Identification and genetic mapping of MAPKK and CDPK kinases involved in ozone and drought stresses in the *Brassica oleracea* genome

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The availability of complete sequence and functional data of many genes in model plant *Arabidopsis thaliana* open new ways for detailed studies of crop plants based on comparative genomic and postgenomic approaches. One of the main beneficiaries of this knowledge are the *Brassica* species because of their close relationship and high similarity of the *Arabidopsis* coding sequences. Earlier comparative analyses suggest that the *B. oleracea* genome is characterised by extensive gene redundancy as remainder of polyploidal origin. Hence, it is necessary the identification specific homologues of each gene in *B. oleracea*. The present work aims at the identifying genes homologous to selected MAPKK (MAPKK-1, -2, -4 and -5) and CDPK kinases involved in ozone and drought stresses responses. Gene probes were chosen on the basis of the *A. thaliana* transcriptome data obtained from the microarray analysis (Agnieszka Ludwikow et al., unpublished). The orthologous genes were mapped in the *B. oleracea* genome by using PCR analysis. Generally, gene specific sequences were selected from the *A. thaliana* genome or directly from known *B. oleracea* genomic fragments to design primers. Additionally, these sequences were screened computationally against the *A. thaliana* whole genome sequence to avoid misidentification of gene families or paralogs. The gene-specific primers were used to amplify genomic DNA from *B. oleracea*. The sequences corresponding to these genes were mapped on *B. oleracea* chromosomes via PCR/segregating population analysis. Detected sequences were located on the existing genetic map of *B. oleracea* and then comparative analysis of the *B. oleracea* and *A. thaliana* genomes was performed. The presence of loci corresponding to the MAPKK and CDPK kinases genes within unique conserved regions in the both genomes support the prediction that loci detected are orthologous. The orthologues identified in the *B. oleracea* genome need further functional verification.
Metabolic profile and transgene stability evaluation

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In order to obtain potato plants with elevated antioxidative properties, we have created plants overexpressing key enzyme of flavonoid synthesis pathway. Obtained plants possessed higher level of flavonoids resulted in higher antioxidative properties. Additionally the transgenic plants were UV protected and have improved resistance against pathogen attack. To assess if the introduction of the transgene caused any other changes, we conducted metabolic profile analyses. It turned out, that parallelly to expected results, some negative changes occurred. The level of starch has decreased, together with the decrease of total yield and the level of glycoalkaloids has increased.

At this point we asked the question on how stable transgenic plant features are, and also whether they all derive from introduces genes construction. In order to answer these questions we performed metabolic profiling in plants grown in the field, in three consecutive years. We have observed that metabolic changes were less and less pronounced in each subsequent year.

The reason for this might be the loss of the transgene, it’s modification (transgene methylation) or it’s silencing by siRNA. Our current investigation is focused on solving this question.

Anti-peptide specific antibodies for the characterization of different glutamate dehydrogenase subunits in yellow lupine

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In plants glutamate dehydrogenase (GDH) is formed by random association of two different kinds of subunits into a hexamer complex. Three genes coding for GDH subunits have been found in Arabidopsis thaliana. Sequence analysis of model legume plants (Medicago truncatula and Glycine max) showed at least two different GDH forms. The main difficulty in studies concerning isoforms of glutamate dehydrogenase is high similarity of subunits. A full-length cDNA encoding GDH1 subunit, cDNA partially encoding GDH2 and two other types of subunits were isolated from yellow lupine by RT-PCR. Amplified DNA fragments were cloned and sequenced (Acc. no. AY681352, AY871066, AY871065, AY871064). Based on the sequence of lupine GDH1 and GDH2 the distinguishing peptide (15AA) was designated to generate the antibody against the GDH1. Recombinant His-tagged fragments of polypeptides of GDH 1, GDH 2 as well as the full-length GDH1 subunits were produced in E. coli. Overexpressed proteins were purified on immobilized nickel ions and probed with polyclonal antibodies raised against GDH1. The results from Western
blots clearly showed that the antibody specifically recognized the GDH1. This antibody was used to further characterize the properties of the enzyme from various lupine organs.

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An insight into the Arabidopsis thaliana transcriptional profile changes initiated by ozone and drought

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During ozone stress biosynthesis of ethylene, salicylic acid and jasmonic acid is induced. Both ozone and drought treatment elevate abscisic acid level and activate ABA signalling. Arabidopsis ecotypes and mutants are available that differ in susceptibility to ozone exposure. WT A. thaliana Columbia and ein2 mutant are ozone tolerant phenotypes while WT A. thaliana Landberg erecta is not resistant to ozone. Taking into consideration that widely described abil mutant was obtained from Ler genotype background, we used T-SALK ABI1 gene insertion line from A. thaliana Col background in our studies. To investigate the role of those hormones upon ozone and drought stress we performed detailed transcriptional analysis of ozone susceptible or tolerant Arabidopsis ecotypes and mutants. For microarray analysis and ozone time course experiment, four-week-old WT Arabidopsis thaliana ecotypes Col [1] and Ler as well as ein2 and abil mutants were subjected to 350 ppb ozone dose treatment. Drought treatment of abil mutant, WT Arabidopsis Col plants and ein2 mutant were performed according to standard protocols. Transcript profiling of ozone- and drought-treated plants utilised Affymetrix GeneChip approach. Ozone time course experiment and validations were performed using quantitative real time RT-PCR. Transcript profiling of ozone-treated plants revealed differences in transcription pattern for genes involved in abscisic acid, ethylene, salicylic acid biosynthesis and signalling pathways in all accesses under investigation. Ozone tolerant ein2 mutant showed lower induction of ABI1 transcript compared to WT Col plants and not necessarily increased ABA biosynthesis. Cluster analysis of ozone-treated WT Arabidopsis Col and abil plants together with drought-treated abil mutant plants reveal some ABA-dependent gene expressions and extensive cross-talk between ozone and drought defence responses. Ozone time course experiment classified ABA signalling as late response in ozone treatment. We confirmed involvement of ABA signalling into defence response upon ozone stress.

References:
Modulators of protein glycosylation and secretion change the pattern of wall (glyco)proteins and affect the functioning of plant cell walls

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Cell walls are essential for plant development and morphogenesis. The majority of wall proteins are glycosylated, either as N- or O-glycans. Following their synthesis in ER and Golgi apparatus, they are secreted into the walls. Various modulators of this process were applied to determine their influence on the wall protein patterns and the functioning of the walls. In suspension-cultured Arabidopsis thaliana cells, qualitative and quantitative differences in wall protein patterns were observed both for proteins secreted into medium and proteins bound in the walls. Proteins changed in their content were identified by mass spectrometry. Application of tunicamycin, an inhibitor of the first enzyme in the N-glycosylation pathway, disturbed correct protein folding and normal functioning of ER. As a result, secretion of misfolded (glyco)proteins together with chaperones, e.g. those from the Hsp70 and Hsp90 families, as well as SHEPHERD protein (responsible for the formation of functional CLAVATA proteins) was observed. On the other hand, changes in the proteolytic degradation pattern of culture filtrate proteins indicate directly the importance of N-glycosylation for the stability of secreted proteins. The effects of 3,4-dehydroproline (affecting O-glycosylation) were less pronounced. Brefeldin A, inhibitor of protein trafficking, disturbed the secretion of proteins to the walls of Lupinus albus plants and in suspension-cultured A. thaliana cells. In Nicotiana tabacum protoplasts, BFA affected the regeneration the walls and the interactions between cytoskeleton and surrounding matrix.

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The proteomic study of white lupine apoplastic fluid from roots

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In this project white lupin ionically bound apoplastic proteins were isolated and analysed using modern MS techniques: MALDI-TOF or LC/ESI/MS/MS. Protein samples were obtained from control plants, plants injected with water or yeast elicitor. To compare those samples, electrophoretic methods, such as 1D or 2D native and/or denaturating conditions electrophoresis were used. Protein spots corresponding to particular proteins were excised from gel, digested with trypsin and analysed with above mentioned mass spectrometric methods. The special approach was used to find
peroxidases, for this purpose we used isoelectrofocusing in native conditions for the first direction and special attempt for SDS electrophoresis for the second dimension.

MALDI-TOF system appeared not to be very useful for analysis of lupine proteins due to the lack of dedicated databases. The use of LC-MS/MS system is more informative, it gives not only informations about masses of peptides (peptide mapping) but also allows to find the exact sequences of those peptides. Thus, it was possible to search existing databases for protein homology and many proteins were identified with good probability. Especially, existing EST databases for Medicago truncatula were of great importance because of its conformity with lupine plant family.

Fourteen cell wall proteins were identified, they belonged to glycoside hydrolases families: 17 (glucan endo-1,3-D-glucosidases) and 18 or 19 (chitinases), other were pectin methylesterases, peroxidases, germins and 1 was similar with soybean trypsin inhibitor family. These proteins are polysaccharide-modifying proteins or could be associated to defense reactions. We found several differences in the profiles of control and stressed plant tissue. Only extracellular proteins were recognized, it could be good evidence for proper preparation of protein extracts. Unfortunately it was not possible to identify all analysed spots (proteins). We do hope that due to the progress in databases completing, it will be possible to find more hits for the analysed proteins in the nearest future.

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**Proteomics of Fagus sylvatica seed dormancy breaking**

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Investigation of proteins, product of genes activated during a complex developmental process as is seed dormancy breakage was the aim of presented research. With seed dormancy breaking the plant hormones are associated: GA, responsible for stimulation of dormancy breaking and germination, and ABA responsible for maintenance of dormancy and inhibition of germination. These studies were carried out on Fagus sylvatica seeds during their stratification and germination. After imbibition in water (control) and in solution of GA, or ABA, beechnuts were subjected to cold stratification (3°C), which breaks dormancy.

Regarding the proteomic approach, proteins of the seeds were separated by 2D-gel electrophoresis, analyzed and identified by mass spectrometry. The influence of hormones was investigated and main protein variations were pointed out. Analysis of the proteins specific only for the GA, (48 spots) or ABA was done (16 spots). The link between the variation of proteins, hormones and dormancy breaking was established.
Phylogenetic analysis of Nod factor receptor NFR5 gene sequences originating from lupines and related legumes

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Establishing of symbiosis of legumes and nitrogen fixing bacteria depends on a molecular dialogue between both symbionts. The signaling molecule, produced by bacteria in the presence of plant root exudates is a lipochitin oligosaccharide, called Nod factor. In nanomolar concentration this compound triggers plant responses which precede cell infection by rhizobia, including calcium spiking, observed a few seconds after Nod factor perception, and de-differentiation of root cortical cells followed by the initiation of nodule organogenesis. Analysis of plant mutants unable to respond to the presence of symbiotic bacteria or purified Nod factor have resulted in identification of putative Nod factor receptor (NFR) proteins. One of these, NFR5 was characterized in *L. japonicus* and *P. sativum* as an intronless gene, coding for a protein composed of an intracellular serine-threonine kinase domain and an extracellular domain with three LysM motifs, connected by a transmembrane segment. Earlier reports that LysM motif–containing domain from *Lactococcus lactis* autolysine binds peptidoglicanes, indicate that the extracellular domain of NFR5 may be involved in Nod factor binding. As the kinase domain of NFR5 protein lacks an activation loop, it is assumed that Nod factor receptor is a heterodimer composed of NFR5 and another receptor-like kinase – NFR1, where NFR5 participates directly in the Nod factor binding.

We obtained a full cDNA sequence of an nfr5 homologue from *Lupinus angustifolius*. Based on sequence conservation we designed specific primers and amplified fragments corresponding to the extracellular (receptor) domain of NFR5 protein on template DNAs extracted from a range of *Lupinus* species representing major lupine phylogenetic groups as well as from related legumes. Our objective was to compare the tree topology of this Nod factor receptor gene with the lupine phylogenies obtained from a set of other sequence loci.
them – CMS-C was discovered over 30 years ago by M. Lapinski in Polish cultivar Smolickie. The use of RAPD markers allowed localizing the main locus for male fertility restoration in CMS-C (Rfc1) on the long arm of 4R chromosome. The aim of presented study was to apply recently developed SCAR markers for more precise mapping of $Rfc1$ gene and checking if these markers are useful for marker assisted selection (MAS). Segregations of ten SCAR markers from 4R chromosome were assessed in two F$_2$ mapping populations derived from crosses between male sterile lines as females and restorer inbred lines as male parents. Results were analyzed in JoinMap 3.0 software allowing to draw map of 4RL chromosome based on data combined from two distinct mapping populations. Composite interval mapping (CIM) analysis was performed for localizing of $Rfc1$ locus on created linkage group.

**Comparative mapping of large chromosomal rearrangements in the Brassica oleracea genome: uncovering the natural history of the species**

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The simplicity of the *Arabidopsis* genome renders from this plant a perfect model for investigation of more complex organisms, especially within the *Brassica* genus (cruciferous oilseeds and leafy vegetables). Comparative mapping approaches that use *Arabidopsis*-derived probes have substantially contributed to our present knowledge about architecture and organisation of *Brassica* chromosomes. However, an unusually high rate of small rearrangements within the family Brassicaceae obscures chromosomal collinearity. That hamper the possibility to simply draw out evolutionary relationships for two genera: *Arabidopsis* and *Brassica*. For these reasons there is a need to introduce more comprehensive strategies for evaluating evolutionary events, which have occurred after *Arabidopsis-Brassica* split. By using comparative chromosome painting in conjunction with genetic mapping, we show that the *Brassica* genome was triplicated during its history, and this triplication proceeded by a few stages. Moreover, a majority of chromosomal rearrangements, that could be detected within *A. thaliana* genome, are common for both genera. In these experiments a few regions of 8.25 Mbp (more than one hundred BAC clones forming three separate contigs) of *Arabidopsis* genome were used as probes for comparative chromosome painting. In addition, as a control of the physical mapping, about 60 *A. thaliana* EST clones for unique genes representing the BAC contigs were applied for genetic mapping of *B. oleracea* chromosomes. The results let us to construct a novel model for the formation of *Brassica oleracea* genome.