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Functional Genomics and Proteomics for Sustainable Agriculture

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a conference commemorating
the 190th birth anniversary of Gregor Mendel

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MENDEL 190
Celebration of G. J. Mendel's Birth Anniversary

MENDEL UNIVERSITY IN BRNO

BOOK OF ABSTRACTS

Functional Genomics and Proteomics for Sustainable Agriculture

a conference commemorating the 190th birth anniversary of Gregor Mendel

Brno 13th – 14th June 2012

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Invited Speakers

Pea entering genomic era, 150 years after Mendel's discovery

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This year we celebrate 190th birth anniversary of J.G. Mendel, man making the foundation of modern genetics. Pea (*Pisum sativum* L.) was the original model organism used in Mendel´s discovery (1866) of the laws of inheritance. However, subsequent progress in pea genomics has lagged behind many other plant species. Also not considered model species, there have been always international community working on various aspects of pea biology, both from practical as well as theoretical points. Although the large size (4.45 Gbp) and repetitive nature (50-60%) of the pea genome has so far restricted its sequencing, comprehensive genomic and post genomic resources already exist. These include BAC libraries, several types of molecular marker sets, both transcriptome and proteome datasets and mutant populations for reverse genetics. The availability of the full genome sequences of three legume species offers significant opportunities for genome wide comparison revealing synteny and co-linearity to pea. A combination of a candidate gene and colinearity approach has successfully led to the identification of not only genes underlying Mendel's traits but also agronomically important traits including quantitative trait loci (QTL). Some of this knowledge has already been applied to marker assisted selection programs, increasing the precision and shortening the breeding cycle. Molecular analysis of pea collections has shown that although substantial variation is present within the cultivated genepool, wild material offers the possibility to incorporate novel traits that may been inadvertently eliminated during domestication. As the next step providing link between phenotype with genotype on a whole genome basis, a process initiated by Mendel about 150 years ago, the association mapping is stepping in. The availability of high throughput ´omics´ methodologies offers great promise not only for basic biology but also for the development of novel, highly accurate selective breeding tools for improved pea genotypes that are sustainable under current and future climates and farming systems. In this review I aim to summarize the status of pea genetics, genomics and molecular biology, as well as current knowledge of Mendel's seven selected traits. The perspective of pea genome sequencing will be shown.

Financial support from the Ministry of Education of Czech Republic ME10006, ME10062, LA08011 and the Bioversity International AEGIS LOA 10/048 projects is acknowledged.

RNAseq transcriptome de novo assembly in pea: a new genomic resource for deciphering the pea genetic variability

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While the demand for protein-rich raw material has constantly increased during the last 40 years, the European domestic production has remained at a low level. Because of their high protein seeds and their unique ability to establish a symbiosis with soil nitrogen fixing bacteria, legumes should play a central role in future sustainable agriculture. Pea, as some other grain legumes, is a strategic crop for Europe but higher and more stable yielding pea varieties are needed to allow for its development. However, molecular tools are lacking to enhance marker-assisted selection and/or gene cloning in pea. The development of tools allowing back and forth comparisons between the legume model species and pea seems highly strategic to enhance gene discovery in pea. A first step towards this aims was to develop a Unigene set for pea. Using Next-Generation sequencing technologies, we sequenced pea cDNA libraries for 20 different plant organs, stages, and nutrition condition. One billion reads corresponding to ca 100 Gb were produced. The these sequences were de novo assembled and a full-length Unigene set was produced. These data revealed the complexity of transcriptomes and allowed identifiying interesting expressional candidates.

Supported by grant from ANR GENOPEA and QualityLegSeed projects and FP6 GLIP Project

Shaping plant body architecture – lessons from pea

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Polar auxin transport in stem is necessary for the control of bud outgrowth by a dominant apex. Following decapitation in pea (*Pisum sativum* L.) the axillary buds establish directional auxin export by subcellular polarization of PIN auxin transporters. Apical auxin application on the decapitated stem prevents this PIN polarization and canalization of laterally applied auxin. These results support a model in which the apical and lateral auxin sources compete for primary channels of auxin transport in the stem to control axillary bud outgrowth and thus shoot branching. Exogenous application of cytokinins, strigolacton or auxin transport inhibitors can stimulate or inhibit bud outgrowth and simultaneously polar auxin transport. Changes in polar auxin transport were followed by *PIN1* and *AUX1* gene expression and localization of PIN1 protein. The cytokinin induced stimulation of bud outgrowth even on intact plants was reflected by earlier elevation of gene expression and PIN1 polarization. Protein synthesis inhibitor cycloheximide prevented any observable outgrowth, but polarization of PIN1 proteins was similar as in outgrowing buds. Despite of NPA application to the buds of decapitated plants they started to grow and their outgrowth was partly inhibited in later stages. This is in accordance with the observed decrease of gene expression and PIN1 localization in longer time.

This work was supported by the project "CEITEC - Central European Institute of Technology" (CZ.1.05/1.1.00/02.0068).

Chromosome genomics reveals the secrets of hereditary information in grasses (Poaceae)

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Chromosome genomics proved to be an elegant method to analyze large and polyploid plant genomes. This approach, which was originally developed in faba bean and garden pea, simplifies the analysis by dissecting nuclear genomes to small parts by flow cytometric sorting of mitotic chromosomes. Recently, the method was modified for economically important grasses, including wheat, barley and rye. In these species, chromosome genomics has been used to develop DNA markers from sub-genomic regions, identify genes and establish virtual gene order for complete sets of chromosomes, as well as to construct physical maps of chromosomes to facilitate map-based cloning and production of reference genome sequences. The sequence information on individual chromosomes enables comparative analyses as well as functional and evolutionary studies between and within chromosome groups. Survey sequencing chromosomes isolated from wild relatives of cultivated cereals provides information to support alien introgression breeding and reveals changes of nuclear genomes that accompanied the evolution and speciation within the grass family.

This work has been supported by the Ministry of Education, Youth and Sports of the Czech Republic and the European Regional Development Fund (Operational Programme Research and Development for Innovations No. CZ.1.05/2.1.00/01.0007).

Evolution, structure and dynamics of plant polyploid genomes

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Allopolyploidy provides for natural experiments in genome perturbation. Genetic changes, subsequent to polyploid formation, can be inferred by studying the descendants of the progenitor species and the allopolyploid. In 1984, McClintock first proposed that these changes might be distinctive: that allopolyploidy can induce 'genomic shock', such a process may produce rapid alteration at the DNA sequence level in addition to significant structural, transcriptional, epigenetic and karyotypic changes. This has led many to envisage a 'genome revolution' where perturbation of progenitor genomes is induced by their unification. We will present examples of genetic and epigenetic changes in associated with allopolyploidy different loci and different systems. Our methodical approached involve next generation sequencing, classical FISH and Southern hybridization methods. We show that the coding and non-coding parts of rDNA units (35S) are unequally homogenized in species that harbour multiple loci indicating that interlocus homogenisation may be rather infrequent in stabilised diploid genomes. However, allopolyploidy can dramatically increase the rate of interlocus homogenization as apparently happened in the case of tobacco allotetraploid in which parental units were largely overwritten by novel units. We will further discuss frequent changes in the organisation and structure of rDNA showing that the 5S and 35S genes may be physically linked and unlinked in different angiosperm lineages. Finally we will introduce a novel on-line *Plant rDNA database* (www.plantrdnadatabase.com) that compiles information on the position and number of rDNA loci in a number of angiosperm, gymnosperm and bryophyte species.

Mendelian factors and their continuous importance in plant breeding

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Plant breeding is the recurrent process which transforms genetic resources into improved cultivars. On a more technical level, plant breeding integrates various sciences and technologies with plant characters and market needs. Agronomic characters, resistances and product quality traits are the three major groups of traits handled in plant breeding programs of many agricultural crops. Throughout most of the 20th century, quantitative traits and their selection on the phenotypic level only have played the major role in crop improvement. Recently, the progress in genomics has allowed for the dissection of quantitative characters into distinct Mendelian factors. As a consequence, genomic selection and QTL-based selection are rapidly gaining in importance for breeding of agronomic characters. Thus, the relevance of Mendelian factors is significantly increasing for yield traits, resistances as well as product quality characters. Moreover, four of the seven characters studied by Gregor Mendel have been exhaustingly characterized down to the molecular level to date. Surprisingly, parallels have been found between important characters of contemporary plant breeding and some of Mendel's traits from his pea experiments: Mendel's tall vs. short (*Le* / *le*) stem length trait is controlled by a gene affecting gibberellic acid metabolism, which parallels the "genes of the green revolution" controlling semidwarf wheat and rice development. Mendel's round vs. wrinkled (*RR* / *rr*) pea seed shape character is caused by a loss of function mutation due to a transposon insertion into a functional gene sequence. Similar mutations have been found to control many important quality features in different crop plants. Finally, Mendel's yellow vs. green (*II* / *ii*) seed color segregation is due to a disruption of the chlorophyll degradation pathway, which causes a "stay green" phenotype; as many plant pathogens are inducing chlorophyll degradation as part of their infection strategy, induction of stay green phenotypes could become a promising future strategy in breeding for tolerance towards a wide range plant diseases.

Applying Mendel's rules on African Crops – sesame breeding in Uganda

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Africa is one of the centers of biodiversity for sesame and it is a high value crop for domestic, regional and international markets. The extent of the regional biodiversity in Uganda as well as other African counties is relatively unknown at this point, so the exploitation of the valuable local diversity for high yielding, pest resistant varieties is hindered by this lack of knowledge. Collecting efforts were conducted in the major sesame growing area to determine the locally available diversity of the crop and to select material suited for the introduction in breeding efforts as well as germplasm conservation strategies.
A set of 200 local varieties and breeding lines were collected in different sites, grown in consecutive field trials and phenotypically characterized using 34 descriptors. In addition biparentally and uniparentally inherited molecular markers were developed and applied in order to assess existing genetic diversity as well as to identify duplications in the germplasm collection. For the geographical and evolutionary differentiation of the different lineages, universal chloroplast regions were used, whereas nuclear microsatellites derived from EST regions serve as tools to investigate putative functional diversity in the collection. In addition these molecular markers were used to analyze the selfing rate of sesame grown in farmer's fields as compared to gene conservation fields.
Results of the analysis of genetic markers or marker combinations discriminating different varieties in combination with phenotypic traits will be presented.

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Proteomic and cell biology approaches to study vesicular traffic in plants

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Considering broad range of functions, vesicular trafficking should play a central role in plant stress adaptation. In this respect, the investigation of vesicular trafficking deserves significant attention. Combined proteomics and cell biological approaches have been used in this study to explore the effects of wortmannin on proteome and endomembrane trafficking in Arabidopsis roots. Wortmannin inhibits phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinases (PI4Ks) in a dose-dependent manner resulting in the inhibition of protein vacuolar sorting and endocytosis.. On the subcellular level, wortmannin caused clustering, fusion and swelling of MVBs leading to the formation of wortmannin-induced multivesicular compartments. Appearance of wortmannin-induced compartments was associated with depletion of TGN as revealed by electron microscopy. This indicates a possible consumption of TGN vesicles by MVBs during wortmannin treatment. On the proteome level, wortmannin caused changes in protein abundance profiles. Proteins affected by this drug belonged to various functional classes. An inhibition of vacuolar trafficking by wortmannin was related to the downregulation of proteins targeted to the vacuole, as showed for vacuolar proteases. A small GTPase, RabA1d, which regulates vesicular trafficking at TGN, was identified as new protein negatively affected by wortmannin. In addition, Sec14 was upregulated and PLD1 alpha was downregulated by wortmannin. Since these proteins are involved in the maintenance of proper phosphatidylcholine and phosphatidylinositol levels in membranes, we propose that they may regulate trafficking route between TGN and MVB in Arabidopsis roots.

This work was supported by grant Nr. P501/11/1764 from the Czech Science Foundation GAČR and by grant Nr. ED0007/01/01 to Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University, Olomouc, Czech Republic.

Cytokinin: on the crossroads to temperature perception?

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Cytokinins (CKs) regulate diverse developmental processes in plants. Recently, we identified novel targets of CK signaling by proteome and phosphoproteome analysis, and our results pointed to a possible link between temperature and CK signaling. To shed new light into the temperature perception and interaction of temperature and CK signaling, we employed 2-DE proteomic analysis on Arabidopsis seedlings treated with cytokinin for 15 min under temperature-shock conditions. We conducted the first proteomic analysis of 15 min temperature-shock responses in plants on the phosphoproteoeme, and proteome fractions (i) enriched in low abundance proteins via proteome equalization and (ii) depleted of RuBisCO. In this large-scale experiment, 148 shock-response proteins were identified. More than 70 % of these temperature-shock response proteins were affected by exogenous cytokinin treatment which seems to partly mimic the effects of heat-shock on the Arabidopsis proteome. CK role in heat perception was also proved in physiological experiments. Further, we studied potential benefits of inducibly-increased endogenous CK levels in temperature-stress conditions. Arabidopsis plants were subjected to 3 different kinds of heat stress for up to 3 hours: heat stress from above (leaves), heat stress from below (roots) and heat stress on the whole plant. In numbers, we found more than 100 responsive proteins and most of these heat-stress responsive proteins exhibit strong modulations by increased CK levels. Moreover, our results indicate interesting interplay between heat-stress perception in roots and leaves.

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The contribution of plant genetic engineering to 21st century sustainable agriculture

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The 21st century is challenged with the problem of a growing world population under changing climate conditions and shrinking environmental resources. In 2050, the world population will reach 9 billion people who will all need drinking water, food, housing and energy. Besides the unresolved political question how to distribute these resources equitably, agriculture was until now able to cope with the requests in food and feed. However, with shrinking agricultural surface and water resources, agriculture will not be able to satisfy the demands with the available technologies. New ways will have to be developed, spanning issues from land management, agricultural practices to improved soil and genetic resources. Plant biotechnology will play a key role in 21st century food security, drinking water availability and health. Besides a fundamental understanding of plant and microbe functioning, crop engineering programs are rapidly needed to be set up to meet global food and energy demands. Modern agriculture will need a new green revolution that will have to function in an ecologically balanced way. I will discuss the current challenges and the panoply of possible technological solutions for a modern sustainable agriculture system.

Transgenic alteration of cytokinin metabolism in order to improve agricultural traits of barley

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Cytokinins (CKs) are ubiquitous phytohormones that have been implicated in development, morphogenesis and many physiological processes throughout plant kingdom. In higher plants, mutants and transgenic plants with altered activity of CK metabolic enzymes or perception machinery pointed to their crucial involvement in plant morphology, productivity and tolerance to various stresses. Furthermore, recent precise metabolomic analyses have elucidated specific occurrence and distinct function of different CKs in various plant species. In cereals, the most abundant CK detected during onthogenesis is cis-zeatin, and dihydrozeatin, which accumulates mostly in dormant seeds. Contrary to trans-zeatin and isopentenyladenine, their biosynthetic origin in plant tissues is still unknown.

Genetic manipulation with enzyme responsible for irreversible CK degradation, cytokinin dehydrogenase, can lead to plants with more proliferated root system and increased grain yield. We prepared several lines of transgenic barley with ectopically overexpressed cytokinin dehydrogenases with natural or artificial cellular compartmentation, as well as lines with silenced gene transcription showing these enhanced agriculturally important traits. Preparation, selection and properties of engineered barley plants will be discussed in more details.

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From Gregor Mendel to Plant Systems Biology

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Approaching genetic complexity in plants - From Mendel's single genes to multi-gene networks

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The search for single genes that control mendelian traits have dominated the field of genetics, particularly since the advent of mutant screens. This endeavor has been very fruitful and tremendous advances have been made. However, it has become clear that many biological processes are not governed by single genes but by networks of genes. The inherent properties of many biological networks such as robustness and the ability for buffering limit the usefulness of approaches focused on single-genes such as mutant screens. In contrast, approaches that use the concepts of quantitative genetics are highly suited to dissect traits that are governed by multiple genes. We have set out to identify novel networks involved in the regulation of developmental processes by exploring the phenotypic space created by natural variation and identifying the causal genes using quantitative genetics. To achieve this, we have established multiple phenotyping platforms at the cellular and organ level for the automated and accurate quantification of multiple root traits from a large number of individuals. Using the phenotypic data of more than 200 accessions to conduct genome wide association (GWA) mapping, we identified several candidate genes that are involved in root growth and development. Furthermore, I will show how natural variation is able to uncover tight connections between separate developmental processes, and will demonstrate that our quantitative methods hold great promise for mutagenesis approaches and might enable network centered mutant screen in the roots.

Beyond Mendel? How forest trees develop and adapt complex traits in changing environments

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With the exception of a few examples, morphological traits and growth characteristics in forest trees could not be traced back to simple Mendelian genes yet. On the other hand, genetic markers show completely regular inheritance and provide a good playground for checking the reality content of population genetic theories, but have shown little correlation with growth and form characters. Yet, decades of provenance tests have also clearly revealed that the adaptation to specific environments is heritable in these trees. In the absence of any practicable crossing tests over several generations, polygenic models have found the best support for explaining these phenomena. How complex, then, are the apparent gene networks that control inheritance in forest trees, and in what way do population genetic aspects like shifting selection in changing climates during long lifetimes, long-range gene flow and overlapping generations interfere with our Mendelian understanding of trait inheritance? Recent results from the study of natural hybrids in *Populus* have advanced our knowledge regarding these gene networks. A hypothesis to corroborate the seemingly discrepant observations will be discussed.

The lab of Berthold Heinze is currently supported by grants from the European Commission, the Austrian Science Fund, and the Austrian Ministry of Agriculture, Forestry, the Environment and Water Management.

Functional genomics in energy plants

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Mendelism and neo-Mendelism of some essential oil components

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Many aromatic and medicinal plants are still in the transitional phase from wild plants to systematic cultivation. Their morphological and chemical polymorphism is therefore rather high and on one side the basis of biosynthetic studies, on the other for targeted breeding: *'A plant breeding program . . . requires a basic knowledge of the inheritance of at least the main EO compounds'*. Targeted breeding as modern approach is based, however, on knowledge of the genetic background of the respective characters. Crossing experiments help to understand the inheritance of characteristics, and molecular biology gives deeper insight into the genetics of secondary plant products both leading to efficient strategies assisted by such techniques. The Mendelian segregation of *Mentha spp.* was exhaustively studied in a fascinating way, followed by several other EO plants, including chamomile and yarrow. Such experiments contributed, for instance, to specific knowledge on the final biosynthetic steps in the formation of thujones in sage, of sabinene hydrate acetate in marjoram or proazulenes and bisaboloids in *Matricaria recutita*. It also became obvious that the mevalonic acid pathway is responsible for the biosynthesis of sesquiterpenes only, while the methyl–erythriol–phosphate pathway was for monoterpenes. In recent years, research on the biosynthesis of EO compounds has been more focused on relevant enzymes within the pathways, on genetic and protein engineering. As a result, many steps to alter EO compounds could be found, and genetically or phytochemically *tailored* plants are to be expected in the future.

Reference: Franz, Ch. and Novak, J.: Sources of Essential Oils, in: Handbook of Essential Oils (KHC. Baser and G. Buchbauer, eds.), p. 39-81. CRC Press 2010

Poster Presentations

Leaf specific thionin gene in *Arabidopsis thaliana* comprises antimicrobial properties

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Plants are continuously exposed to a variety of pathogenic organisms including bacteria and fungi. Plants have evolved different defense mechanisms such as phytoanticipins and phytoalexins, PR-proteins, and antimicrobial peptides (AMPs). Plant AMPs are usually cysteine rich and basic peptides and include thionins, defensins, and lipid transfer proteins. In the present study, *At1g66100 (Thi2.4),* a gene from the model plant *Arabidopsis thaliana* which is a member of the thionin family was studied. To evaluate the expression in different tissues, promoter::GUS fusion lines were studied. These lines showed strong expression in seedling, rosette leaves, hypocotyl and root. Overexpression lines in the Col0 background were produced to study a possible antimicrobial effect of this AMP. Three candidate overexpression lines were screened on the basis of quantitative polymerase chain reaction from twenty homozygous lines. Quantitative RT-PCR showed that this gene was strongly up-regulated against *Pseudomonas syringae* DC3000 strain. The data from qRT-PCR and promoter::GUS fusion lines demonstrated slight up-regulation of Thi2.4 in 5 and 15 days old feeding sites of beet cyst *Heterodera schachtii*. The overexpression lines and a T-DNA insertion mutant line were compared with the wild type plants after infection with the pathogenic strain *Pseudomonas syringae* DC3000. The mutant *thi2.4* was more susceptible than the wild type while overexpression lines were significantly more resistant. Similar results were found after infection with the *H. schachtii.* Infection tests with *Fusarium graminearum* and *Botrytis cinerea are* currently under study.

pMAA-Red: a new vector for fast visual screening of transgenic lines at the seed stage

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The production of transgenic plants, either for the overproduction of the protein of interest, for promoter::reporter lines, or for the sillencing of genes is an important prerequisite in modern plant research but is also very time-consuming. We have produced additions to the pPZP family of vectors. Vector pPZP500 (derived from pPZP200) is devoid of NotI sites and vector pPZP600 (derived from pPZP500) contains a bacterial kanamycin resistance gene. Vector pMAA-Red contains a promoter*Pdf2.1*::DsRed marker (DsRed as fluorescent selectable marker for plants instead of conventional antibiotics) and a CaMV::GUS cassette within the T-DNA and is useful for the production of promoter::GUS lines and overexpression lines. In addition to the seeds, *Pdf2.1* promoter delivers DsRed into the nematode *Heterodera schachti feeding* sites called syncytia; the infection assay with *H. schachti* does not show significant difference between pMAA-Red construct line and wild type. So overexpression of RsRed in the syncytia has no effect on nematodes development. Transgenic seeds show red fluorescence which can be used for selection and the fluorescence level is indicative of the expression level of the transgene. The advantage is that plants can be grown on soil and that expression of the marker can be screened by eye which saves time and resources. It concludes that the vector pMAA-Red allows for fast and easy production of transgenic Arabidopsis plants with a strong expression level of the gene of interest.

Proteome and phosphoproteome analysis of butenolide treated *Arabidopsis thaliana* plants

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Butenolide/karrikins constitute a chemically defined family of plant growth regulators. They were first discovered in 2003 in smoke from wildfires (Flematti G. R. et al. 2004 and Van Staden J. et al. 2004). Wildfire smoke can positively affect germination and post-germination stage(s) resulting in increased seedling vigour (Soós V. et al. 2009). To date, six members of butenolide/karrikin family have been found in smoke, KAR_1 and its five naturally occurring analogues (KAR_2 – KAR_6), (Flematti G. R. et al. 2009). KAR_1 has been established as the major germination stimulant present in smoke (Chiwocha, S. D. S. et al. 2009, Light, M. E. et al. 2009). Smoke stimulates germination of numerous plant species including *Arabidopsis thaliana*. In this work we decided to use *A. thaliana* plants treated with KAR_1 to analyse changes on the protein and phosphoprotein levels. We employed total protein TCA-acetone precipitation for total protein extraction (Méchin et al. 2006) and the PhosphoProtein Purification Kit (QIAGEN) was used to obtain phosphoproteins. We then employed 2D electrophoresis using SDS polyacrylamide gels and gel image analysis (Decodon delta 2D) to analyse protein expression changes. Chosen spots with significant changes underwent in-gel tryptic digestion and identification by MALDI-TOF/TOF MS and MASCOT peptide mass fingerprint search. We identified 32 spots with changed protein expression from phosphoproteome analysis and 42 spots from total proteome analysis. Our work was focused on investigation of responses to KAR_1 on expression of phosphoproteins and total proteins in *Arabidopsis thaliana.* Our aim is to reveal the possible target(s) of karrikin action and further to understand the mechanism(s) of its action.

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Dynamic model for cell-fate Determination and Patterning in the Vascular Bundles of *Arabidopsis thaliana*

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Cellular patterning in the root and shoot apical meristems of *Arabidopsis thaliana* (Arabidopsis) has been widely studied from experimental and modelling perspectives. However, less is known about cellular patterning in the vascular meristem, which gives rise to vascular tissues and underlies radial plant growth. We study the process of cellular determination and patterning of the three basic vascular cell types (procambial, xylem and phloem cells) in the shoot vascular meristem, which is organized in vascular bundles. A set of molecular elements involved in this process has now been identified and partially characterized, but it is not yet clear how the regulatory interactions among these elements, in conjunction with cell-to-cell communication processes, give rise to the steady patterns that precede cell-fate determination within vascular bundles. In order to explore the dynamics of pattern formation within vascular bundles in Arabidopsis, we put forward a spatiotemporal dynamic model integrating the interactions between molecules (genes, peptides, mRNA and hormones) that have been reported to be central in this process. When some informed assumptions are considered, the model reproduces the hormonal and molecular profiles and spatial patterns expected for the three regions within vascular bundles. In order to test the model, we simulated mutant and hormone-depleted systems and compared the results with the available reported phenotypes. Overall, the proposed model provides a formal framework to integrate the currently available experimental data and a dynamic account of how the collective action of the hormones, genes, and other molecules may result in the specification of the three main regions and cell types within vascular bundles. Finally, the model renders novel predictions that can be experimentally tested, and provides a reference for further studies comparing the dynamics of patterning and meristem maintenance in plants.

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Maize germination: a proteomic analysis

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Germination of seeds is a complex physiological process which has a tremendous effect on plant growth and development. It is triggered by several stimuli, including imbibition of water. However, exact molecular mechanisms on proteome level are still to be elucidated. The role of light stimulus and the effect of imbibition (24 h) and early germination (48 h) on *Zea mays* seeds proteome was investigated by 2D gel analysis. In proteome maps, significant differences were reproducibly observed for 50 and 34 protein spots in light and dark cultivated seeds, respectively. Further, it was found that absence of light stimulus is not manifested during imbibition, but has a negative effect on number of responsive proteins in the second phase of germination.

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The cytokinin metabolic network at the cellular level – better access with new tools

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Active cytokinin (CK) levels are the result of the combinatorial regulation of its biosynthesis, reversible and irreversible metabolic interconversion, and terminal degradation. While approaches involving exogenous application, increased biosynthesis and more efficient degradation have yielded insights into the roles of CK in general, the significance of many of the subtler conversions have remained largely intractable. We have developed the zeatin-O-glucoside (ZOG)-specific β-glucosidase Zm-p60.1 as a molecular tool to specifically harness the reversibility of the O-glucosylation step in order to ask questions about the achievement and/or maintenance of CK homeostasis at the cellular level.

When transgenic tobacco plants over-expressing Zm-p60.1 in the apoplast were grown on medium containing zeatin, they did not accumulate fresh weight as observed in other subcellular variants of Zm-p60.1. However, when grown on medium without hormone, transgenic plants were significantly heavier than wild-type three weeks after sowing. The enzyme was histochemically localized in cotyledons, leaves and ectopic structures at the base of the hypocotyl using an indigogenic substrate for β-glucosidase. Molecular and histological analyses of the phenotype will be presented.

Database searches were conducted to look for tobacco orthologs to Arabidopsis response regulators, the effector molecules in cytokinin signal transduction. We queried EST databases using the receiver domain amino acid consensus sequence. Three suitable candidates were found and the expression of these genes in the transgenic variants mentioned above will be presented.

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Identification and characterization of pea resistance genes for *Pea seed-borne mosaic virus* (PSbMV) and other potyviruses

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The genus Potyvirus is one of the largest and most diverse genera of plant viruses. *Pea seed-borne mosaic virus* (PSbMV), a member of this genus, causes severe losses in field pea and other legumes. These losses can be prevented by growing resistant varieties. The eukaryotic translation initiation factor 4E was shown to be involved in natural resistance against several potyviruses in plants. In pea, resistance to the common P1 strain of PSbMV is conferred by a single recessive gene (*eIF4E*), localized on linkage group VI (*sbm-1* locus), candidate homologues *eIF4E(iso)* gene was mapped closely to *sbm- 2* locus (linkage group II). We have analyzed variation in the *eIF4E(iso)* genomic sequences from pea lines, reported as donors of resistance. Mutations of *eIF4E(iso)* between resistant and susceptible genotypes have been identified but not yet functionally assigned. From the comparison of *sbm-1* genotype to phenotype of F_2 plants, there was a 26% discrepancy between the PCR and ELISA-based assays with susceptible heterozygous plants missed (Smýkal *et al*. 2010). We analyzed by qRT-PCR expression of both resistant and sensitive *eIF4E* alleles in F_1 heterozygous plants, sensitive and resistant homozygotes after PSbMV infection, together with evaluation of viral concentration. The novel *eIF4E* alleles detected in wider pea germplasm as well as TILLING mutants (Dalmais *et al.* 2008) are being tested for various potyviruses. It can be expected that information gain in this study might be extended to other legume species, particularly to economically important species such as chickpea, lentil, faba bean or soybean where potyviruses cause problems.

Reference: Dalmais M, Schmidt J, Le Signor C *et al* (2008) Genome Biol 9: R43; Smýkal P, Šafářová D, Navrátil M, Dostalová R (2010) Mol Breeding 26: 425-438.
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Identification of cytokinin receptors of *Brassica napus*

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Cytokinins (CK) belong to a group of plant hormones playing crucial role in plant growth and development. CK signal is transduced through a multistep phosphorelay where the phosphoryl group is transferred between His and Asp residues. Of at least three gene families, taking part in the CK signal transduction, sensor histidine kinases (HKs) have been well-studied and shown to be important morphological factors in *Arabidopsis*. As *Brassica napus* shares a high degree of nucleotide identity in coding regions with *Arabidopsis*, we used sequences of *A.thaliana* HK genes (*AHKs*) to search for their orthologs in *B. napus* (*BnHKs*). Experimental screen of the *B. napus* genomic library resulted in identification of redundant number of overlapping BAC clones which hybridized with PCR-generated probes derived from sequences of phylogenetically conserved AHK structural domains. 5 selected clones have been sequenced and defined as CK receptors by computational analysis. Based on the prediction, gene-specific primers have been designed and coding- as well as untranslated cDNA regions cloned. Results of the predicted functional domains of five BnHK proteins and, based on the comparison of genomic and cDNA sequences, results of *BnHK* genes' structure analysis are presented. Four orthologs of AHK2 and one ortholog of AHK3 have been identified this way.

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Light and cytokinin interactions in regulating elongation of the Arabidopsis hypocotyl

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Light and cytokinins participate in the regulation of a number of similar growth and developmental responses in plants. Recently, we have uncovered a role for cytokinins in the stimulation of hypocotyl elongation in Arabidopsis seedlings grown under decreased white light intensity (20 $\mu mol.m^{-2}.s^{-1}$). The CK effect on hypocotyl length was due to cell elongation. The stimulation of hypocotyl elongation was observed for all four principal CKs (*t*-Z, iP, BA, TDZ) and was dose-dependent in the nanomolar range. The stimulatory effect of the CKs was antagonized by PI-55. Mutant and transgenic plant analysis indicated that the canonical two-component response pathway is necessary for this process with prevailing contribution of cytokinin receptors AHK2 and AHK3. This CK action was independent of ethylene signaling and partially inhibited by IAA. Here we show that this response is, in part, ecotype-dependent. Further, we examined the effect of cytokinin (*trans*-zeatin, *t*-Z) in light receptor mutants grown in blue, red and far-red light. Low but statistically significant stimulation of hypocotyl elongation by cytokinin was observed in blue light (20 $\mu mol.m^{-2}.s^{-1}$), and was found to be cryptochrome and HY5 dependent. In red light (20 $\mu mol.m^{-2}.s^{-1}$), cytokinin caused phytochrome-dependent inhibition of hypocotyl elongation. In far red light, cytokinin stimulated hypocotyl elongation in a phytochrome A-dependent manner to an extent comparable to that seen at decreased white light intensity. Finally, cytokinin treatment caused almost the same relative stimulation of hypocotyl elongation in decreased white light (20 $\mu mol.m^{-2}.s^{-1}$), and combined blue and red light (10 $\mu mol.m^{-2}.s^{-1}$ each). The results suggest as yet unrecognized links between cytokinin and light signaling.

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Developing protein tools for bioengineering the plant hormone system

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Plant biotechnology is critical for addressing the challenge of food security in the near future. Virtually all aspects of plant growth and development including key factors enabling the development of usable plant biotechnology are controlled by the plant hormone system.
The maize β-D-glucosidase Zm-p60.1 releases active cytokinins from their storage/transport forms, and its over-expression in tobacco disrupts zeatin metabolism. The role of the active-site microenvironment in fine-tuning Zm-p60.1 substrate specificity has been explored, particularly in the W373K mutant, using site-directed random mutagenesis to investigate the influence of amino acid changes around the 373 position. Two triple (P372T/W373K/M376L and P372S/W373K/M376L) and three double mutants (P372T/W373K, P372S/W373K and W373K/M376L) were prepared. Their catalytic parameters with two artificial substrates show tight interdependence between substrate catalysis and protein structure. P372T/W373K/M376L exhibited the most significant effect on natural substrate specificity: the ratio of hydrolysis of *cis*-zeatin-O-β-D-glucopyranoside versus the *trans*-zeatin-O-β-D-glucopyranoside shifted from 1.3 in wild-type to 9.4 in favor of the *cis*- isomer. The P372T and M376L mutations in P372T/ W373K/M376L also significantly restored the hydrolytic velocity of the W373K mutant, up to 60% of wild-type velocity with *cis*-zeatin-O-β-D-glucopyranoside. These findings reveal complex relationships among amino acid residues that modulate substrate specificity and show the utility of site-directed random mutagenesis for changing and/or fine-tuning enzymes. Preferential cleavage of specific isomer-conjugates and the capacity to manipulate such preferences will allow the development of powerful tools for detailed probing and fine-tuning of cytokinin metabolism *in planta*.

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Bud outgrowth in pea can be based on the competitive canalization of auxin

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Polar auxin transport in stem is necessary for the control of bud outgrowth by a dominant apex. Following decapitation in pea (*Pisum sativum* L.) the axillary buds establish directional auxin export by subcellular polarization of PIN1 auxin transporters. Application of auxin efflux inhibitor (NPA, TIBA) to the second axillary bud of decapitated plants reduces bud outgrowth. Inhibition of outgrowth of the second axillary bud in these plants caused outgrowth of the first bud, which is associated with changes in expression profiles of *PIN1* and *DRM1* genes. These results support the competitive canalization theory, by which canalization of auxin from the lateral auxin source is possible only if the primary source is removed or weakened. Length of the decapitated stem stump may affect timing of changes in expression of *PIN1* and *DRM1* genes and hence the timing of initiation bud outgrowth after removal of the dominant apex. The signal for axillary bud outgrowth therefore could be the auxin decrease or depletion in the stem.

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Increased Levels of Cytokinins Induce HR-like Cell Death

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Cytokinins (CKs) can, among others, positively regulate shoot development and delay onset of senescence. However, recently opposite effects of CK action, namely promotion of programmed cell death, and cytotoxic effects of over-expression of the CK-biosynthetic gene *ipt* in tobacco, were recognized. Here we investigated effects of *ipt* expression in tobacco in detail. We show that hypersensitive response like cell death in expanded tobacco leaves proceeds shortly after *ipt* induction – the first lesions accompanied with huge wilting being observed in app. 60 hours after induction of *ipt* expression, and lesions can spread over the entire leaf area within 4 days after induction. Formation of visible lesions was preceded by increase in reactive oxygen species and membrane damage. Concomitantly with increase in ROS levels, transcript levels of genes involved in light phase of photosynthesis (FNR1 and CAB), and xanthophylls metabolism (VDE) were markedly down-regulated and genes involved in defence responses (PR-1b and PR-Q) were up-regulated as revealed by RT-qPCR analysis. Furthermore, we show that the increase in CKs is followed by changes in levels of stress-related hormones.

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Decreased Glutathione Levels and Induction of Sulphur Starvation-marker Genes after Cytokinin Action in *Arabidopsis*

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Cytokinins (CKs) are plant hormones implicated in many aspects of plant growth and development. Among others, CKs affect uptake and metabolism of nutrients, including sulphur (S). While CK action causes reduction of sulphate uptake, a stimulatory effect of CKs on S assimilation was suggested. To study resulting impact of contradictory effects of CKs on S uptake and assimilation we determined total glutathione (GSH) levels and expression of low-S-marker genes.

GSH pool, which is indicative for sulphur nutritional status, was decreased after treatment of 12-days-old seedlings with CK benzyladenine (BA) for 24 h. BA induced a decrease in GSH more effectively than sulphate deprivation, showing that CK-induced decrease in GSH is not solely due to reduced sulphate uptake. Furthermore, neither the expression of key enzymes of S assimilation and GSH biosynthesis or feeding experiments with GSH precursors (sulphate, cysteine and O-acetylserine) showed any indication that CKs disturb GSH formation. Notably, experiments with GSH biosynthesis inhibitor L-buthionine sulfoximine (BSO) strongly suggested that CKs induce consumption of GSH as i) BA induced GSH decrease more effectively than blocking GSH formation by BSO showing that CKs accelerate GSH consumption and ii) the effect of BA and BSO on GSH levels in a simultaneous treatment was additive suggesting again that CKs impact GSH amounts not on the level of formation but on the level of consumption.

In accordance with decrease of GSH level, BA induced S starvation-marker genes including isoforms of *adenosine 5'-phosphate reductase1-3*, *serine-O-acetyltransferase 3;2*, *sulphate transporter 4;2*, *putative thioglucosidase At2g44460*, *sulphur deficiency-induced 1*, *serine hydroxymethyltransferase 7*, *ChaC-like protein* (*Chac-c*), *low-sulphur-induced 1* and *low-sulphur-induced 2* (*LSU2*) at the mRNA level.

GSH is a signal molecule that represses genes responding to S starvation. However, a decrease in GSH alone to the extent observed after CK action is not a sufficient signal for strong induction of the S starvation-response as shown in seedlings challenged with BSO. We suppose that levels of other metabolites with a signalling role in low-S-response (ie. O-acetylserine) may be changed after CK action.

Early induction of *Chac-c* and *LSU2* was observed after CK treatment. Interestingly, both these genes are possibly implicated in GSH degradation. Thus, *chac-c* and *lsu2* mutants will be used to follow the cause of CK-induced decrease of GSH.

The links of CKs to GSH level and S-starvation responsive genes indicate an important role for CKs in sulphur nutrition. Thus increased levels of CKs could negatively impact the nutritional value of crops and stress tolerance due to an imbalance in S status.

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Interaction of plant hormones in Arabidopsis development under low light intensity

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Cytokinins as light are one of the master regulators of development. It is known, that plant response pathways for light and cytokinins are in interaction. Hypocotyl elongation is one of results of shade avoidance response to low photosynthetic photon flux density (PPFD). The role of several plant hormones (e.g. auxin, gibberellins) in regulating hypocotyl growth under low PPFD is already well described. Recently, by physiological experiments, it was revealed the involvement of cytokinin in the hypocotyl response to low PPFD. Now, we would like to uncover molecular mechanism of this process. Quantitative PCR with specific hydrolysis probes is used to analyse the expression profile of genes potentially involved in cytokinin interaction with light and other plant hormones which is important for the regulation of hypocotyl elongation under low PPFD. On the basis of experiment with wild-type or basic mutant (e.g. mutants in cytokinin receptors - *Arabidopsis* histidine kinase mutants, photoreceptor mutants - phytochrome A,B mutants) *Arabidopsis thaliana* seedlings grown under standard (100 µmol m^{-2} s^{-1}) or decreased (20 µmol m^{-2} s^{-1}) light intensity we will make selection of genes, whose next analysis will lead to clarification of the role of cytokinin in the shade avoidance response.

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PX1 regulates protoxylem cell fate via RNA processing

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Plant hormones, cytokinin, as well as auxin, have been proposed as important regulators of *Arabidopsis* vascular cell fate. Protein AHP6, a member of cytokinin transduction cascade, has been demonstrated to play a critical role during protoxylem development. In order to put AHP6 in a broader molecular context, we made a genetic screen, using *AHP6p:GFP* as a marker of cytokinin activity in protoxylem tissue.

We isolated mutant *px1*, which shows a decreased and erratic *AHP6p:GFP* activity, accompanied with aberrant protoxylem formation. In addition, *px1* displays pleiotropic phenotype, including defective gravitropism, abnormal cotyledonal development and an altered sensitivity to auxins and auxin efflux inhibitors. *PX1* codes for a weak allele of an embryonic lethal gene (EMB) which is, based on homology to known *Drosophila* systems, possibly involved in splicing.

In order to elucidate the molecular function of PX1 complex, we performed a tandem affinity purification (TAP) experiment. We identified two RNA processing proteins and a regulator of ubiquitin degradation pathway. In addition, in order to find EMB downstream targets, we are sequencing the px1 transcriptome, exploring the importance of alternative splicing during protoxylem formation.

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Modulation of heat-stress responsive proteome in transgenic *Arabidopsis thaliana* plants with inducibly-increased levels of endogenous cytokinins.

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Cytokinins (CK) are phytohormones with important role in plant growth and development and they are known to participate in number of plant signaling pathways. In our recent work we found link between temperature perception and early responses to CKs and in this follow-up project we studied the effect of inducibly-increased CK levels on temperature-stress responses. Plants were subjected to 3 different kinds of heat stress for up to 3 hours: heat stress from above (leaves), heat stress from below (roots) and heat stress on the whole plant. We analyzed heat stress induced responses by 2-D gel electrophoresis followed by MALDI-TOF/TOF protein identification in root proteome and Rubisco-immunodepleted leaf proteome in plants with increased endogenous CK levels, and wild-type (Col.). In numbers, we found more than 100 responsive proteins and most of these heat-stress responsive proteins exhibit strong modulations by increased CK levels. Moreover, our results indicate interesting interplay between heat-stress perception in roots and leaves.

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Designing molecular tools for the discovery and modulation of plant hormone systems

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Plant life is limited to one location. Their development and growth is influenced by external factors such as weather or soil quality. Plant can partially compensate effects of the external environment by changing the level of active plant hormones resulting to the change čímž of physiological parameters in their body. The amount of phytohormones is dynamically directed by a series of complex processes, in which all sub-steps (starting from biosynthesis continuing with the activation of phytohormones and ending with degradation) can not do without the presence of plant enzymes.
This work deals with the study of a particular enzyme, β-glucosidase Zm-p60.1 coming from corn. Zm-p60.1 is an enzyme that is part of cytokinin metabolism and can convert the inactive glycosylated form of cytokinin to its active form. Zm-p60.1 is one component that regulates the level of active hormones. Altered activity or specificity of this enzyme provides a tool which would be possible to use for fine modulation of the cytokinin level in the plant and thereby affect individual processes such as cell division, differentiation, root growth, sprout growth and other processes. Protein engineering offers a means to change substrate specificity of this enzyme.
Substrate specificity of the enzyme Zm-p60.1 is determined by amino acid composition in the vicinity of active center. The W373position in Zm-p60.1 is involved in stacking interaction with aromatic system on the substrate. By structural folding of 33 glycosidases from GH1 family was found that the position of W373 is highly preserved. Likewise, by the multiple sequence comparison of characterized enzymes from GH1 family, it was found that the W373 is sequentially preserved with a few exceptions where in this position occur other amino acids. By saturation mutagenesis was created a library containing 20-mutants at position W373. On artificial substrates was carried out preliminary test studying the effects of mutations on the activity of mutant enzymes. None of the mutants had higher activity than the original wild enzyme form of Zm-p60.1 (W373W). As the second most active appears W373H followed by group of 4 mutants W373F, W373G, W373T and W373Y with significantly lower activity. Other mutants had little or no activity.
New variants of functional β-glucosidase Zm-p60.1 will be further studied to determine changes in substrate specificity towards cytokinin conjugates. If such a change will be found, a new glucosidases could serve as a tool for studying the hormonal system of plants.

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Proteome regulation in *Arabidopsis* reveals shoot- and root-specific effects of cytokinins on hormonal homeostasis

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Shoot and root reveal specific and sometimes even contrary responses to plant hormones cytokinins (CK). However, the molecular mechanisms underlying the shoot and root specificity of CK-mediated regulations remain mostly unclear. We show that differential CK-mediated proteome regulation underlies the shoot and root specificity of CK response at the hormonal metabolism. Overall, we identified 43/18 differentially regulated proteins in the shoot and 31/21 in the root in the early/delayed response, respectively. We demonstrate that CK predominantly regulate proteins involved in carbohydrate and energy metabolism in the shoot and in protein synthesis and destination in the root. In the root we identified 3 out of 4 enzymes of the ethylene biosynthetic pathway to be upregulated in the early CK response, suggesting that majority of the ethylene biosynthetic pathway is under strong control of CK in the root. On the other hand, in the shoot we identified several abscisic acid (ABA)-related proteins to be upregulated by CK. To substantiate potential crosstalk of BAP with metabolism of other hormones, we analyzed changes of the endogenous hormone levels after CK application. In a good accordance with our proteomic data, we found prompt upregulation of 1-aminocyclopropane-1-carboxylic acid, the rate-limiting precursor of ethylene biosynthesis in the root, while ABA was upregulated in the early response of the shoot. The functional analysis of *atms1* and *aco2* mutants confirmed involvement of diverse steps of ethylene biosynthesis in the root response to CK. In conclusion, we propose tissue-specific proteome response and CK-mediated hormonal crosstalk as important factors contributing to the long-known specificity of CK regulations over shoot and root development.

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