Benzylaminopurines belong to group of fytohormones, cytokinins. These compounds naturally occur in plants and exhibit high biological activity. Synthetic compound 6-(2-hydroxy-3-methoxybenzylamino)purine riboside (2-OH-3-OCH_3-BAPR) structurally also belongs to this group. Surprisingly, we have proved its high cytotoxicity against CEM and HL-60 leukaemia cell lines. To study this highly promising compound in more details, rapid and highly efficient method for its purification from complex samples is needed. Therefore, we have prepared monoclonal antibody against 2-OH-3-OCH_3-BAPR. Immobilization of pure antibody on the immunomagnetic beads and optimalization of their separation will be described. The developed method will be further employed for putative isolation of natural di- and tri-substituted benzylaminopurines and their ribosides from different plant materials as well as for studies of pharmacokinetic and metabolism of 2-OH-3-OCH_3-BAPR in murine-blood samples.

P7-4: New, sensitive method for enzyme kinetic study of rare glucosides

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The maize β-glucosidase Zm-p60.1 is important for regulation of plant development due to its role in the targeted release of free cytokinins from cytokinin-O-glucosides, their inactive storage forms. Enzyme kinetics studies using these rare substrates close to physiological concentrations are difficult due to: *a)* Available methods are mainly suited for end-point kinetics. *b)* These methods are not sufficiently sensitive when using rare glucoside substrates. We developed a glucose assay using a system comprising enzymes b-glucosidase, glucose oxidase and horseradish peroxidase, with the new substrate N-acetyl-3,7-dihydroxyphenoxazine-Amplex UltraRed reagent. A calibration curve was constructed for resorufin and validation carried out by comparing our method with the standard spectrophotometric method using p-nitrophenyl-β -D-glucopyranoside. Compared to the other methods tested (MALDI, ELISA, microcalorimetry, peroxidase/glucose-oxidase-coupled reaction with o-dianisidine) this method is more sensitive, precise, and accurate. The main achievement of our method is rapid procedure (up to 2.5 s) suited for continuous kinetics and its utility in enzyme kinetics studies of rare glucoside substrates. (Supported by GA CR 203/02/0865, MSM0021622415, AVOZ50040507.)

P7-5: New chromatographic and mass spectrometric approach for cytokinin analysis

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Application of new analytical methods makes possible new direction in plant hormone research. Ultra performance liquid chromatography (UPLC) is new technique using high pressure and hybrid particles as column packing. A chromatographic separation of 20 cytokinins (*trans/cis*-zeatin, dihydrozeatin, isopentenyladenine, benzyladenine, meta/ortho-topolin groups) on C18 column by HPLC/MS was developed (Novák et al., 2003). Run time of published analysis was 50 min. Using UPLC analysis run time was reduced by up to seven times. Retention time stability ranged between 0.1-0.4 % RSD and injection accuracy between 1.5-5.0 % RSD. Combination of quick separation with fast scanning triple quadrupole mass spectrometer was tested. The method based on capillary liquid chromatography with quadrupole-time of flight mass spectrometry was also used for sensitive identification. Exact mass determination gave us a possibility to do more accurately identifications of cytokinins and their metabolites. Accurate masses of parent ions and their fragments were calculated and used for determination of elementary structure with fidelity 10 ppm and better. Sensitivity of quantitative analysis were comparable with HPLC/MS, shorter dynamic range was replaced by more perfect way of structural determination.

P7-6: The use of imunoaffinity extraction in auxin analysis in plants

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The study of auxin metabolism is a task comprising the two main problems: high content of ballast compounds and low levels of analyte. To meet the task, masterly instrumentation, *e.g.* MS/MS, is often employed. Here we suggest the use of immunoaffinity extraction (IAE) based on polyspecific antibodies against 3-indolylacetic acid (IAA) as an efficient tool for a compound-specific purification of pre-purified plant extracts, which reduces requirements for analytical instrumentation. Polyclonal anti-IAA antibodies were obtained from rabbits immunized with a conjugate of IAA coupled to BSA through carboxyl group and characterized in ELISA using IAA methyl ester as standard. The antibodies exhibited high cross reactivities with methyl esters of amide conjugates and other IAA-related compounds; they were therefore coupled to Affi-Gel and immunoaffinity purification procedure was developed. We used the analytical protocol combining solid-phase extraction, immunoaffinity extraction and HPLC with fluorimetric and/or mass detection for auxin analysis in *Arabidopsis thaliana*.

P7-7: Quantitative detection of zeatin O-glucosyltransferase gene (ZOG1) transcript in tobacco under drought

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Drought stress changes plant development and metabolism considerably, promoting senescence and chlorophyll degradation in older leaves and stimulating nutrient transport to and chlorophyll retention by young leaves. As cytokinins (CKs) and abscisic acid are involved in the regulation of these processes, their levels were determined in individual leaves of tobacco (*Nicotiana tabacum* L). CK metabolites were analyzed in WT plants as well as plants with increased level of CK storage forms through constitutive expression of the *Phaseolus lunatus* zeatin O-glucosyltransferase (*ZOG1*) gene. Significantly higher levels of CK deactivation and storage forms were found in older leaves of both the wild type and 35S::*ZOG1* plants under severe stress, which correlated with lower chlorophyll content. The amount of stress perceived by the plant will be followed in individual leaves by quantification of stress marker (*e. g.* dehydrin) expression. Reference genes showing steady expression levels under drought stress in tobacco were selected by quantitative RT-PCR with FastStart DNA Master SYBR Green I and LightCycler device (Roche). We found *NtAct9, NtL25* and *18S* as suitable genes to normalize *ZOG1* and stress marker gene expression. (Supported by GA CR 522/04/0549.)

P7-8: Effects of elevated endogenous cytokinin levels on the *Arabidopsis thaliana* proteome

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The plant hormone cytokinin (CK) plays a crucial role in plant development from germination to senescence. The exact mechanisms of CK action in regulation of plant development are the subject of current intense research. We report a classical proteome-based approach as an attempt to reveal more information about CK action in the model plant, *A. thaliana.* Total proteins were extracted from whole wild-type plantlets as well as from the transgenic pOp-*ipt*::LhGR line in which the production of endogenous CK is inducible by dexamethasone (DEX). Plants were grown for 15 days under long-day conditions on MS media, in parallel with and without 2.5 mM DEX. Image analysis of 2-D gels and subsequent statistical evaluation revealed significant changes in protein expression between plants with elevated CK levels and controls, whereas no major effects of DEX on the protein maps were observed. Out of the differentially expressed protein spots a total of 55 were characterized by MALDI-TOF MS analysis. Among them, there are several interesting candidates whose potential relationship to CK metabolism and action will be discussed. (Supported by the Ministry of Education of the Czech Republic MSM143100008, MSM0021622415, AS CR AVOZ50040507 and by the scholarship IN-CO Czech 2002-04.)