Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

To introduce the Research Report, I will state some summary data describing the Institute's activities during the evaluated period of 2010–2014. The employees of the Institute of Experimental Botany published 565 articles in journals with impact factors. This number, which corresponds to a 50% increase in comparison to the previous 5-year period, indicates that descriptions of results and directions on the following pages will need to be limited to only the most important and highest-quality matters, while passing lightly over many other results that are undeniably also noteworthy and of very high quality.

During the evaluated period, we published in the most prestigious journals: Nature (3x), Science (5x), Cell, Nature Biotechnology, Nature Genetics, Nature Chemical Biology (2x), Nature Communications (3x), Developmental Cell (4x), Genes & Development, Trends in Plant Sciences (2x), Genome Biology (2×), Progress in Lipid Research, Proceedings of the National Academy of Sciences of the USA (6x), Plant Journal (15x), Plant Cell (12x), Plant Physiology (11x), New Phytologist (14x) and others.

Few institutes of the Academy of Sciences have fulfilled its motto "Science for Practice" as impeccably as has the Institute of Experimental Botany. During 2010–2014, we acquired 29 patents in many countries including the US and the EU and 35 times registered breeding rights to newly bred apple varieties resistant to scab. We sell licences to grow these varieties all over the world, and revenue from these licences is an important component of the IEB's budget.

I consider the number and quality of outputs to be impressive especially in relation to the Institute's size and the quantity of institutional financial support.

The most important results of Institute of Experimental Botany are listed below:

Research team **Centre of Plant Structural and Functional Genomics** focuses on molecular organization and evolution of plant genomes. The research involved three groups of plants: (a) cereals from the tribe *Triticeae* (wheat, barley and rye); (b) forage grasses (fescue, ryegrass and their hybrids); and (c) banana. In cereal genome analysis, the work involved refinement of a protocol for construction of large insert BAC libraries from flow-sorted chromosomes, and the team remains the only one in the world that can construct this type of DNA libraries. These unique resources are making the biggest impact in the development of ready to sequence physical map of bread wheat. The team is one of the key members of the International Wheat Genome Sequencing Consortium (IWGSC) and after a huge effort, they constructed BAC libraries from all chromosomes of bread wheat (Šafář et al. 2010; ID 349181). The availability of chromosome BAC libraries permitted estimation of the fidelity of BAC contig assembly using genomic libraries (Luo et al. 2010; ID 347998). The availability of chromosome BAC libraries from homoeologous chromosomes enabled characterization of changes in genic sequences during wheat genome evolution, including the rates of non-collinear gene insertion and gene duplication (Bartoš et al. 2012; ID 382219).

The suitability of chromosome genomics for producing reference sequence of the wheat genome was definitely confirmed in a collaborative project in which the first reference sequence of (the largest) wheat chromosome (3B) was produced after sequencing BAC clones from its physical maps. This result was published in Science (Choulet et al. 2014; ID 433903) and provided so far the most detailed information on genome structure of bread wheat. To date, members of IWGSC constructed physical maps from 16 out of 21 wheat chromosomes, including the map of chromosome 6A (Poursarebani et al. 2014; ID 432116). During the process of constructing physical maps, BAC contigs are ordered and oriented predominantly using genetic markers. However, genetic linkage maps suffer from poor resolution in (peri)centromeric regions. To tackle this problem, the team developed a FISH technique to localize cDNA probes shorter than 3.5 kb on plant mitotic metaphase and prometaphase chromosomes, and demonstrated that it could be a valuable tool to order physical map and provide a complementary approach to genetic mapping for chromosome regions with limited or no recombination (Karafiátová et al. 2013; ID 424633).

Despite some limitations, genetic linkage maps remain one of the main tools in gene mapping and positional gene cloning projects. These projects benefit from high density maps, and an attractive approach to construct such maps is to develop new markers specifically from target genome regions. In collaborative projects, the team demonstrated that this can be done using DNA from flow-sorted chromosomes (Shatalina et al. 2013; ID 393905). For example, they showed that the Diversity Arrays Technology (DArT) can be coupled with chromosome sorting to increase the density of genetic maps for specific chromosomes or chromosome arms of wheat. Since a small amount of chromosomal DNA (5 ng) is needed to develop DArT markers, this approach can be readily applied to any crop, for which chromosome sorting is available (Wenzl et al. 2010; ID 349830).

Sequencing DNA amplified from flow-sorted chromosomes by next generation sequencing (NGS) technologies is another powerful application of chromosome genomics developed by the team. Sequencing DNA of flow-sorted chromosomes of barley allowed assembling a majority of its genes and establishing their putative linear order along all chromosomes. This was the first blueprint of a diploid *Triticeae* genome and provided a framework for the study of *Triticeae* genome evolution and the development of novel strategies in cereal breeding (Mayer et al. 2011; ID 365200). In a similarly novel study, a virtual linear gene order models were established for all chromosomes of rye. A genome-wide high-density comparative analysis of synteny between rye, barley and model grass genomes indicated that introgressive hybridizations and/or a series of whole-genome or chromosome duplications played a role in rye speciation and genome evolution (Martis et al. 2013; ID 423097).

In collaboration with other groups, the team demonstrated the utility of chromosome sequencing for the analysis of complex genome of bread wheat in several studies (Berkman et al. 2011, ID 365378; Berkman et al. 2012, ID 380686; Wicker et al. 2011, ID 365266; Vitulo et al. 2011, ID 366036; Fluch et al. 2012, ID 380689; Lucas et al. 2012, ID 380697; Lucas et al. 2014, ID 441332; Akhunov et al. 2013, ID 394040; Ma et al. 2013, ID 423953; Tanaka et al. 2014, ID 432722). For example, sequencing both arms of chromosome 4A (Hernandez et al. 2012, ID 380583) facilitated construction of an ordered gene map of the chromosome, embracing over 85% of its genes. The work provided new insights into the evolutionary dynamics between homoeologous chromosomes and syntenic chromosomal regions and enabled precise localization of translocation and inversion breakpoints on the chromosome. Following these pioneering studies, IWGSC decided to sequence all chromosomes of bread wheat using NGS technology. This large-scale exercise was successfully completed in 2014 and the results were

published in Science (Mayer et al. 2014, ID 433366). The work provided novel insights into the genome biology of a polyploid species and the results obtained will facilitate faster gene isolation, genetic marker development, and precise breeding of the important crop.

The research in forage grasses (*Festuca* spp., *Lolium* spp. and their hybrids), focused on the analysis of their genome structure and evolution at cytogenetic and DNA level. With the aim to increase the resolution of genome analysis and achieve higher throughput, the team developed DArT markers specific for fescue and ryegrass. Validation of the DArTFest array confirmed its suitability to determine genome constitution in *Festuca* x *Lolium* hybrids at high resolution and its ability to discriminate between Festulolium cultivars. The results also confirmed the potential of the array to follow changes in genome composition of *Festuca* x *Lolium* hybrids during successive generations (Kopecký et al. 2011, ID 364120). The DArTFest array was also used to saturate genetic maps of *F. pratensis* and *L. multiflorum* and identify markers associated with traits of interest. Three genomic loci associated with freezing tolerance co-localized with chromosome segments and QTLs previously implicated in freezing tolerance. Moreover, sequencing the markers enabled comparison of genome structure of both species with the genomes of rice and *Brachypodium* and revealed their syntenic relations (Bartoš et al. 2011, ID 364761). These results again confirmed the potential of the DArTFest array in genetic studies of the Festuca-Lolium complex. The annotated DArTFest array resources could accelerate further studies and improvement of desired traits in Festuca-Lolium species.

Given the lack of detailed information on genome structure of *F. pratensis*, the team applied their chromosome genomics approach to generate a draft genome sequence assembly. As the first step, they flow sorted chromosome 4F and sequenced it by NGS technology. This provided the first insight into the composition of the fescue genome, permitted estimation of gene content, enabled the construction of virtual gene order of the chromosome, and facilitated detailed comparative analysis with the sequenced genomes of rice, *Brachypodium*, sorghum and barley. The analysis of chromosome sequences enabled identification of new tandem repeats, which were mapped using FISH and were found suitable as cytogenetic markers for karyotyping *F. pratensis*, *Lolium* species and their hybrids. This was the first report on dissection of a complex and large forage grass genome using chromosome sorting (Kopecký et al. 2013, ID 422267).

The research on *Musa* focused on two areas: genetic diversity and phylogeny, and genome structure. The team has been appointed by Bioversity International to serve as Musa Genotyping Centre and genotyping and phylogenetic analyses are part of this mandate (Christelová et al. 2011a, ID 365578). As the classification of species from the *Musaceae* (banana) family and their phylogenetic inter-relationships remain controversial, the team studied evolutionary relationships using 13 species and DNA sequences obtained from a set of unlinked nuclear genes. The study contributed significantly to the classification of the *Musaceae* family species. The first estimates for the divergence times of the four sections of genus *Musa* were obtained and the study provided a substantial insight into the course of speciation within the Musaceae. An understanding of the main phylogenetic relationships between banana species provided data to fine-tune the taxonomy of *Musaceae* (Christelová et al. 2011b, ID 365203).

The team obtained the first detailed information on internal transcribed spacer (ITS) sequence diversity in the genus *Musa* and characterized the structure and diversity of the ITS region in 87 representatives of the family *Musaceae*. Phylogenetic reconstruction based ITS sequences showed

that the genus is divided into two distinct clades. A need to identify putative pseudogenic ITS sequences, which may have negative effect on phylogenetic reconstruction at lower taxonomic levels was shown. Independent evolution of parental rDNA in hybrids enabled determination of genomic constitution of hybrids using ITS sequences (Hřibová et al. 2011, ID 365197).

In order to provide more insights into the *Musa* genome structure and evolution, the team characterized genomic organization of two main banana DNA satellites together with other DNA sequences in nineteen accessions of *Musa*, including inter-specific hybrids. The study expanded the number of individual chromosomes which can be identified cytogenetically and provided tools to support determination of genomic constitution in inter-specific hybrids (Čížková et al. 2013, ID 394043).

In order to provide the knowledge needed to apply molecular and genomics tools in improvement of banana and contribute to the understanding of *Musa* evolution, the team participated in a large-scale sequencing project, which produced a draft sequence of the 523-megabase genome of *M. acuminata*. The banana genome sequence was the first of its kind for a monocotyledon outside Poales and represented an essential bridge for comparative genome analysis in plants. The study revealed three rounds of whole-genome duplications in the *Musa* lineage, which were followed by gene loss and chromosome rearrangements, resulting in little synteny conservation between lineages. The results of the work, which also led to discovery of conserved noncoding sequences predating monocotyledon–eudicotyledon divergence, were published in Nature (D'Hont et al. 2012, ID 381157).

The research in the **Laboratory of Hormonal Regulations in Plants** was concentrated predominantly on two groups of plant signalling compounds (phytohormones) – auxins and cytokinins (including their interactions with other phytohormones, e.g. abscisic acid and ethylene). Team has focused on both qualitative and quantitative aspects of processes involved in establishment and modulation of their homeostasis (i.e. metabolism and transport), on their signalling and control of physiological processes (namely stress reactions).

In the field of auxin, main interest was to characterize mechanisms of action of auxin transporters, residing on plasma membrane (PM) and on endomembranes, as well as to reveal related auxin-metabolizing processes.

Team have contributed significantly to discovery of the so far unknown PILS (PIN-LIKES) family of putative auxin transporters that – similar to PIN5 - localize to endoplasmic reticulum (ER). Changes in their expression result in changes in spectrum of auxin metabolites in cells, thus determining the accessibility of free auxin for the nuclear auxin-signalling pathway (Barbez et al. ID 380744). However, not only PIN5 and PILSes can be found on endomembranes. Team has participated on characterization of another non-canonical member of PIN family of auxin transporters: PIN8, which is expressed in male gametophyte of *Arabidopsis thaliana* and plays a crucial role in pollen development and its functionality. PIN8 co-localizes with PIN5 on the ER and is also involved in control of auxin homoeostasis and metabolism (Ding et al. 2012, ID 380680). We have also participated on characterization of the PM-localized auxin transporter *At*ABCB4 from the superfamily of ABC transporters, and on revealing its unique function: Depending on intracellular auxin level, ABCB4 is able to transport auxin into cells or from cells. This is the first finding of a bi-directional, 'substrate'-concentrationdependent transport of a low-molecular compound across the PM in plants (Kubeš and

Yang et al. 2012, ID 380687). The paper also provides a likely explanation for the herbicidal activity of synthetic auxin 2,4-D.

We contributed fundamentally to revealing the so far unknown activity to facilitate auxin uptake to cells of the nitrate 'transceptor' NRT1.1. This PM-protein possesses two unique characteristics: it serves as a sensor as well as transporter for nitrate. As it is able to transport auxin as well, it connects availability of a crucial mineral nutrient with auxin-controlled root development. This is the first observation of a direct hormonal regulation via nutrient availability driven by one particular protein species in plants (Krouk et al. 2010, ID 343963). The work on characterization of kinetic properties of NRT1.1 continues in relation activities of phosphorylated and non-phosphorylated forms of NRT1.1 at amino acid residue T101 that differ significantly in both signalling functions and transport functions (Bouguyon et al., Nature Plants, in press, http://dx.doi.org/10.1038/nplants.2015.15).

We have also collaborated on revealing of the so far unknown interaction between auxin efflux carriers PIN1 and PIN2 and dynamin-related proteins (DRPs) at the cell plate that is necessary for proper polar PIN positioning in interphase cells and, concomitantly, for auxin-mediated development (Mravec et al. 2011, ID 364093), and on characterization of reversible ubiquitylation of the auxin efflux carrier PIN2 that is important for PIN2 endocytosis and its delivery into cell's lytic compartment. However, we have shown that such ubiquitylation does not interfere with PIN2 auxin efflux activity at the plasma membrane (Leitner et al. 2012, ID 380702).

We have contributed significantly to characterization of the role of major auxin degradation product, 2-oxindole-3-acetic acid (oxIAA), in control of auxin homeostasis (Pěnčík et al. 2013, ID 423096), and to better understanding of the mode of action of some auxin transport inhibitors (Laňková et al., 2010, ID 347619; Yin et al. 2014, ID 430993). We have designed and experimentally verified the first mathematical model of transport processes involved in auxin accumulation on a single cell level providing estimates of key transport parameters for the synthetic auxin 2,4-D (Hošek et al. 2012, ID 381050).

Auxin, after its interaction with the auxin receptor ABP1, inhibits the clathrin-mediated internalization of PINs via non-transcriptional mechanism (Robert et al. 2010, ID 350344). Then, we have further characterized a link between activity of ABP1 in regulation of dynamics in the PM and activity of PIN auxin transporters (Čovanová et al. 2013, ID 421122).

In the field of cytokinins (CKs), we have performed and published as yet the most comprehensive characterization of a specific class of CKs, *cis*-zeatins (Gajdošová et al. 2011, ID 365271). We have demonstrated the abundance of *cis*-zeatin(Z)-type CKs throughout the plant kingdom and their biological activities in CK bioassays as well as have specified their uptake, accumulation and metabolic pathways in plants. In addition, we have participated in demonstration of *cis*Z-types as the predominant CK forms in dry and/or imbibed seeds of various plant species (Stirk et al. 2012, ID 380691; Stirk et al. 2012, ID 380751). We have shown an involvement of *cis*Z-type CKs in plant responses to fungal infection (Behr et al. 2012, ID 381049) as well as to abiotic stresses (Dobrá et al. 2010 ID 350307; Kosová et al. 2012, ID 380692; Macková et al., 2013, ID 399220). Using potato and centaury plants constitutively overexpressing the CK oxidase/dehydrogenase (CKX) gene from *Arabidopsis thaliana* and grown *in vitro*, we have demonstrated a diminished growth and/or reduced morphogenetic potential in association with considerably lower contents of *cis*Z- than *trans*Z-type CKs (Raspor et al. 2013, ID 380750; Trifunović et al. 2013, ID 421942, respectively). Based on these findings,

we have hypothesized the conceivable function of *cis*-zeatins as delicate regulators of CK responses in plants under growth-limiting conditions.

We have contributed to the description of molecular function of the *Pseudomonas syringae* pv. *tomato* effector HopQ1 that activates CK signalling and enhances concentrations of bioactive CK forms by stimulation of LOG (*lonely guy*) biosynthetic pathway and have demonstrated the importance of CKs in plant-microbe interactions (Hann et al. 2014, ID 431074). We have discovered that the involvement of SlZF2 in delayed senescence and improved salt tolerance of tomato plants correlates with maintaining photosynthesis, increasing polyamine and ABA biosynthesis/signalling and modifying ratio between *trans*- and *cis*-zeatin-type cytokinins (Hichri et al. 2014, ID 429597).

The impact of cytokinin down-regulation, both constitutive and root-targeted, on the drought and/or heat stress tolerance was characterized in tobacco (Macková et al. 2013, ID 399220). Apart of hormonal responses, stress tolerance was evaluated by expression levels of dehydration marker genes *ERD1* and *P5CSA*. The results suggest that high stress tolerance of *35S:AtCKX1* transformant is given predominantly by morphological changes (suppressed growth rate of dwarf shoots, enhanced root system).

Complex phytohormone analysis allowed us to characterize hormonal cross-talk during the individual phases of the response to cold stress (e.g., in alarm phase or acclimation) in leaves and crowns of winter and spring wheat genotypes (Kosová et al. 2012, ID 380692). The cold response of the proteome was described in detail (Kosová et al. 2013, ID 421944). The up-regulation of active cytokinin content at the onset of transition from vegetative to generative developmental phase indicated decisive role of cytokinins in this process (Vanková et al. 2014, ID 429933).

Participation of jasmonic acid, hormone generally associated with the defence against herbivore and necrotroph attack, was demonstrated during severe dehydration of the resurrection plant *Haberlea rhodopensis* (Djilianov et al. 2013, ID 399223). We have also contributed to evaluation of cytokinin regulatory roles in drought, salt and ABA responses, and ABA biosynthesis (Nishiyama et al. 2011, ID 365273), to understanding the metabolic reorganisation prior to early drought responses in lupine (Pinheiro et al. 2011, ID 365274), to complex analysis of soybean response to drought stress (Le et al. 2012, ID 382217), to characterization of hormonal changes underlying improved performance of somaclonal finger millet line (Radchuk et al. 2012, ID 382086), to study of cytokinin functions in grapevine berry initiation (Crane et al. 2012, ID 365275), to elucidation of the effects of ABA on nuclear genes encoding chloroplast-localized proteins in the basal and apical segments of wheat leaves (Yamburenko et al. 2013, ID 422266), and to identification of the new role of ascorbate peroxidase 6 in germination control and seed stress tolerance in relation to hormonal cross-talk (Chen et al. 2014, ID 433740).

We have also contributed to understanding of the phytohormone co-action in control of some developmental processes: e.g. floral formation (gibberellins, auxin and mutual changes in contents of *cis*- and *trans*-zeatin - Quinet et al. 2014, ID 433556), apical hook development (auxin and ethylene - Vandenbussche et al. 2010, ID 343964; Žádníková et al. 2010, ID 347625), organogenesis (cytokinins and auxin – Marhavý et al. 2011, ID 366038), and in response of plants to shading (Ananieva et al. 2011, ID 368552).

Finally yet importantly, within our research we have optimized some methods and designed new ones. After acquisition of high performance liquid chromatograph coupled with mass spectrometer (LCMS) in 2009, we have optimized the extraction of plant samples and HPLC/MS analytical procedure, so that we are now able to reduce sample amount to tens to few hundreds mg of fresh weight and to simplify extraction and purification procedures using fewer steps and utilizing micro-volumes of elution solvents allowing us to process larger sample numbers. We can perform simultaneous and highly reliable determinations of multiple CK forms together with other plant hormones and their metabolites in one single sample (Dobrev and Vankova 2012, ID 387792; Djilianov et al. 2013, ID 399223). We have designed a new method to detect carrier-driven activities, based on the transient, single-cell-based system. Relative changes in signalling output of the auxin responsive promoter element DR5 were used to indirectly visualize auxin carriers' activity. This single-cell-based system can be useful to investigate also other hormonal (e.g. cytokinin – via TCS) signalling and transport pathways (Barbez et al. 2013, ID 395429).

We have uncovered yet unknown and unexpected effects of the FM (Fei Mao) styryl dyes. Until then, FM dyes have been considered reliable tools for tracking of plant endocytosis; however, we showed that routinely used concentrations of FM 4-64 and FM 5-95 trigger transient re-localization of PIN and ABCB proteins and FM 1-43 affects their activity. Our results emphasize the need for circumspection during in vivo studies of membrane proteins performed using simultaneous labelling with FM dyes (Jelínková et al. 2010, ID 343962).

The research of the **Laboratory of Cell Biology** was aimed to the selected molecular regulatory modules of plant cell polarity and morphogenesis operating mostly cortically at the plasma membrane, and at the interface of secretory pathway, lipid membrane and cytoskeleton.

A major progress was made in the characterization of plant exocyst complex culminating in the publication of comprehensive reviews (Žárský et al. 2014, ID 423950 and Synek et al. 2014, ID 429989).

Analysing exo84b and exo70A1 *Arabidopsis* mutants we for the first time showed both crucial contribution of exocyst to plant cell division and detailed dynamics implying exocyst especially in phragmoplast initiation and then final fusion and maturation of the new cell wall. Using co-immunoprecipitation interaction of EXO84 subunit with other exocyst subunits was proven (Fendrych et al. 2010, ID 351017). Later, for the first time for any eukaryotic cell type, we contributed to a report on a co-ordination between TRAPP and exocyst complex in *Arabidopsis* cytokinesis (Rybak et al. 2014, ID 429525). In the expansion phase TRAPP dominates the process, while during new cell wall insertion and maturation exocyst is maximally present (Rybak et al. 2014, ID 429525).

We described for the first time (including yeast and animals) the eukaryotic exocyst complex dynamics at the cytoplasmic membrane (Fendrych et al. 2013, ID 398129). For the first time we showed the importance of exocyst complex for the recycling and cellular localization of PIN auxin efflux carriers and regulation of polar auxin transport (Drdová et al. 2013, ID 395460).

We also described unknown developmentally regulated reduction in seed coats deposition in siliques of the first inflorescence (Kulich et al 2010, ID 350472). The discovery that EXO70B1-containing exocyst subcomplex plays a role in the Golgi-independent autophagic transport into the vacuole, was possibly the most important findings from the laboratory over the last years (Kulich et al. 2013, ID 421002). The exo70B1 mutant has spontaneous leaf lesions/hyper sensitive reaction due to over

accumulation of salicylic acid (Kulich et al. 2013, ID 421002). On this pathway is dependent an import of anthocyanins into the vacuole, and possibly some other molecules including salicylic acid (SA). It is possible, that autophagy related endomembrane transport is a hidden and so far neglected branch of the plant membrane trafficking (Kulich and Žárský 2014, ID 429326).

An active role of plant exocyst complex in defense against both bacterial and fungal pathogens points also to a direct interaction between the SNARE protein, SNAP33 with EXO70B1 and EXO70B2 (Pečenková et al., 2011, ID 367701).

We contributed to the follow up discovery of EXO70B exocyst subunits as a subject to specific E3 ligase ubiquitination-dependent regulatory degradation in biotic interactions (Stegmann et al.2012, ID 389143). Also extraordinary evolutionary dynamics of exocyst subunits indicates an involvement in the plant-microbe interactions (Cvrčková et al. 2012, ID 441887).

We contributed a first report on RAB GTPases prenylation modifying complex in plants based also on genetic analysis (Hála et al 2010, ID 350388). We also contributed to the characterization of ROP6 activity regulation by phosphorylation (Fodor-Dunai et al. 2011, ID 367374). An important contribution of ROP GTPases activity we observed also in case of the NADPH oxidase activity regulation in pollen tubes (Potocký et al. 2012, ID 381547).

We made connection to the interaction and regulation between F-/or G-actin and phospholipase D activity (PLD). F-actin binding domain on Arabidopsis phospholipase D beta (PLD) was pinpointed and characterized. This interaction stimulates PLD activity and production of membrane lipid phosphatidic acid (PA); while interaction with monomeric G-actin inhibits PLD activity and PA production (Pleskot et al. 2010, ID 350391). We reported detailed insight into the molecular mechanism of heterodimeric F-actin capping protein (CP) interaction with membrane lipids phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidic acid (PA) explaining enhanced affinity of plant CP to PA (Pleskot et al. 2012, ID 390490).

We documented specific localization of phosphatidic acid (PA) in subapical pollen tubes domain specifically overlapping with phosphatidylinositol (4,5)-bisphosphate (PIP2) signal at the very tip (Potocký et al 2014, ID 429592). The progress in this new field of PLD/PA/F-actin but also microtubuli regulation was summarized in a review (Pleskot et al. 2013, ID 399937). This along with a report on PA turnover (Pleskot et al. 2012, ID 380699) and PA effect on NADPH oxidase activity in pollen tubes (Potocký et al. 2012, ID 381547) and especially recently developed computational molecular dynamics approaches (Pleskot et al. 2012, ID 390490) opens both conceptually as well as methodologically new avenues for the analyses of membrane lipids-proteins interface functioning in cell polarity regulation not only on the pollen tube model.

The **Laboratory of Plant Reproduction** is working on two main topics: 1) genomics and transcriptomics of plant mitochondria and mitochondrial-nuclear interactions including cytoplasmic male sterility (CMS), and 2) floral induction and the evolution of flowering-related genes in Chenopodium.

We revealed an extreme polymorphism in mtDNA in Central European populations of *S. vulgaris*. We also found that specific mtDNA haplotypes were associated with specific transcripts of the genes atp1 and cox1 (Elansary et al. 2010, ID 344002). We analyzed in detail the transcription of

the atp1 gene. We found that the sequences upstream from this gene, including putative regulatory motifs, varied among mt haplotypes. We examined the atp1 transcript in the KOV mt genome by means of RNA circularization and discovered three 5‘ ends, created by the combination of transcription initiation and RNA processing. Surprisingly, the utilization of a particular transcription start site depended on the nuclear background, which suggested the involvement of nuclear genes in transcription initiation in plant mitochondria (Muller and Storchova 2013, ID 395063).

Our findigs have documented prominent differences in mt genomes at the within-species level in *S. vulgaris*. We have previously contributed Southern hybridizations confirming intramolecular mt genomic recombinations in *Silene latifolia* in the assembly of the first completely sequenced mt genome in the genus *Silene* (Sloan et al. 2010, ID 350206). We generated complete sequences of four mt genomes of *S. vulgaris* and revealed an extreme level of mt genomic rearrangements affecting not only gene order, but also gene content. We also performed Southern hybridization, confirming an autonomous physical structure of the smallest subgenome predicted by bioinformatic approach (Sloan et al. 2012, ID 384239).

We confirmed the florigenic function of CrFTL1 by the complementation of ft mutants in *Arabidopsis*. The CrFTL1 gene is expressed during the day. A dark-light transition following a permissive period of darkness is necessary for its activation. This requirement is unique among investigated short-day plants, which express FT homolog at night and do not need a dark-light transition for its activation (Drabešová et al. 2014, ID 429593).

The **Laboratory of Virology** contributed to the understanding of plant viral diseases and to development of new and innovative ways to control them. Main focus was on the biotechnological use of plant viruses, especially on transient expression of proteins of therapeutic value.

We designed strategy for production of polyclonal antibodies against both structural and nonstructural recombinant proteins of potato-infecting viruses. Recombinant viral proteins expressed in bacteria showed interesting potential as an alternative source of antigens for raising specific antibodies to plant viruses, which can be produced in large quantities and can be adapted for specific applications (Čeřovská et al. 2010, ID 347697, Plchová et al. 2011, ID 364101 and Čeřovská et al. 2012, ID 380694). We have successfully expressed $L2_{108-120}$-epitope derived from minor capsid protein HPV-16 L2 fused to the N-terminus of PVX CP in plants. The PVX chimeric particles displaying immunoreactive epitopes moved systemically in experimental plants and achieved high yields (Čeřovská at al. 2012, ID 380685). Mutagenized E7 oncoprotein (E7ggg) from HPV-16 was fused to the C-terminus of PVX CP and successfully expressed in plants (Plchová at al. 2011, ID 364095).

We have also designed a novel virus vector based expression platform, which can be used in plant molecular farming (Czech patent application CZ201100843-A3).

The research of the **Laboratory Stress Physiology** was focused on two major areas: nitrosative and oxidative stress and antioxidant defence either during ageing or in stress response.

The first summary study of nitric oxide and reactive nitrogen species (RNS) metabolism during natural and stress induced leaf senescence was published (Pavlikova et al. 2014, ID 435451). We also contributed on antioxidant defence in tobacco with CK depletion either during ageing or in stress responses (Lubovska et al. 2014, ID 433743 and Mackova et al. 2013, ID 399220).

Station of apple breeding for disease resistance is active in a long term project on breeding of high quality apple varieties resistant to scab caused by *Venturia inaequalis* (the most widespread and harmful apple disease).

The first source of resistance against scab used in the breeding was *Malus floribunda* bearing the resistance gen *Vf*. The gen can be transferred to the progeny simply by crossing. Recently this type of monogenic resistance has been overcome by new races of the fungus. This initiated to turn our attention to look to a scab resistance encoded polygenicaly. According to resistance against the new scab races two different clones were identified and used for crossing which resulted in selection of two promising varieties: Admiral (late variety protected by Community Plant Variety Right (CPVR) in the EU, ID 383392, ID 383404, ID 438938) and Allegro (early variety protected by CPVR in Czech Republic which will be extended to the whole EU, ID 438945).

The results obtained were transferred into practice by conclusion of License Agreements with the buyers for planting and apple production. The royalties are paid to the licensor (IEB). In the past years, our Topaz was the most planted scab resistant variety in Europe. Topaz is very popular amongst organic fruit growers, more than 400 000 trees per year have been planted on a total surface of 1000 ha in the year 2014 (ID439085). Variety UEB 32642, known under the trademark Opal and registered in more than 40 countries (Plant Patent in USA and CPVRs in EU, ID 438904), is very popular mainly in USA and Chile. In the years 2010 to 2014 the total amount of Opal trees sold exceeded 1.3 million.

In the years 2010 to 2014 16 Licensee Agreements were concluded for varieties with scab resistance *Vf* newly released for commercialization. Of particular importance is the contract with an American Society for scab resistant varieties with compact columnar growth habit for the territory of the whole world. The total number of trees of UEB varieties sold in year 2013 reached more than 1.2 million.

The **Laboratory of Plant Biotechnologies** has been focused predominantly on the area of plantxenobiotic interactions, as well as on the area of plant secondary metabolites.

Toxicity of cadmium, cobalt, copper, zinc, nickel, lead, chromium and arsenic was compared in 23 *Linum usitatissimum* cultivars (Soudek et al. 2010, ID 357036). In order to find a suitable plant for phytoremediation of soils heavily polluted with Cd, Cr, Cu, Pb or Zn, we compared responses of different plant species (Soudek et al. 2010, ID 357039, Soudek et al. 2011, ID 370674, Soudek et al. 2014, ID 429931). The improvement of the metal accumulation capacities was also achieved by addition of chelating agents (Petrova et al. 2012, ID 380679). We tested radionuclide (uranium, thorium and radium) up-take by plants in contaminated areas (Soudek et al. 2011, ID 370666) or in model experiments (Soudek et al. 2010, ID 348487, Soudek et al. 2011, ID 370676, Soudek et al. 2013, ID 399221, Soudek et al. 2014, ID 433558).

Plants can also substantially diminish other types of pollutants, e.g. dust particles, which represent major environmental problem in urban areas (Soudek et al. 2012, ID 38252).

Serious soil and water contamination is caused also by organic xenobiotics. We characterized the impact of 2,4,6-trinitrotoluen (TNT) on gene expression using microarrays (Landa et al. 2010, ID 350208). The up-regulated genes included nitrate reductase, several glycosyltransferases and ABC

transporters. The up-take and transformation of nitroglycerine and ethylene glycol dinitrate from wastewater were optimized for different plants (Podlipná et al. 2010, ID 350475).

Wide use of pharmaceuticals represents serious, gradually increasing contamination. That is especially true for pain releasing drugs (diclofenac, ibuprofen and acetaminophen) (Kotyza et al. 2010, ID 348000). In case of hydroponic plants grown on contaminated water, the best effectiveness of ibuprofen removal was found with the use of *Phragmites*. Similar situation was in case of benzimidazole anthelmintics, the drugs against parasitic worms (Podlipná et al., 2013, ID 399919).

We evaluated the potential effect of zinc oxide (nZnO), fullerene soot (FS) or titanium dioxide (nTiO2) nanoparticles on gene expression in *Arabidopsis thaliana* roots using microarrays. Nanoparticles nZnO and FS caused wide changes of the transcriptome, especially the elevation of abiotic and biotic stress responsive genes (Landa et al. 2012, ID 389142).

They also focused our effort on characterization of natural products with anti-inflammatory and antiproliferative properties, which could be used as natural substituents of synthetic pharmaceuticals (Landa et al. 2013, ID 395042, Hošek et al. 2011, ID 367668, Landa et al. 2014, ID 433742, Langhansová et al. 2014, ID 432566, Landa et al. 2012, ID 380682, Tesařová et al. 2011, ID 367700, Halamová et al. 2010, ID 351528, Kutil et al. 2014, ID 429537, Pastorková et al. 2013, ID 395455).

The effectivity of anticancer drugs may be substantially increased by their conjugation with specific targeting compounds. Synthesis of the conjugate of cytostatic paclitaxel with an analogue of the gonadotropinreleasing hormone increased the antiproliferative effect of paclitaxel substantially (Přibylová et al. 2011, ID 370667). The non-hydrolyzable alkylcarbonate analogues of O-acetyl-ADP-ribose were synthesized to obtain effective inhibitors of sirtuins, histone deacetylases, which are required for gene silencing (Dvořáková et al. 2013, ID 399579).

Recently, labelling of physiologically active compounds with fluorescent dye proved to be useful in the visualization of the compound localization *in vivo*. Fluorescent labelling of the brassinosteroid receptor enabled to follow dynamics of its subcellular distribution and regulation of brassinosteroid signal transduction (Irani et al. 2012, ID 378975).

Researchers at the **Laboratory of Growth Regulators** (LGR) deal mainly with cytokinins, but recently also with other groups of plant growth regulators. One globally renowned contribution of the LGR in this field is the expansion of a number of cytokinins, especially the aromatic cytokinins and their olomoucine-derived derivatives. Olomoucine was the first in a line of anti-tumor agents derived from cytokinins. Development of other, more effective inhibitors of cyclin-dependent kinases, key enzymes of cell division cycle like bohemine, roscovitine, olomoucine II and others followed. Roscovitine was licensed by Cyclacel Pharmaceuticals, Inc. and under its commercial name, Seliciclib® (www.cyclacel.com) is completing phase IIB clinical trials for cancer treatment in Europe and in the USA.

In the agricultural research area, using an inhibitor of cytokinin oxidase/dehydrogenase called INCIDE they discovered how to increase the amount of endogenous cytokinins, which supports growth. Owing to this discovery, we were able to increase the yield of a number of agricultural crops and plant stress resistance.

Recently, they developed a product which makes the skin youthful aids in the treatment of skin diseases. Cytokinins, which also retard ageing in humans were used in this development. Two international patents were issued for these discoveries. These were licensed to the American company Pyratine Plc in California which incurred the costs for patent protection. The product with the trade name Pyratine is derived from the first cytokinin kinetin (www.pyratine.com). This not only treats skin roughness, wrinkles, and pigmentation, it is also effective for treating various forms of acne. LGR focuses on scientific research and teaching in the field of experimental biology, especially in the preparation of new, purine-based growth regulators with potent biological activities, the development of relevant analytical methods, the study of the functions and effects on growth and developmental process in normal and tumor cells, including the development of anti-tumor agents derived from plant hormones.

Research in the **Isotope Laboratory** has been focused at the three basic directions: 1) investigation of medicinally important plant products, 2) development of heterocyclic derivatives of synthetic origin (purinebased compounds), including radiolabeling and 3) preparation of cytokinin derivatives, mostly with radiolabeling.

Publication activity of the team (LRR and IL) is enormous, only the main research areas and selected publications from almost three hundred papers are listed below:

New phytohormone standards, probes and labelled derivatives: both laboratories have a long standing experience in organic synthesis, labelling of phytohormones and production of heavy and radioactively labelled phytohormones (Walla et al. 2010, ID349821, Voller et al. 2010, ID 349823, Pertry et al. 2010, ID349831, Béres et al. 2010, ID 351018, Vícha et al. 2010, ID359282, Tarkowski et al. 2010, ID359308, Zelenka et al. 2011, ID369950, Karady et al. 2011, ID 374545, Brcko et al. 2012, ID 375171, Irani et al. 2012, ID 378975, Novák et al. 2012, ID384241, Svačinová et al. 2012, ID385990, Béres et al. 2012, 385991, Kripach et al. 2013, ID 397254, Urbanová et al. 2013, ID 399103, Zhang et al. 2014, ID 429940, Floková et al. 2014, ID433242, Aremu et al. 2014, 433341).

New compounds modulating phytohormone perception, biosynthesis and degradation: We have contributed to studies of transgenic plants with cytokinin receptor loss-of-function mutations (Jeon et al. 2010, ID 359299, Vescovi et al. 2012, ID 382525), plants with mutations in cytokinin-synthesizing genes (Kopečná et al. 2013, ID 423120, Lindner et al. 2014, ID 431517), or cytokinin-deficient plants with genetically enhanced cytokinin degradation (Kowalska et al. 2010, ID 359304, Frébortová et al. 2010, ID359306, Werner et al. 2010, ID370793, Bartrina et al. 2011, ID 368587, Long et al. 2012, ID 382517, Rosar et al. 2012, ID 388866, Macková et al. 2013, ID 399220, Trifunovic et al. 2013, ID 421942, Mřížová et al. 2013, ID 423952, Kollmer et al. 2014, ID 429595). Many growth regulatory compounds regulating both the perception and metabolism of brassinosteroids and cytokinins were developed during the last five years (Kopečný et al. 2010, ID 359309, Mik et al. 2011, ID 368599, Mik et al. 2011, ID 368655, Aremu et al. 2012, ID 374918, Reusche et al. 2013, ID 399219), and for patents see (Lenobel et al. 2010, ID351020, Doležal et al. 2010, ID 440436, Szüčová et al. 2011, ID 368578, Doležal et al. 2011, ID 369539, Zatloukal et al. 2011, ID 380684, Szüčová et al. 2011, ID 427107, Doležal et al. 2012, ID 380681, Doležal et al. 2012, ID 380683, Szüčová et al. 2012, ID 382905, Szüčová et al. 2012, ID 427106, Doležal et al. 2012, ID 427581, Doležal et al. 2012, ID 427629, Szüčová et al. 2013, ID 425881, Szüčová et al. 2013, ID 425882, Szüčová et al. 2014, ID 3438081, Doležal et al. 2014, ID 438177 and www.espacenet.com).

Chemical modulators of kinases (cytokinin-derived): we have continued development of increasingly effective CDK inhibitors, leading to the discovery of several other potent compounds with various structural motifs (Hyun et al. 2010, ID 357894, Gucký et al. 2010, ID 357898, Jedinák et al. 2011, ID 368591, Janeczko et al. 2011, 368590, Zatloukal et al. 2013, ID 395483, Gucký et al. 2013, ID 399222, Imramovský et al. 2013, ID 423094, Jorda et al. 2014, ID 433741, Vilkauskaite et al. 2014, ID 436360). Potential of CDK inhibitors in different therapeutic areas was also reviewed several times (Kryštof and Udrijan 2010, ID 359286, Kryštof et al. 2010, ID 359307, Kryštof et al. 2012, ID 382222, Jorda et al. 2012, ID 382312). New generations were prepared following well-established methods, including our previously described syntheses of purines, pyrazollo[4,3- d]pyrimidines, 8-azapurines and arylazopyrazoles (for patents see Moravcová et al. 2010, ID 349828, Moravcová et al. 2010, ID 349834, Moravcová et al. 2010, ID 352737, Moravcová et al. 2010, ID 352738, Moravcová et al. 2012, ID 380690, Meijer et al. 2012, ID 427611, Moravcová et al. 2013, ID 425883 and www.espacenet.com).

Natural phytochemicals as potential drug candidates: Betulin and saponins based on betulin scaffold possess interesting biological properties which have been extensively studied and reviewed in the last years. We have been able to identify an entirely new compound with much higher cytotoxic activity than was described for the original structure (Cmoch et al. 2014, ID 429932, Cmoch et al. 2014, ID 433373). New anticancer saponins derived from OSW-1 - OSW-1, has a low toxicity for normal cells but inhibits the growth of a variety of malignant tumor cells and is 10-100 times more potent than clinically applied anticancer agents, such as adriamycin, cisplatin, camptothecin, and taxol. Potent analogues of OSW-1 were prepared (Maj et al. 2011, ID 368598).

Steroid plant growth regulators (brassinosteroids and ecdysteroids): we have developed a number of new brassinosteroid (BR) analogues over the last 20 years which have been tested in different plant human cell bioassays. New anticancer and other properties of the compounds have been discovered (Hniličková et al. 2010, ID 347182, Svobodová et al. 2010, ID 349942, Steigerová et al. 2010, ID 359313, Irani et al. 2012, ID 378975, Kvasnica et al. 2012, ID 384104, Steigerová et al. 2012, ID 386424, Rárová et al. 2012, ID 388867, Kvasnica et al. 2014, ID429538).

Since 2008, different MS were gradually introduced by our laboratory in plant hormone analysis (cytokinins, auxins, JAs, ABAs, gibberellins, brassinosteroids, phenolics, intact cytokinin nucleotides in human K-562 leukemia cells, 2-methylthio-cytokinin derivatives produced by the phytopathogenic actinomycete *Rhodococcus fascians*, roscovitine oxidation products, isoflavonoids and other phenylpropanoids) (Béres et al. 2010, ID 351018,Tarkowski et al. 2010, ID 359308, Karady et al. 2011, ID 374545, Prokudina et al. 2012, ID 382519, Novák et al. 2012, ID 384241, Svačinová et al. 2012, ID 385990, Urbanová et al. 2013, ID 399103, Pěnčík et al. 2013, ID 423096, Floková et al. 2014, ID 433242). The analysis of cytokinin nucleotides by capillary zone electrophoresis with diode array and mass spectrometric detection as well as LC-NMR was also introduced (Béres et al. 2012, ID 385991, Horník et al. 2013, ID 424009). The ability of LC-NMR to detect simultaneously free and conjugated phytosterols in natural extracts was also tested. The results of qualitative and quantitative analyses were in a good agreement with the literature data (Horník et al. 2013, ID 424009). Mass spectrometry has developed towards increasingly higher sensitivity and selectivity in the analyses, which now makes it possible to perform tissue and cell specific quantification of phytohormones and different phytohormone metabolites – for example, simultaneous profile of the majority of known auxin precursors and conjugates/catabolites (auxin metabolome) in small amounts of Arabidopsis tissue was determined (Novák et al. 2012, ID 384241, Pěnčík et al. 2013, ID 423096). For minute tissue samples,

miniaturization of the extraction and purification steps was improving the sensitivity of analytical method, since it can minimize analyte losses due to adsorption to surfaces and/or increase analyte recovery in the solid phase extraction (SPE) step (Svačinová et al. 2012, ID 385990).

Since its establishment the **Laboratory of Signal Transduction** has been focused upon phospholipid signalling. During the evaluated period the LST has been focused upon investigating the role of non-specific phospholipase C (NPC) in plant development and stress responses.

In three articles, they reported upon the role of NPC in responses of plants to aluminium toxicity. In the first one (Pejchar et al. 2010, ID 349924) they showed biochemically that a significant decrease of DAG in cells treated with $AlCl_3$ was caused by an inhibition of NPC activity. In the next article (Pejchar et al. (2015). Frontiers in Plant Science 6: 66), they referred about the impact of Al on the expression, activity, and function of the NPC4. In the third article (Pejchar and Martinec, Plant Signaling & Behavior, in press), they hypothesize that the activity of NPC is affected by Al-induced changes in plasma membrane's physical properties. Salt stress was another stress that was studied in connection with Arabidopsis NPCs (Kocourková et al. 2011, ID 369946). Their observations clearly demonstrated a role for NPC4 in the response of Arabidopsis to salt stress. Possible role of NPCs in plant development and hormone signalling was studied as well (Wimalasekera et al. 2010, ID 349937). It was shown that at least one NPC is a plant signalling enzyme in BL signal transduction. The first review about non-specific phospholipase C in plants was published (Pokotylo et al. 2013, ID 395057).

The majority of findings published by the **Laboratory of Biologically Active Compounds** (LBAC) can be assigned to three fields: 1) somatic embryogenesis in conifers; 2) endogenous polyamines, phenolic compounds and phytohormones; and 3) abiotic stresses. They uprooted the dominant opinion that use of the actindepolymerizing drug latrunculin B conclusively arrests the development of somatic embryos in conifers (Schwarzerová et al. 2010, ID 356952, Vondráková et al. 2014, ID 432115). Another study comprises proteomic, transcriptomic and morphologic analyses, as well as monitoring of endogenous abscisic acid and carbohydrate levels during pine somatic embryo development (Morel et al. 2014, ID 433191). The role of endogenous polyamines has been studied both during the preparation of embryogenic culture of Norway spruce prior to cryopreservation and during culture regrowth after cryopreservation (Vondráková et al. 2010, ID 357035).

They also examined the effect of drought and heat stress on polyamines metabolism (Cvikrová et al. 2012, ID 379082, Cvikrová et al. 2013, ID 423949).

The research in the **Laboratory of Pathological Plant Physiology** (LPPP) has been focused on plant–microbe interactions. We have been studying the signaling pathways implicated in plant defence responses and induced resistance against pathogens.

In the natural pathosystem *Brassica napus* with its hemibiotrophic fungal pathogen Leptosphaeria maculans, we have reported an unusual cooperation of SA and ethylene (ET) signaling contributing to the resistance (Šašek et al., 2012, ID 382093). An unusually complex phytohormone signaling was also reported in the interaction of *B. napus* with *Sclerotinia sclerotiorum* (Nováková et al. 2014, ID 433554). The role of reactive oxygen species (ROS) in the interaction B. napus – L. maculans was reported (Jindřichová et al. 2011, ID 429974). We have also studied the action of inducers of plant resistance and reported that β-aminobutyric acid (BABA) had in addition to its priming activity also a direct antifungal activity (Šašek et al. 2012, ID 382522). Furthermore, our search for resistance-

inducing compounds led to two studies dealing with a trisaccharidic pathogen-associated molecular pattern from *L. maculans* (Kim et al. 2013, ID 397255) and an antimicrobial peptide anoplin (Jindřichová et al. 2014, ID 429974).

The second research field studies an interconnection between the SA signaling pathway and a phospholipid signaling system. Upon a long-term collaboration with Dr. Eric Ruelland (Université Paris) and prof. Olga Valentová (UCT Prague) we are mainly focused on the role of phosphatidylinositol 4-kinases (PI4Ks) and phospholipase D (PLD) in plant defense. Our work revealed that PI4Kβ1 and PI4Kβ2 are negative regulators of SA biosynthesis and act upstream of EDS1 (Šašek et al. 2014, ID 432723, Janda et al. 2014, ID 441351). Another study showed an interconnection between the SA pathway, actin cytoskeleton and PLD signaling (Matoušková et al. 2014, ID 429540).

The **Laboratory of Pollen Biology** focused on the identification of pollen-expressed transcription factors (TF) involved in the regulation of male gametophyte development (Reňák et al. 2012, ID 379285). They localized the AtREN1 protein specifically to the nucleolus that suggests its likely involvement in ribosomal RNA biogenesis therefore linking heat stress response with translation (Reňák et al. 2014, ID 429991). The results revealed a role of the auxin transport in male gametophyte development in which the distinct actions of ER-localized PIN transporters maintained the auxin levels optimal for pollen development and pollen tube growth (Ding et al. 2012, ID 380680).

They conducted the first comprehensive developmental transcriptomic analysis of the tobacco male gametophyte representing the first plant species shedding bicellular pollen (Hafidh et al. 2012, ID 382528; Hafidh et al. 2012, ID 391243). A model outlining the network of posttranscriptional control with a focus on the role of stored RNPs was proposed (Hafidh et al. 2011, ID 380676; Hafidh et al. 2013, ID 440716).

They showed for the first time the dynamics of protein phosphorylation and dephosphorylation associated with early stages of pollen germination (Fíla et al. 2012, ID 384621).

They also scouted for new genes thought to be potentially involved in DSB repair (Böhmdorfer et al. 2011, ID 365270, da Costa-Nunes et al. 2014, ID 440714).

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Station of apple breeding for disease resistance

2.1. Applied research

This is a continuation of a long term project on breeding of high quality apple varieties resistant to scab caused by *Venturia inaequalis* the most widespread and harmful apple disease.

The first source of resistance against scab used in the breeding was *Malus floribunda* bearing the resistance gen *Vf*. The gen can be transferred to the progeny simply by crossing and the presence of the gen in plants is evidenced by means of specific molecular markers. A resistance hybrid can have a commerce potential only when their growing/bearing characteristics and fruit qualities fulfil the requirements of the grower and the market. To achieve this there are usually several progeny generation needed. At present in most of the resistant varieties the scab resistance is encoded monogenicaly by the gen *Vf*.

Recently this type of monogenic resistance has been overcome by new races of the fungus. This initiated to turn our attention to look to a scab resistance encoded polygenicaly. According to resistance against the new scab races two different clones were identified and used for crossing which resulted in selection two promising varieties: Admiral (late variety protected by Community Plant Variety Right in the EU) and Allegro (early variety protected by Plant Variety Rights in Czech Republic which will be extended to the whole EU). The polygenic resistances are usually durable in plants. An efficient use of the polygenic resistance in apple breeding will require developing molecular markers to identify the genes involved.

The results obtained were transferred into practice by conclusion of License Agreements with the buyers for planting and apple production. The royalties are paid to the licensor. The amount of the trees sold depends mainly on the qualities of the variety, but also on their publicity and fruit selling organizations. From the License Agreements concluded before 2010 the most successful are the varieties Topaz with its red mutation Red Topaz and the variety sold under its Trademark Opal. In the past years Topaz was the most planted scab resistant variety in Europe. Topaz is very popular amongst organic fruit growers, more than 400 000 trees a year have been planted on a total surface of 1000 ha in the year 2014.

Very popular, mainly in USA and Chile is the variety UEB 32642 known under the Trademark Opal which is registered in more than 40 countries. In Europe an international society fruit.select was grounded from 5 members of plant nurseries.

In USA the independent society Perishables Group rated Opal by the highest note “excellent” based on a large scale testing by the consumers. The variety is protected by Plant Patent in USA and by Community Plant variety Right in EU and later was applied for UPOV protection in a number of countries (Australia, Brazil, Chile, South Africa, Canada, Morocco, Mexico and New Zealand).

In USA Opal apples are exclusively sold by the society First Fruits from which a portion of all sales of Opal apples is awarded to charitative purposes. In 2015 the estimate to be donated makes 150 000 $. In the years 2010 to 2014 the total amount of Opal trees sold exceeded 1.3 million.

In the years 2010 to 2014 16 Licensee Agreements were concluded for varieties with scab resistance *Vf* newly released for commercialization. Of particular importance is the contract with an American Society for scab resistant varieties with compact columnar growth habit for the territory of the whole world. Columnar varieties released were protected in the EU 8 varieties and in USA 2 varieties. This type of trees is sold for home gardens. In the years 2010 to 2014 about 300 000 trees were sold in Netherlands, Germany, USA, Switzerland and Czech Republic.

The total number of trees of UEB varieties sold in year 2013 reached more than 1.2 million.

2.2. Basic research

During the evaluated period we also started basic research towards understanding the mechanism of apple scab infection and the role of apple scab resistant genes in this process. In 2010 we therefore started a formal cooperation between our team and the laboratory of Prof. Timothy Hall, IDMB, Texas A&M University. The project was funded by NSF from the US part and by the Ministry of Education, Youth and Sports form the Czech part, under project Contact (ME10038, Project No. 1). Apart of participating in such excellent research and obtaining valuable results, we could learn new powerful techniques such as next generation sequencing (NGS) that can be later used in our lab for apple research.

2.2.1. Epigenetic control of Phaseolin expression

The goal of this joint project was to gain insight to the complex mechanisms governing eukaryotic gene expression. The biological system that we used is primarily the phaseolin (phas) gene that encodes a bean (Phaseolus vulgaris) seed-storage protein. During seed development (embryogenesis), phas is expressed at exceptionally high levels but it is fully repressed in all vegetative tissues (roots, stems, leaves). In the past, a detailed understanding of the signals and mechanisms that achieve these extreme states were reported. Step 1 involves chromatin remodeling (initiated by the B3 domaincontaining transcription factor, PvALF), that permits formation of a potentiated or poised transcription complex. In Step 2 transcriptional activation is initiated by an ABA-induced signal transduction cascade. This system provides a nice example of the interaction and coordination of epigenetic (chromatin) and genetic (transcription factor) events that regulate gene expression. Our focus was therefore on the events and processes involved in the transition of promoter activity from the silent to the highly active state. The previous studies on phas in Hall's lab have contributed to major advances in plant molecular biology, including the first demonstration of introns in a plant gene and the first functional transfer of a developmentally regulated gene from one species to another.

We used a heterologous in vivo PvALF/phas-gus expression system in transgenic Arabidopsis leaves in conjunction with the powerful RNA-Seq approach to capture transcriptional landscapes of phas promoter expression. Expression of over 2000 genes from 11 functional categories coincided with changes in the transcriptional status of the phas promoter. Gene network analysis of induced genes and amiRNA-mediated loss-of-function genetic assays identified transcription factors RLT2 and AIL5 as key players essential for phas transcription. PvALF binding to the RLT2 and AIL5 promoter regions was

confirmed by EMSA. RLT2 and AIL5 knock-down lines displayed reduced expression of several endogenous seed genes, suggesting that these factors are involved in activation of endogenous Arabidopsis seed-storage gene expression. Overall, the identification of novel factors involved in phas activation provides important insight into the two-step transcriptional regulation of seed -specific gene expression.

Our lab participated equally in all experiments. S. Kertbundit took part in all wet laboratory experiments, especially plant tissue culture, RNA isolation and library construction as well as in qPCR. M. Juricek performed bioinformatical analysis of obtained results from the next generation sequencing. The project ended in 2012 and it was evaluated as “Excellent”. The informal cooperation continued also in 2013 when joint article was published.

2.2.2. Apple basic research

Our main goal in the current research is to understand the mechanism of the polygenic resistance against apple scab which will lead to better understanding of *Venturia* infection a thus learn how to prevent this fungus to overcome apple scab resistance in the future. In order to achieve this goal, we plan to evaluate differential RNA expression of the apple transcriptome using RNA-Seq method before and after inoculation with *Venturia* while using different resistant (monogenic and polygenic) varieties of apple and different isolates of *Venturia inaequalis*, including the one that is able to overcome monogenic resistance to apple scab based on *Vf* gene.

The first part of this research was aimed to develop reliable method of isolating suitable cultures of Venturia and also developing methods for reliable infection of different apple varieties under greenhouse condition. We succeeded and suspensions of washed conidia from apple scab infected leaves were isolated and used for inoculation of rooted apple plantlets of obtained from tissue culture. Golden delicious variety was used in these experiments and it will serve as a control. After different periods of time, youngest developed leaves were cut away, rinsed with sterile distilled water and used for RNA isolation which was then sent for NGS.

Recently, we also developed a sensitive Real-Time PCR method for apple phytoplasma detection using 23S phytoplasma RNA and specific MGB-probe.

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Hromonal Regulation of Plants

Research in our Laboratory (Laboratory of Hormonal Regulations in Plants, LRHP) was funded by **grant projects no 2-20 (Appendix 3-1, team number 2; total funding in 2010-2014 2,860 M€).** We concentrated predominantly on two groups of plant signalling compounds (phytohormones) – *auxins and cytokinins*, including their interactions with other phytohormones, e.g. abscisic acid and ethylene. We have focused on ***both qualitative and quantitative aspects of processes involved in establishment and modulation of their homeostasis (i.e. metabolism and transport) as well as on their signalling and control of physiological processes (namely stress reactions)***. In the following text, our research activities in 2010-2014 are summarized briefly (papers published within the period 2010-2014 are in bold).

In the field of auxin, our main interest was to characterize ***mechanisms of action of auxin transporters***, residing on plasma membrane and on endomembranes, as well as to reveal related ***auxin-metabolizing processes***:

We have continued to study intracellular putative auxin transporters (following our previous co-authored article by Mravec et al. Nature 459:1136, 2009, where intracellular localization of PIN5 auxin transporter was identified). We have contributed significantly to discovery of the so far unknown PILS (PIN-LIKES) family of putative auxin transporters that – similar to PIN5 - localize to endoplasmic reticulum (ER). Changes in their expression result in changes in spectrum of auxin metabolites in cells, thus determining the accessibility of free auxin for the nuclear auxin-signalling pathway (Barbez et al., NATURE 2012, ID 380744). However, not only PIN5 and PILSes can be found on endomembranes. We have participated in characterization of another non-canonical member of PIN family of auxin transporters: PIN8, which is expressed in male gametophyte of *Arabidopsis thaliana* and plays a crucial role in pollen development and its functionality. PIN8 co-localizes with PIN5 on the ER and is also involved in control of auxin homoeostasis and metabolism (Ding et al., NATURE COMMUNICATIONS 2012, ID 380680).

We have also participated in characterization of the plasma membrane-localized auxin transporter *At*ABCB4 from the superfamily of ABC transporters, and on revealing its unique function: Depending on intracellular auxin level, ABCB4 is able to transport auxin into cells or from cells. This is the first finding of a bi-directional, 'substrate'-concentration-dependent transport of a low-molecular compound across the plasma membrane in plants (Kubeš and Yang et al., PLANT JOURNAL 2012, ID 380687). The paper also provides a likely explanation for the herbicidal activity of synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D).

We contributed fundamentally to revealing the so far unknown activity to facilitate auxin uptake to cells of the nitrate 'transceptor' NRT1.1. This plasma membraneprotein possesses two unique characteristics: it serves as a sensor as well as transporter for nitrate. As it is able to transport

auxin as well, it connects availability of a crucial mineral nutrient with auxin-controlled root development. This is the first observation of a direct hormonal regulation via nutrient availability driven by one particular protein species in plants (Krouk et al., DEVELOPMENTAL CELL 2010, ID 343963). The work on characterization of kinetic properties of NRT1.1 continues e.g. in evaluation of the activities of phosphorylated and non-phosphorylated forms of NRT1.1 at amino acid residue T101 that differ significantly in both signalling and transport functions (Bouguyon et al., Nature Plants, in press, http://dx.doi.org/10.1038/nplants.2015.15).

We have also collaborated on revealing of the so far unknown interaction between auxin efflux carriers PIN1 and PIN2 and dynamin-related proteins at the cell plate that is necessary for proper polar PIN positioning in interphase cells and, concomitantly, for auxin-mediated development (Mravec et al., CURRENT BIOLOGY 2011, ID 364093), and on characterization of reversible ubiquitylation of the auxin efflux carrier PIN2 that is important for PIN2 endocytosis and its delivery into cell's lytic compartment. However, we have shown that such ubiquitylation does not interfere with PIN2 auxin efflux activity at the plasma membrane (Leitner et al., PNAS 2012, ID 380702).

We have contributed significantly to characterization of the role of major auxin degradation product, 2-oxindole-3-acetic acid (oxIAA), in control of auxin homeostasis (Pěnčík et al., PLANT CELL 2013, ID 423096), and to better understanding of the mode of action of some auxin transport inhibitors (Laňková et al., JOURNAL OF EXPERIMENTAL BOTANY 2010, ID 347619; Yin et al., NEW PHYTOLOGIST 2014, ID 430993).

In *Arabidopsis thaliana* (ecotype Landsberg erecta) suspension-cultured cells (LE line) we characterized carrier-mediated uptake (influx) and efflux for native auxin indole- 3-acetic acid (IAA) and synthetic auxins, naphthalene-1-acetic acid (NAA) and 2,4-D. In contrast to tobacco cells, only a small proportion of NAA is metabolized in LE cells. This makes the LE line favourable for measurements of auxin transport kinetics on a single cell level, distinct from well-established tobacco BY-2 cells (Seifertová et al., JOURNAL OF PLANT PHYSIOLOGY 2014, ID 429938).

Using single-cell-based tobacco system, we were able to track the course of auxin accumulation inside the cells and thus describe the activity of auxin influx and efflux carriers as well as contribution of auxin diffusion and metabolism. Based on this data, we have designed and experimentally verified the first mathematical model of transport processes involved in auxin accumulation at a single cell level providing estimates of key transport parameters for 2,4-D. Such specified and quantified auxin transport parameters represent unique data necessary for precise characterization of auxin transport at tissue level (Hošek and Kubeš et al., JOURNAL OF EXPERIMENTAL BOTANY 2012, ID 381050).

We also investigated the so far unknown ***relationship(s) between nontranscriptional auxin signalling and cellular auxin efflux:***

In 2005, auxin was shown to inhibit its own efflux from cells via inhibition of endocytosis of some plasma membrane proteins (our co-authored article by Paciorek et al., Nature 435: 1251, 2005). In a direct follow up, we contributed significantly to reveal the underlying mechanism: Auxin, after its interaction with the auxin receptor ABP1, inhibits the clathrin-mediated internalization of PINs via non-transcriptional mechanism (Robert et al., CELL 2010, ID 350344). Then, we have further characterized

link between the activity of ABP1 in regulation of dynamics of proteins in the plasma membrane and activity of PIN auxin transporters (Čovanová et al., PLOS ONE 2013, ID 421122).

Using numerous auxin analogues, we determined their physiological activity, and investigated their modes of action. Results demonstrated similar but not identical structure-activity relationships, and highlighted some synthetic auxin analogues that display a non-canonical behaviour. Such compounds can serve as useful tools for studies of distinct mechanisms involved in the auxin mode of action (Simon et al., NEW PHYTOLOGIST 2013, ID 426212).

In the field of cytokinins, we have performed and published as yet the most comprehensive characterization of a specific class of *cis*-zeatin-type cytokinins (Gajdošová et al., JOURNAL OF EXPERIMENTAL BOTANY 2011, ID 365271). We have demonstrated their abundance throughout the plant kingdom and their biological activities in cytokinin bioassays as well as have specified their uptake, accumulation and metabolic pathways in plants. In addition, we have participated in demonstration of *cis*-zeatin-type cytokinins as the predominant cytokinin forms in dry and/or imbibed seeds of various plant species (Stirk et al., JOURNAL OF PLANT PHYSIOLOGY 2012, ID 380691; Stirk et al., JOURNAL OF PLANT GROWTH REGULATION 2012, ID 380751). We have also shown an involvement of cis-zeatin-type cytokinins in plant responses to fungal infection (Behr and Motyka et al., MOLECULAR PLANT-MICROBE INTERACTIONS 2012, ID 381049) as well as to abiotic stresses (Dobrá et al., JOURNAL OF PLANT PHYSIOLOGY 2010; Kosová et al., JOURNAL OF PLANT PHYSIOLOGY 2012, ID 380692; Macková et al., JOURNAL OF EXPERIMENTAL BOTANY 2013, ID 399220). Using potato and centaury plants constitutively overexpressing the cytokinin oxidase/dehydrogenase (CKX) gene from *Arabidopsis thaliana* and grown *in vitro*, we have demonstrated a diminished growth and/or reduced morphogenic potential in association with contents of *cis*-zeatin-type cytokinins that were considerably lower than *trans*-zeatintype cytokinins (Raspor et al., JOURNAL OF PLANT GROWTH REGULATION 2013, ID 380750; Trifunović et al., PLANT CELL TISSUE ORGAN CULTURE 2013, ID 421942, respectively). Based on these findings, we have hypothesized the conceivable function of *cis*-zeatin-type cytokinins as delicate regulators of cytokinin responses in plants under growth-limiting conditions.

We have contributed to the description of molecular function of the *Pseudomonas syringae* pv. *tomato* effector HopQ1 that activates cytokinin signalling and enhances concentrations of bioactive cytokinin forms by stimulation of LOG (*lonely guy*) biosynthetic pathway and have demonstrated the importance of cytokinins in plantmicrobe interactions (Hann et al., NEW PHYTOLOGIST 2014, ID 431074). We have characterized two tomato cytokinin biosynthetic genes, *SlIPT3* and *SlIPT4*, in homologous as well as heterologous system and determined their different spatiotemporal expression patterns during tomato plant development, *in vitro* enzymatic activity and regulation of both genes under salt stress conditions. Moreover, we have found an improved tolerance of tomato plants to salinity resulting from the *SlIPT3* overexpression (Žižková et al., BMC Plant Biol. 2015, DOI 10.1186/s12870-015-0415-7). We have been involved also in the identification and functional characterization of the first tomato repressor-type zinc finger transcription factor, *SlZF2*, demonstrating its important role in control of plant development and salinity response. We have discovered that the involvement of *SlZF2* in delayed senescence and improved salt tolerance of tomato plants correlates with maintaining photosynthesis, increasing polyamine and abscisic acid biosynthesis/signalling and modifying ratio between *trans*- and *cis*-zeatin-type cytokinins (Hichri et al., PLANT PHYSIOLOGY 2014, ID 429597).

We have proved a positive effect of enhanced expression of cytokinin biosynthetic gene under senescence-inducible promoter (*SAG12:IPT*) on suppression of leaf senescence and enhancement drought-tolerance of cassava plants (which provide a source of dietary carbohydrate for over 600 million of people - Zhang et al., JOURNAL OF INTEGRATIVE PLANT BIOLOGY 2010, ID 348004). We have extended to rice our previous finding on wheat that grain formation strategy of cereals is based on fast translocation of metabolites to developing grains after pollination (Rubia et al., JOURNAL OF PLANT GROWTH REGULATION 2014, ID 429986).

Part of our activities was devoted to elucidation of ***functions of phytohormones in responses to abiotic stresses***, predominantly drought, heat and cold:

We evaluated the role of osmolyte proline by comparing the drought and/or heat stress responses in tobacco plants with constitutively elevated proline content (Dobrá et al., JOURNAL OF PLANT PHYSIOLOGY 2010, ID 350307). Hormonal profiles during the early heat stress response (cytokinin up-regulation and abscisic acid down-regulation) demonstrated that stimulation of transpiration is a crucial mechanism to maintain the leaf temperature lower than the environment, at least until defence mechanisms could be activated. Determination of expression profiles of two proline biosynthetic and three proline degradation genes revealed crucial role of *NtP5CSA* in the stress and *NtPDH2* during rehydration, as well as differential regulation of proline content in shoots and roots (Dobrá et al., JOURNAL OF PLANT PHYSIOLOGY 2011, ID 365268). Polyamine changes in the proline overexpressor and wild-type were described with our contribution (Cvikrová et al., PLANT SCIENCE 2012, ID 379082; Cvikrová et al., PLANT PHYSIOLOGY & BIOCHEMISTRY 2013, ID 423949).

The impact of cytokinin down-regulation, both constitutive and root-targeted, on the drought and/or heat stress tolerance was characterized in tobacco (Macková et al., JOURNAL OF EXPERIMENTAL BOTANY 2013, ID 399220). Apart of hormonal responses, stress tolerance was evaluated by expression levels of dehydration marker genes *ERD1* and *P5CSA*. The results suggest that high stress tolerance of *35S:AtCKX1* transformant is given predominantly by morphological changes (suppressed growth rate of dwarf shoots, enhanced root system). Thorough analysis of antioxidant enzymes (ascorbate peroxidase, catalase and superoxide dismutase) revealed that expression of cytosol isoforms is stimulated upon stress conditions, when chloroplast isoenzymes are suppressed (together with the photosynthesis) (Lubovská et al., JOURNAL OF PLANT PHYSIOLOGY 2014, ID 433743).

Complex phytohormone analysis allowed us to characterize hormonal cross-talk during the individual phases of the response to cold stress (e.g., in alarm phase or acclimation) in leaves and crowns of winter and spring wheat genotypes (Kosová et al., JOURNAL OF PLANT PHYSIOLOGY 2012, ID 380692). The cold response of the proteome was described in detail (Kosová et al., JOURNAL OF PROTEOME RESEARCH 2013, ID 421944). The up-regulation of active cytokinin content at the onset of transition from vegetative to generative developmental phase indicated decisive role of cytokinins in this process (Vanková et al., ENVIRONMETAL AND EXPERIMENTAL BOTANY 2014, ID 429933).

Participation of jasmonic acid, hormone generally associated with the defence against herbivore and necrotroph attack, was demonstrated during severe dehydration of the resurrection plant *Haberlea rhodopensis* (Djilianov et al., JOURNAL OF PLANT GROWTH REGULATION 2013, ID 399223).

We have also contributed to evaluation of cytokinin regulatory roles in drought, salt and abscisic acid responses, and its biosynthesis (Nishiyama et al., PLANT CELL 2011, ID 365273), to understanding the metabolic reorganisation prior to early drought responses in lupine (Pinheiro et al., JOURNAL OF EXPERIMENTAL BOTANY 2011, ID 365274), to complex analysis of soybean response to drought stress (Le et al., PLOS ONE 2012, ID 382217), to characterization of hormonal changes underlying improved performance of somaclonal finger millet line (Radchuk et al., JOURNAL OF EXPERIMENTAL BOTANY 2012, ID 382086), to study of cytokinin functions in grapevine berry initiation (Crane et al., PLANTA 2012, ID 365275), to elucidation of the effects of abscisic acid on nuclear genes encoding chloroplast-localized proteins in the basal and apical segments of wheat leaves (Yamburenko et al., JOURNAL OF EXPERIMENTAL BOTANY 2013, ID 422266), and to identification of the new role of ascorbate peroxidase 6 in germination control and seed stress tolerance in relation to hormonal cross-talk (Chen et al., PLANT PHYSIOLOGY 2014, ID 433740).

We have also contributed to understanding of ***phytohormone co-action in control of some developmental processes****:* e.g. floral formation (gibberellins, auxin and mutual changes in contents of *cis*- and *trans*-zeatin - Quinet et al., JOURNAL OF EXPERIMENTAL BOTANY 2014, ID 433556), apical hook development (auxin and ethylene - Vandenbussche et al., DEVELOPMENT 2010, ID 343964; Žádníková et al., DEVELOPMENT 2010, ID 347625), organogenesis (cytokinins and auxin – Marhavý et al., DEVELOPMENTAL CELL 2011, ID 366038), and in response of plants to shading (Ananieva et al., PLANT GROWTH REGULATION 2011, ID 368552).

We have also published several (mostly invited) ***reviews*** where we summarized and evaluated the present state-of-the-art in topics related to our expertise:

- ***On auxin in general:*** We have discussed what is auxin (Simon and Petrášek, PLANT SCIENCE 2011, ID 364098) and what are its possible applications (Skůpa et al., In: Nick P and Opatrný Z (eds): APPLIED PLANT CELL BIOLOGY: CELLULAR TOOLS AND APPROACHES FOR PLANT BIOTECHNOLOGY 2014, ID 436993). We were invited to edit the monograph summarizing the present knowledge on auxin and its role in plant development, where leading scientists in the field contributed with particular chapters (Zažímalová E, Petrášek J, Benková E (eds): AUXIN AND ITS ROLE IN PLANT DEVELOPMENT 2014, ID 438215).

- On auxin transport and transporters: We have not only summarized the present state-of-the-art in auxin transporters research but also tried to explain (from both functional and evolutionary points of view) why there are so many different types of auxin transporters in plants (Zažímalová and Murphy et al. COLD SPRING HARBOR PERSPECTIVES IN BIOLOGY 2010, ID 343966; Petrášek et al., In Geisler M. and Venema K. (eds). TRANSPORTERS AND PUMPS IN PLANT SIGNALING 2010, ID 350474).

- On subcellular compartments and processes related to auxin action: Plasma membrane (Malínská and Zažímalová, CURRENT PROTEIN AND PEPTIDE SCIENCE 2011, ID 360742) and methods used for studying endocytosis (Malínská et al., In Otegui MS (ed.): PLANT ENDOSOMES - METHODS AND PROTOCOLS 2014, ID 433745).

- On cytokinins and their role(s) in stress responses (Ha et al., TRENDS IN PLANT SCIENCE 2012, ID 380693; Kocsy et al., PLANT SCI. 2013, ID 399927; Vaňková, In Tran LS and Pal S (eds): PHYTOHORMONES: A WINDOW TO METABOLISM, SIGNALING AND BIOTECHNOLOGICAL APPLICATIONS 2014, ID 441628).

- *On abscisic acid*, particularly its signalling (Vaňková et al., In Ahmad P and Prasad MNV (eds): ABIOTIC STRESS RESPONSES IN PLANTS: METABOLISM, PRODUCTIVITY AND SUSTAINABILITY 2012, ID 382532).

Finally yet importantly, within our research we have ***optimized some methods and designed new ones:***

After acquisition of high performance liquid chromatograph coupled with mass spectrometer (LCMS) in 2009, we have optimized the extraction of plant samples and HPLC/MS analytical procedure, so that we are now able to reduce sample amount to tens to few hundreds mg of fresh weight and to simplify extraction and purification procedures using fewer steps and utilizing micro-volumes of elution solvents allowing us to process larger sample numbers. We can perform simultaneous and highly reliable determinations of multiple cytokinin forms together with other plant hormones and their metabolites (auxins, abscisic acid, jasmonates, salicylic acid, gibberellins) in one single sample (Dobrev and Vankova, METHODS IN MOLECULAR BIOLOGY 2012, ID 387792; Djilianov et al., JOURNAL OF PLANT GROWTH REGULATION 2013, ID 399223). Based on this expertise, we have been involved in studies of metabolic fate and dynamics of labelled hormones at cellular level (e.g. Barbez et al., NATURE 2012, ID 380744; Simon et al., NEW PHYTOLOGIST 2013, ID 426212; Seifertova et al., JOURNAL OF PLANT PHYSIOLOGY 2014, ID 429938) as well as hormonal metabolite identification and transport dynamics (Hošek and Kubeš et al., JOURNAL OF EXPERIMENTAL BOTANY 2012, ID 381050).

We have designed a new method to detect carrier-driven activities, based on the transient, single-cell-based system. Relative changes in signalling output of the auxin responsive promoter element DR5 were used to indirectly visualize auxin carriers' activity. This single-cell-based system can be useful to investigate also other hormonal (e.g. cytokinin – via TCS) signalling and transport pathways (Barbez et al. BMC PLANT BIOLOGY 2013, ID 395429).

We have uncovered yet unknown and unexpected effects of the FM (Fei Mao) styryl dyes. Until then, FM dyes have been considered reliable tools for tracking of plant endocytosis; however, we showed that routinely used concentrations of FM 4-64 and FM 5-95 trigger transient re-localization of PIN and ABCB proteins and FM 1-43 affects their activity. Our results emphasize the need for circumspection during *in vivo* studies of membrane proteins performed using simultaneous labelling with FM dyes (Jelínková et al. PLANT JOURNAL 2010, ID 343962).

We stimulated and participated in development of methods for in vivo measurement of pH in microscopic biological samples of µm or µl size. The methods are based on self-referenced ratiometric fluorescence intensity measurements using pH-sensitive transducer immobilised on the tip of optical fibres. They were successfully used for *in vivo* determination of pH in plant cells and xylem exudates (Kašík et al., ANALYTICAL AND BIOANALYTICAL CHEMISTRY 2010, ID 349751; Kašík et al., MATERIALS SCIENCE AND ENGINEERING C 2013, ID 424363).

We optimized some cell culture- and auxin homeostasis-related methodologies, and their description was published as invited book chapters in two volumes of Series „Methods in Molecular Biology", by Springer Science+Business Media, Totowa:Humana Press: Petrášek et al., In: Hicks G and Robert S (eds.): PLANT CHEMICAL GENOMICS, METHODS AND PROTOCOLS 2014, ID 429994; Simon et al., In: Hicks G and Robert S (eds.): PLANT CHEMICAL GENOMICS: METHODS AND PROTOCOLS 2014, ID

430550; Seifertová et al., In Žárský V and Cvrčková F (eds): PLANT CELL MORPHOGENESIS: METHODS AND PROTOCOLS 2014, ID 432114.

We have also participated in preparation of the nomenclature for elements of the two-component pathway in plants involved e.g. in cytokinin signaling (Heyl et al., PLANT PHYSIOLOGY 2013, ID 427104).

Our Laboratory is involved in ***broad international collaboration***, e.g. with:

- Prof. Jiří Friml a Dr. Eva Benková (VIB, Univ. Ghent, Belgium and IST Austria, Klosterneuburg, Austria): Transport of auxin and its role in plant development; sharing experimental material and joint papers (e.g. Vandenbussche et al. 2010, ID 343964; Žádníková et al. 2010, ID 347625; Robert et al. 2010, ID 350344; Ding et al. 2012, ID 380680; Simon et al. 2013, ID 426212; Čovanová et al. 2013, ID 421122; Zažímalová et al. (eds.) 2014, ID 438215; Grones et al., J. Exp. Bot., accepted).

- Prof. Angus Murphy a Dr. Wendy Peer (Purdue Univ., Indiana, USA, from 2013 Univ. of Maryland, Maryland, USA): Mechanism of auxin transport; sharing experimental material, joint papers (Zažímalová and Murphy et al. 2010, ID 343966; Kubeš and Yang et al. 2012, ID 380687). • Prof. Alain Gojon and Dr. Philippe Nacry (Institut de Biologie Intégrative des Plantes, CNRS/INRA, Montpellier, France): Nitrate transporter NRT1.1 and its role in auxin transport; sharing of experimental material and joint papers (Krouk et al. 2010, ID 343963; Bouguyon et al. Nature Plants, in press 2015).

- Dr. Jürgen Kleine-Vehn, (VIB, Univ. Ghent, Belgium and Univ. BOKU, Vienna, Austria): Transport of auxin and its mechanisms; sharing experimental material and joint papers (e.g. Jelínková et al. 2010, ID 343962; Robert et al. 2010, ID 350344; Zhang et al. 2011, ID 360769; Barbez et al. 2012, ID 380744; Barbez et al. 2013, ID 395429).

- Prof. Dr. Thomas Schmülling (Freie Universität Berlin, Dahlem Centre of Plant Sciences, Institute of Biology/Applied Genetics, Berlin, Germany): stress tolerance of tobacco plants over-expressing cytokinin oxidase/dehydrogenase gene under different promoters (35S or WRKY6) (Macková et al. 2013, ID 399220).

- Prof. Stanley Lutts, Dr. Imène Hichri and Dr. Muriel Quinet (Earth and Life Institute, Université catholique de Louvain, Louvain-la-Neuve, Belgium): Molecular mechanisms involved in hormonal control of plant development and salinity response; joint projects (Wallonie-Bruxelles International: Rhéa 2011/35047, 2011-2013 and WBI/14-3, 2014-2016), sharing experimental material and joint papers (Hichri et al. 2014, ID 429597; Quinet et al. 2014, ID 433556; Žižková et al. 2015).

We also closely collaborate with prof. Martin Hof and his team (J. Heyrovský Institute of Physical Chemistry CAS, Prague): Advanced fluorescence microscopy (raster image correlation spectroscopy (RICS), cross correlation Number & Brightness Analysis, FLIM/FRET).

Based on our expertise in cell cultures and our unique collection of cell lines overexpressing or silencing genes involved in auxin and cytokinin metabolism, transport and signalling, as well as our skills in biochemical, bioanalytical and cytological methods, we contribute to international collaborative research with these experiments and their evaluation and interpretation:

- Measurements of auxin and cytokinin (and possibly other hormones) movement across the plasma membrane using single-cell-based plant assay system, and determination of kinetic behaviour of auxin and cytokinin transporters;

- Tracking intracellular dynamics of proteins related to auxin and cytokinin mode of action (e.g. auxin and cytokinin transporters, ABP1 auxin receptor);

- Mass-spectrometry-based methodology for multiple hormone analysis and hormone metabolic profiling;

- Determination of activity of enzymes involved in hormonal metabolism.

General comment:

In the period 2010-2014, ***some researchers from our Laboratory were/are involved in management of the Institute:***

♣ Zažímalová – Director of the Institute (2007-2012)

♣ Vaňková – Chair of the Executive Board of the Institute (since 2012)

♣ Hoyerová – Scientific Secretary of the Institute (2007-2014)

♣ Petrášek – Head of IEB Imaging Facility (Malínská - administrator). On behalf of the IEB, Petrášek prepared application for Research infrastructure „National research infrastructure for biological and medical imaging“ (Czech-BioImaging), that will represent one of the nodes of the European project Bioimaging, connecting prominent European imaging workplaces. National project CzechBioimaging was positively evaluated among other research infrastructures, and negotiations with the Ministry of Education, Youth and Sports of the Czech Republic are now in the process.

Such managerial activities were/are very much time demanding and so they could have affected research in our Laboratory indirectly.

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Cell Biology

In our Laboratory of Cell Biology (LCB) at the IEB we are interested in the selected molecular regulatory modules of plant cell polarity and morphogenesis operating mostly cortically at the plasma membrane, and at the interface of secretory pathway, lipid membrane and cytoskeleton.

Between years 2010 and 2014 our laboratory made a major progress in the characterization of plant exocyst complex culminating in the publication of comprehensive review on the current state of arts in this field (Žárský et al. 2014 / ID 423950). Other more general aspects of exocyst as a cortical cytoplasmic membrane and cell polarity organizer in eukaryots we summarized in another review (Synek et al. 2014 / ID 429989). Analysing exo84b and exo70A1 Arabidopsis mutants using genetics and microscopy (both CLSM and electron microscopy) we for the first time not only show crucial contribution of exocyst to plant cell division, but show detailed dynamics implying exocyst especially in phragmoplast initiation and then final fusion and maturation of the new cell wall. Using co-immunoprecipitation interaction of EXO84 subunit with other exocyst subunits was proven (Fendrych et al. 2010 / ID 351017). Later in collaboration with the laboratory of Farhah Assaad at TUM, Munich, Germany we contributed to a report on a co-ordination between TRAPP and exocyst complex in Arabidopsis cytokinesis. This uncovered direct communication between these two complexes for the first time for any eukaryotic cell type (Rybak et al. 2014 / ID 429525). Both vesicle tethering complexes participate in the initiation of cytokinesis - assmebly of phragmoplast. In the expansion phase TRAPP dominates the process, while during new cell wall insertion and maturation exocyst is maximally present (Rybak et al. 2014 / ID 429525). The idea of this paper was established during the discussions at "Exocytosis in animals, fungi and plants" SEB Cell symposium 2011 in London organized by Viktor Žárský. The contribution of Laboratory of cell biology to this report was decisive - considerable part of work on exocyst part of this story was done in IEB/Prague including crucial indication for direct TRAPP-exocyst subunits interaction.

Using high resolution Total Internal Reflection Fluorescent Microscopy/Variable Angle Microscopy /TRIF/VAEM) we described for the first time (including yeast and animals) the eukaryotic exocyst complex dynamics at the cytoplasmic membrane (Fendrych et al. 2013 / ID 398129). Along with the expected proof of significant co-localization with exocytotic v-SNARE, half life and co-localization of exocyst subunits indicate, that there is also possible vesicles independent dynamics of the assembled complex at the membrane (Fendrych et al. 2013 / ID 398129).

Phenotypic deviations of our first Arabidopsis exocyst mutant exo70A1 showing reduced apical dominance and impaired root and root hairs growth (Synek et al. 2007) prompted us to look into the possible participation of exocyst in polar auxin transport regulation. Using genetics, microscopy, pharmacology and mobility of radioactive auxin assays we for the first time show and proof the importance of exocyst complex for the recycling and cellular localization of PIN auxin efflux carriers

and regulation of polar auxin transport (Drdová et al. 2013 / ID 395460; one set of radioactive IAA transport experiments was done in the laboratory of Angus Murphy at Purdue, USA).

Our efforts to study functions of exocyst using phenotypic analyses of Arabidopsis TDNA mutants and in parallel comparative analyses of ests resulted in the uncovering of an unrecognized and therefore un-annotated gene duplication of SEC10 locus (Vukasinovic et al. 2014 / ID 429536).

Important evidence for the exocyst engagement in cell wall biogenesis was gained using Arabidopsis seed germination and imbibition. In this report we have genetically and cytologically documented specific participation of vesicles tethering complex exocyst in cell wall pectins deposition on the model of seed coat maturation in Arabidopsis. New plant specific interactor of exocyst RHO1 was also described - possibly a first candidate for negative regulator of plant secretory pathway. We also described unknown developmentally regulated reduction in seed coats deposition in siliques of the first inflorescence (Kulich et al 2010 / ID 350472).

Despite the non-conspicuous journal where we published it, we consider our discovery that EXO70B1-containing exocyst subcomplex plays a role in the Golgi-independent autophagic transport into the vacuole as possibly the most important one from our laboratory over the last years (Kulich et al. 2013 / ID 421002). The exo70B1 mutant has spontaneous leaf lesions/hyper sensitive reaction due to over accumulation of salicylic acid (Kulich et al., 2013 / ID 421002). On this pathway is dependent an import of anthocyanins into the vacuole, and possibly some other molecules including salicylic acid (SA). It is possible, that autophagy related endomembrane transport is a hidden and so far neglected branch of the plant membrane trafficking (Kulich and Žárský 2014/ ID 429326).

Our report showing for the first time an active role of plant exocyst complex in defense against both bacterial and fungal pathogens points also to a close homolog - EXO70B2 (Pečenková et al., 2011/ ID 367701). Along with EXO70H1 we describe their transcriptional pathogen inducibility and demonstrate, that respective Arabidopsis mutants are significantly more sensitive to *Pseudomonas syringiae* and produce abnormal defensive papillae in response to *Blumeria graminis*. In exo70B2 mutants infected with powdery mildew, local cell wall thickenings (papillae) on the site of contact with germinating spores accumulated abnormal vesicular compartments, suggesting a defect in vesicle tethering or fusion, but without effects on the penetration efficiency of the fungus (Pečenková et al. 2011/ 367701). Working on phytopathogen interactions context of exocyst complex we discovered also a direct interaction between the SNARE protein, SNAP33 with EXO70B1 and EXO70B2 (Pečenková et al., 2011/ 367701).

In collaboration with the Marco Trujillo lab at the IPB Leibnitz inst. in Halle, Germany we contributed to the follow up discovery of EXO70B exocyst subunits as a subject to specific E3 ligase ubiquitination-dependent regulatory degradation in biotic interactions (Stegmann et al.2012 / ID 389143). Also extraordinary evolutionary dynamics of exocyst subunits, especially EXO70s - possibly driven by competition between host and parasite - indicates an involvement in the plant-microbe interactions (Cvrčková et al., 2012 / ID 441887).

In collaboration with Andrea Genre from University of Torino we contributed to the analysis of the symbiosis between plant hosts and arbuscular mycorrhizal fungi by showing participation of exocyst on the perifungal membrane biogenesis during the symbiosis establishment (Genre et al. 2012

/ ID 382534). These discoveries are also a major basis for our current research on exocyst supported by CSF.

As exocyst was discovered as an effector of RAB and RHO GTPases we are studying in our lab also some aspects of their cell biology related to the regulation of secretory pathway. RAB GTPases are post-translationally modified by Geranylgeranyl transferase. Using LOF Arabidopsis mutant in one of two beta-subunits of this transferase we contributed a first report on RAB GTPases prenylation modifying complex in plants based also on genetic analysis (Hála et al 2010 / ID 350388). Along with expected biochemical defect in betasubunit Arabidopsis mutant resulting in a agravitropic shoot, it uncovered a surprising developmental aspect of RAB function in switch between etiolation and photomorphogenesis, supported by our transcriptomic data used to interpret this result (Hála et al 2010 / ID 350388).

In collaboration with the laboratory of Attila Feher at the BRC, Szeged, Hungary (and also Antje Berken at University in Bochum) we contributed by an experiment in pollen tubes to the characterization of ROP6 activity regulation by phosphorylation (Fodor-Dunai et al. 2011 / ID 367374). An important contribution of ROP GTPases activity (along with other factors) we observed also in case of the NADPH oxidase activity regulation in pollen tubes (Potocký et al. 2012 / ID 381547).

We have also adopted over last years moss *Physcomitrella patens* as an ideal model to study exocyst functions in cell polarity and morphogenesis (supported partly by the EU project ITNPLANTORIGINS to VZ at Charles Univ.) and are preparing first data to be published (Brejšková et al. in prep.; Rawat et al. in prep.).

These exocyst and GTPases related reports were supported by our projects - 21, 22, 24, 26, 27 and 28.

Another crucial aspect of cell polarity reciprocally connected to the targeted secretion is the functioning of cytoskeleton - especially a F-actin one. Crucial F-actin nucleators in plants are formins - studied in collaboration with the group of Fatima Cvrčková at Charles University, Prague. New specific interaction domain (GOE) in plant class I F-actin nucleators formins was unexpectedly uncovered during Matyáš Fendrych visit to the laboratory of Patrick Hussey (Durham university, UK) mediating interaction with microtubular cytoskeleton (Deeks et al. 2010 / ID 350394). This new link between F-actin and microtubular cytoskeletons is crucial to understand their interaction at the endomembranes including cytoplasmic membrane, as GOE-domain formins are anchored into the endomembranes via transmembrane domain. First author Matyáš Fendrych (a shared first authorship with Michael Deeks) is a member of the Laboratory of cell biology and contributed major part of experimental work (great deal of it while visiting collaborating lab of Patrick Hussey in Durham). Viktor Žárský participated in preparing the collaborative project, planning experiments, interpreting data and writing.

Our major interest in Laboratory of Cell Biology however is the regulatory relationship between secretory pathway, membrane lipids and cytoskeleton. Between years 2010 and 2014 our laboratory in collaboration with the laboratory of Chris Staiger at Purdue made a major progress in the analysis of plant actin cytoskeleton interaction with membrane lipid phosphatidic acid (PA) and phospholipase D (PLD).

We made connection first to the interaction and regulation between F-/or G-actin and phospholipase D activity (PLD). Using pollen tube as a model the F-actin binding domain on Arabidopsis phospholipase D beta (PLD) is pinpointed and characterized. This interaction stimulates PLD activity and production of membrane lipid phosphatidic acid (PA); while interaction with monomeric G-actin inhibits PLD activity and PA production (Pleskot et al. 2010 / ID 350391). This report is an outcome of collaborative project coordinated from Laboratory of cell biology with Laboratory of signal transduction (IEB - P. Pejchar and J. Martinec) and Institute of Chemical Technology/ICT (J. Linek, Z. Novotná and O. Valentová) - most of the experimental work initiated originally at the ICT (J. Linek, Z. Novotná and O. Valentová) was done in the Laboratory of cell biology by Roman Pleskot and Martin Potocký with cloning/expression help of P.Pejchar. Based on published data on F-actin capping dimer inhibition by PA (Staiger laboratory - see further) we proposed and experimentally tested in pollen tubes a specific positive feed-back loop between F-actin and PLD activity (Pleskot et al. 2010 / ID 350391).

Using combination of experimental evidence (initiated in the lab of C. Staiger and finished in our Laboratory of cell biology) and recently developing methods of molecular dynamics computation (our Laboratory of cell biology) we report detailed insight into the molecular mechanism of heterodimeric F-actin capping protein (CP) interaction with membrane lipids phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidic acid (PA) explaining enhanced affinity of plant CP to PA (Pleskot et al. 2012 / ID 390490). This publication was initiated by the discovery of C. Staiger lab (at the Purdue Univ. USA), that plant F-actin heterodimeric capping protein (CP) has enhanced affinity to phosphatidic acid as compared to preference of animal CP for phosphatidylinositol (4,5)-bisphosphate. All of the actual work on molecular dynamics computation and finalization of point mutated proteinslipids interactions were done by our Lab of cell biology (by cloning and binding assays assisted P. Pejchar/Lab. of signal transduction IEB).

The understanding of phosphatidic acid (PA) enriched membrane domains functioning in plant cells is hampered by a lack of useful PA marker. Based on the specific PA binding protein domain of yeast SNARE protein Spo20 (from the laboratory of Nicolas Vitale at the University in Strasbourgh) we established and verified fluorescent marker for sub-population of PA in plant cells. We document specific localization of PA in subapical pollen tubes domain specifically overlapping with phosphatidylinositol (4,5)-bisphosphate (PIP2) signal at the very tip. Using this probe extremely rapid turn-over rate of PA and its function in endocytosis and cytoskeleton regulation are corroborated (Potocký et al 2014 / ID 429592). This result is almost fully outcome of the Laboratory of cell biology experimental work with the contribution of P. Pejchar from the Lab. of signal transduction IEB . Our collaborators from abroad contributed starting PA binding Spo20 domain (N. Vitale. Strasbourg Univ.) and planning and writing of the report (B.Kost, Univ. of Erlangen - part of collaborative/visiting project with M. Potocky).

The progress in this new field of PLD/PA/F-actin but also microtubuli regulation was summarized in a joint review with the laboratory of Chris Staiger (Pleskot et al. 2013 / ID 399937). This along with a report on PA turnover (Pleskot et al. 2012 / ID 380699) and PA effect on NADPH oxidase activity in pollen tubes (Potocký et al. 2012 / ID 381547) and especially recently developed computational molecular dynamics approaches (Pleskot et al. 2012 / ID 390490) opens both conceptually as well as methodologically new avenues for the analyses of membrane lipids-proteins interface functioning in cell polarity regulation not only on the pollen tube model.

Work on the membrane lipids related topics and cytoskeleton was supported by our projects 21, 23, 24, 25 and 29.

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Plant Reproduction Laboratory

The Laboratory of Plant reproduction was established in 2007, its history is therefore short. It represents a young team considering an average age of lab member (except for the head of the laboratory Helena Štorchová, who is a senior scientist). The lab is working on **two main topics** using advanced approaches of molecular biology.

(1) The first field of interest is the genomics and transcriptomics of plant mitochondria and mitochondrial-nuclear interactions including cytoplasmic male sterility (CMS).

Plant and animal mitochondrial (mt) genomes are very distinct, despite of sharing a common eubacterial ancestor. They carry a similar set of genes, but differ in structure, size, evolutionary rate, control of transcription, and even in their genetic codes. Whereas animal mt genomes are short, compact and conserved in structure, plant mt genomes are large and highly rearranged.

We selected *Silene vulgaris* (bladder campion or maiden's tear) as a study species to better understand plant mt genome structure and evolution, because it has been developed as a model for the study of gynodioecy, a plant reproduction system characterized by the co-ccurrence of females and hermaphrodites, and controlled by the interaction of mitochondrial and nuclear genes. Gynodioecy is frequent among angiosperms (about 10% of species). CMS is important in agriculture, because it can be exploited to ensure the production of hybrid seed. A suitable model, like *Silene vulgaris*, to investigate these phenomena is therefore necessary.

Initially, we revealed an extreme polymorphism in mtDNA in Central European populations of S. vulgaris. Indeed, the mt genome changes so rapidly, that differences arise between not only among full siblings, but even among the branches of the same individual. We also found that specific mtDNA haplotypes were associated with specific transcripts of the genes *atp1* and *cox1* (ID 344002: Elansary et al. BMC Plant Biology 2010). To further investigate the cause of this remarkable within-species transcript size variation, we analyzed in detail the transcription of the *atp1* gene. We found that the sequences upstream from this gene, including putative regulatory motifs, varied among mt haplotypes. We examined the *atp1* transcript in the KOV mt genome by means of RNA circularization and discovered three 5‘ ends, created by the combination of transcription initiation and RNA processing. Surprisingly, the utilization of a particular transcription start site depended on the nuclear background, which suggested the involvement of nuclear genes in transcription initiation in plant mitochondria (ID 395063: Muller and Storchova, Plant Molecular Biology 2013).

Our findigs have documented prominent differences in mt genomes at the within-species level in *S. vulgaris* and led to the development of a joint effort with Douglas R Taylor (Univ. Virginia, USA) and Daniel B Sloan (now Colorado State Univ., USA) aiming to sequence and compare several mt genomes in this species. We have previously contributed Southern hybridizations confirming

intramolecular mt genomic recombinations in *Silene latifolia* in the assembly of the first completely sequenced mt genome in the genus *Silene* (ID 350206: Sloan et al. 2010). Together with our colleagues from Virginia, we generated complete sequences of four mt genomes of *S. vulgaris* and revealed an extreme level of mt genomic rearrangements affecting not only gene order, but also gene content. This within-species mtDNAvariation is by far the highest so far described. We additionally contributed the 454 sequencing and assembly of KOV and MTV mt genomes. We also performed Southern hybridization, confirming an autonomous physical structure of the smallest subgenome predicted by bioinformatic approach (ID 384239: Sloan, Muller et al. New Phytologist 2012).

A closer inspection of mt genomic sequences identified several open reading frames (ORF), composed of the pieces of known mt genes and putatively encoding highly hydrophobic proteins. These features suggested that the ORFs might have been candidate genes for CMS. The ORF discovered in the MTV genome was identical with the previously described *Bobt* gene exhibiting a significantly higher expression in females than in hermaphrodites which is characteristic for CMS genes (ID 380745: Storchova et al. PloS ONE 2012). Interestingly, some mt genomes harbored two or three CMS candidates, in contrast to KOV which contained none, despite a clear indication of CMS. In conclusion, *S. vulgaris* represents an ideal plant to study mitochondrial-nuclear interactions owing to its enormous variation in mt genomes associated with gene losses, rearrangements and with the origin of novel genes. Whether or not such mt diversity is mirrorred by nuclear genomes, represents a topic of current and future research.

With five distinct completely sequenced mt genomes of *S. vulgaris* available, we are now adopting a transcriptomic approach to analyze global transcript profiles and editing events (conversion of C to U in RNA) in male sterile and male fertile plants. We would like to know modes of gene expression across entire mt genomes and their modifications in distinct nuclear backgrounds. Finally, we aim to understand the control of mt trancription in various tissues and genders and the roles of nuclear genes involved in male fertility. *Silene vulgaris* research was supported by the following grants (2-4) GA521/09/0261 Dynamic changes of mitochondrial DNA and transcription profiles in *Silene vulgaris*, (4-4) ME09035 The molecular genetic analysis of the candidate genes responsible for cytoplasmic male sterility in *Silene vulgaris*, and (1-4) LC06004 Integration of research activities to study the plant genome.

(2) Our second interest is the floral induction and the evolution of flowering-related genes in Chenopodium (goosefoot)

Unlike *S. vulgaris*, a recent addition to our lab, *Chenopodium rubrum* has been a traditional model plant in our institute for decades and detailed experimental protocols of its floral induction have long been elaborated. *C. rubrum* is a short-day weedy plant which may be induced to flowering by a single period of darkness at seedling stage with two cotyledons and no true leaves. This feature makes possible to achieve synchronous growth and to study the regulatory genes participating in a signaling pathway, which is less complex than in other models such as *Arabidopsis* or rice.

The proper timing of flowering is an essential requirement for plant survival and adaptation. It is triggered by the *FLOWERING LOCUS T LIKE1 (CrFTL1)* gene in *C. rubrum*, the homolog of the *FT* gene of *Arabidopsis* (Cháb et al. Planta 2008). We confirmed the florigenic function of CrFTL1 by the complementation of ft mutants in *Arabidopsis*. The *CrFTL1* gene is expressed during the day. A dark-light transition following a permissive period of darkness is necessary for its activation. This

requirement is unique among investigated short-day plants, which express *FT* homolog at night and do not need a dark-light transition for its activation (ID 429593: Drabešová et al. Journal of Experimental Botany 2014).

FTL genes serving as key inducers of flowering have been so far discovered in each angiosperm species under study. They underwent duplications in the course of plant evolution and the paralogous copies often obtained a novel function. For example, the *BvFT1* paralog is a floral inhibitor in sugar beet, which belongs to the same family Amaranthaceae as *Chenopodium*. We had discovered the second copy of *FTL* in *C. rubrum* (*CrFTL2*- Cháb et al. 2008) two years before Pin et al. (2010) published their study on sugar beet BvFT1 and BvFT2. The *CrFTL2* and *BvFT1* are the homologs and arose by the duplication occurring before or in the course of the origin of the family Amarantaceae. Unfortunately, Pin et al. (2010) chose the number 1 (*BvFT1*) for the sugar beet homolog of *CrFTL2*, which leads to the problems with names of newly discovered orthologous genes. Our study was the first, so we continue to use the number 2 to *CrFTL2* homologs. The function of CrFTL2 is so far enigmatic. It is not a pseudogene, because its transcript level is high. *CrFTL2* shows an accelerated evolution rate and its expression pattern as well as overexpression in Arabidopsis are not consistent with any function in the process of flower induction (ID 429593: Drabešová et al. Journal of Experimental Botany 2014). This research was supported by the grant (6-4) GAP506/12/1359 The control of flowering in *Chenopodium* investigated by the transcriptomic approach.

We noticed that the third intron of the *FTL1* and *FTL2* genes was a sensitive genetic marker capable of resolving complicated phylogenetic relationships among closely related *Chenopodium* species. We used these *FTL* introns to solve the origin of quinoa (*C. quinoa*), an important crop of South America. Quinoa is allotetraploid, but only one parent (related to recent American *Chenopodium* diploids) was known. We found that the second parent of quinoa was related to *C. suecicum* or *C. ficifolium*, which are recently distributed in Europe and Asia. Most likely, their relative grew in America in the past. We are collaborating with Brigham Young University in Provo (USA) on quinoa research. The BYU colleagues confirmed our conclusion with three additional markers, published independently. Our paper on the origin of quinoa was accepted in November 2014, but it will be published in 2015 (Storchova et al. Genetic Resources and Crop Evolution 2015). We are now continuing to apply *FTL* markers in a broad taxonomic survey of *Chenopodium* in collaboratin with the Institute of Botany CAS in Pruhonice (the joint project (7-4) GA13-02290S The role of hybridization and polyploidization on the evolution in *Chenopodium album* aggregate: From biosystematics to gene expression).

During the last three years we have constructed the reference transcriptome of *C. rubrum* based on 454 sequencing and Illumina HiSeq. We adopted bioinformatic tools in transcriptomic assembly, most recently the Evigene pipeline. We are currently analyzing differential gene expression to identify additional genes involved in floral induction in *C. rubrum*.

(3) Collaborative research

Our laboratory supports other teams at IEB to adopt the methods of molecular biology in plant research. Helena Storchova has mainly collaborated with Radka Vankova (Laboratory of Hormonal Regulations in Plants) and designed RT qPCR essays to estimate the expression of cytokinin-related genes (ID 365268: Dobrá et al. 2011; ID399220: Macková et al. 2014). She also helped to adapt qPCR for the study of mycorrhiza in the Institute of Botany CAS in Pruhonice (ID 381085: Krak et al. 2012; ID

397604 Janoušková et al. 2013) in frame of the joint project (3-4) GA526/09/0838 Coexistence of native and inoculated arbuscular mycorrhizal fungi in the roots of host plants.

Recently, lab members introduced a transcriptomic approach at the University of South Bohemia in České Budějovice in the course of collaborative research on aquatic rootless plant *Utricularia vulgaris* (the manuscript was accepted in December 2014, but published this year – Bárta et al. BMC Plant Biology 2015). This collaboration was supported by the project (5-4) GAP504/11/0783 Hunters or gardeners? Probing plant-microbe interactions in rootless carnivorous Utricularia from a transcriptomic perspective.

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Virology and Laboratory of Stress Physiology

The team consists of two research units, one studying the interaction of plants with viruses while the second unit interactions with abiotic stressors. At the end of 2012 the laboratory moved to new premises of the institute with state of the art lab-space and most importantly equipped with the adequate plant growth facility. During the evaluation period Team 5 solved projects no. 37-41.

The first unit – the **Laboratory of Virology** contributed to the understanding and management of plant viral diseases and to development of new and innovative ways to control them. Main focus was on the biotechnological use of plant viruses, especially on transient expression of proteins of therapeutic value.

Laboratory of Virology reported the strategy for production of polyclonal antibodies against both structural and non-structural recombinant proteins of potato-infecting viruses. Recombinant viral proteins expressed in bacteria showed interesting potential as an alternative source of antigens for raising specific antibodies to plant viruses, which can be produced in large quantities and can be adapted for specific applications. The obtained polyclonal antibodies could be used not only for virus detection but also for the study of viral replication cycle (Cerovska et al., J. Phytopatol., 2010 – ASEP ID 347697; Plchová et al., J. Phytopatol., 2011 - ASEP ID 364101 and Cerovska et al., J. Phytopatol., 2012 - ASEP ID 380694; project no. 37). In cooperation with the Potato Breeding Institute in Havlickuv Brod we have developed transgenic potato cultivars with increased resistance to Potato Leafroll Virus (PLRV) and set of primers for quantitative detection of viral load in infected material (industrial designs CZ 23384, CZ 23383 (ASEP ID 383245 and 383248; project no. 38).

Biosafe and efficient transient gene expression in plants could give a fast, flexible and reproducible technique for high-level production of useful proteins. In these projects we focused on the expression of the heterologous proteins in plant host cells at quantities sufficient for practical use. Particularly we have explored possibility to transiently express epitopes of *Human papillomavirus* type 16 (HPV-16) to prepare experimental vaccine against HPV-16. *Human papillomavirus* type-16 (HPV-16) along with other high risk papillomaviruses are responsible for approximately half a million new cervical cancer cases every year. Current vaccines provide limited cross-protection between various HPV types and could only be used as prophylactic treatment prior to the HPV infection. Experimental vaccines are being developed to address either one or both of these issues.

For the transient expression of HPV-16 antigens we used viral vector based on *Potato virus X* (PVX). It has been shown that coat protein (CP) of plant viruses represents an ideal, highly ordered, multivalent scaffold to be used for the display of immunogenic epitopes. So far the PVX based vector has been utilized in two ways – (1) for the production of non-fused fulllength proteins/peptides or (2) for proteins/peptides fused to the N-terminus of PVX-CP and their presentation on the surface of viral

particles. In our work we have used both well established means for epitope presentation as well as novel strategies developed in our team.

We have successfully expressed $L2_{108-120}$-epitope derived from minor capsid protein HPV-16 L2 fused to the N-terminus of PVX CP in plants. The PVX chimeric particles displaying immunoreactive epitopes moved systemically in experimental plants and achieved high yields (Cerovska at al., J. Biosci., 2012 - ASEP ID 380685; project no. 39).

Also C-terminus PVX CP was used for peptide presentation for the first time. Mutagenized E7 oncoprotein (E7ggg) from HPV-16 was fused to the C-terminus of PVX CP and successfully expressed in plants (Plchová at al., Prot. Exp. Purif., 2011 - ASEP ID 364095; project no. 39).

The same approach was used for expression of HPV-16 $L2_{108-120}$ epitope on the C-terminus of PVX CP (Cerovska at al., J. Biosci., 2012- ASEP ID 380685; Hoffmeisterova et al., Biol. Plant 2012- ASEP ID 381055; project no. 39). To display another HPV-16 antigens on PVX particles mutagenized oncoprotein E6 (E6GT) was transiently expressed as C-terminal fusion with PVX CP using the same system previously designed for E7ggg. The chimeric protein accumulated in inoculated leaves of *N. benthamiana* plants and formed viral particles (Cerovska et al., PCTOC, 2013 - ASEP ID 396719; project no. 39 and 41).

To obtain more reliable system for antigens presentation on the surface of viral particles for experimental vaccine development we started with expression system improvement by searching for new fusion positions located within PVX CP. Based on spatial structure model of the PVX CP subunit in a virion we inserted HPV E7 epitope (aa 44-60) together with 6xHis or StrepII tag into different putative surface exposed loops (Plchova, PCTOC, in press).

Furthermore, we determined whether PVX induces DNA damage in nuclei, evaluated by the comet assay, and mutations on leaves, evaluated by the somatic mutation assay, in PVX inoculated tobacco leaves (Cerovska et al., Biol. Plant., 2014 – ASEP ID 438179; project no. 41).

We have also developed a novel virus vector based expression platform, which can be used in plant molecular farming. The system is based on in planta encapsidation of defective plant virus by coat protein expressed in trans. It has the advantage that the production plants can be efficiently inoculated without the use of Agrobacterium and at the same time the virus cannot be further transmitted by secondary infection. This system has been described in Czech patent application CZ201100843-A3.

In cooperation with the scientist at the Danforth Plant Science Center and USDA, St. Louis USA we have successfully developed a system for controlled and tissues specific protein expression in soybean seed (Semenyuk et al., Transgenic Res., 2010 - ASEP ID 348486).

The research of our second unit, the **Laboratory Stress Physiology**, was focused on two major areas: nitrosative and oxidative stress and antioxidant defence either during ageing or in stress response. In the first area of investigation we have published the first summary study of nitric oxide and reactive nitrogen species (RNS) metabolism during natural and stress induced leaf senescence (project no. 40). Contrary to the majority of data concerning NO metabolism that do not consider nitrogen from the point of view of a macronutrient, we followed also the ratio of individual compounds of nitrogen during ageing and senescence (Pavlikova et al., J. Plant Physiol. 2014 - ASEP ID 435451).

NO metabolism was also related to elevated cytokinin (CK) levels in leaves. Enhanced CK did not affect a decrease in NO content with leaf age. Simultaneously also nitrate reductase activity declined even if this is not probably the crucial source of NO. On the other hand significant increase of nitrated protein tyrosine was observed with leaf age what represents elevated nitrosative stress and CK mitigated nitrosative stress. Here, detected higher activity of nitrosoglutathione reductase could prevent NO production from Nitrosoglutathione (Prochazkova et al., Plant Soil Env. 2012 - ASEP ID 381458).

Beside the natural leaf senescence it was induced by Zn contamination as zinc is the element playing important role in nitrogen utilization. Zn caused increased nitrosative stress, NO content elevation was apparent in younger leaves and CK promoted this phenomenon. Leaves with increased CK were more resistant to Zn stress reflected in lower declines of photosynthetic and transpiration rates. Further this tolerance was associated with maintenance of accumulation of free amino acids playing role in stress adaptation i.e. methionine and γ- aminobutyrate. On the other hand asparagine, major compound associated with senescence was kept lower in leaves with enhanced CK. (Pavlikova et al., J. Plant Physiol. 2014 - ASEP ID 435451; Ecotoxicol. Environ. Safety 2014 - ASEP ID 429992)

DNA damage (single and double strand breaks, alkali labile sites, incomplete excision repair sites and DNA cross-links) was detected during both in natural and stress induced senescence by the Comet or Single cell gel electrophoresis assay. We succeeded in the application and modification of the Comet assay in plant senescence research (Prochazkova et al., Biol. Plant. 2013 - ASEP ID 399938).

The second branch of our research was focused on antioxidant defence in tobacco with CK depletion either during ageing or in stress response. Tobacco overexpressing CKX1 were exposed to drought and/or heat shock. Effect of down regulation of CK levels on antioxidant enzymes differs in transgenic plants with different promoters and may be mediated rather by the altered habit, resulting in improved stress resistance. This is associated with diminished stress impact on photosynthesis and changes in source/sink relations. (Lubovska et al., J. Plant Physiol. 2014 - ASEP ID 433743; Mackova et al., J. Exp. Bot. 2013 - ASEP ID 399220).

Similarly, we noticed higher tolerance also in tobacco with inserted CKX2 gene subjected to drought stress. In these plants CK depletion is partially alleviated by increased antioxidant enzymes i.e. glutathione reductase (GR) in leaves, as well as ascorbate peroxidase (APX) and superoxide dismutase (SOD) in roots. (Biol. Plant. 2010 - ASEP ID 348435). Although CKs inhibit ageing, we found that transgenic tobacco with CK depletion (CKX2) surprisingly aged more slowly than WT. We revealed that such transgenes have more effective antioxidant system on level of total activity (GR) and higher activity of SOD and APX particularly in old leaves as well as of certain isoenzymes (SOD, APX) (Mytinova et al., Plant Growth Regul. 2011 - ASEP ID 365377).

We also participated in project with Faculty of Science, Charles University, focused on explaining different tolerance of maize genotypes to drought stress. In short term drought stress the tolerant maize genotype maintains opened stomata thus photosynthesis can continue operating. This enables, among others, proteosynthesis of protective proteins. The number and level of these compounds were lower in sensitive genotype. Such risky strategy can contribute to drought tolerance. Here we also studied differences in antioxidant capacity on the level of activities (Benesova et al., PLoS ONE 2012 - ASEP ID 382487)

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Pollen Biology

The Laboratory of Pollen Biology IEB has been established in the 1990's since then we have continuously dedicated our research to the fundamental research of plant reproductive development, sexual plant reproduction and genome stability. In this area, our laboratory is internationally recognized and we have performed research leading to several pioneering and highly cited publications, namely our priority results on pollen developmental transcriptomics (in a broader sense the first study of effectively single-cell global gene expression profiling and its developmental dynamics in plants). These results also contributed to the establishment of the current paradigm in male gametophyte research towards asking and answering specific gene-oriented questions. Moreover, novel strategies to manipulate the gametophyte development and function are of current interest in agriculture and breeding. This is related to the introduction of new model species like Physcomitrella, hops and tomato. The former Laboratory of Molecular Farming and DNA Repair joined in 2013 the Laboratory of Pollen Biology as a self-reliant Group of DNA Repair. The group continued on developing new approaches to study DNA damage and cell response to it as DNA repair of afflicted DNA lesions and possible consequences like induced mutations.

Laboratory of Pollen Biology consists of three groups that are closely interconnected as documented by joint participation on grants and publications. In the evaluated period, the research of the Laboratory of Pollen Biology was funded by 16 projects (projects 42-57; Appendix 3-1; team number 6; total funding in 2010-2014 1,589 M€) awarded by the Czech Science Foundation (9 projects) and the Ministry of Education, Youth and Sports of the Czech Republic (7 projects) as well as in the Management Committees of several COST Actions supported by European Science Foundation-COST. On top of this, A. Gibalová was a PI in one more MEYS-financed project (COST) with the Charles University affiliation.

In the years 2010-2014 we extended our previous activities and our research was focused mainly on several aspects of pollen development, pollen communication with female tissues and genome stability. Since our research represents a continuous effort, here we sometimes refer also to our results and activities before and after the evaluation period.

1) Regulation of Arabidopsis pollen development

Screen and functional analyses of male gametophytic transcription factors

Male gametophyte development leading to the formation of a mature pollen grain is precisely controlled at various levels, including transcriptional, post-transcriptional and posttranslational, during its whole progression. The pollen development thus represents a fragile and vital phase of plant ontogenesis and pollen was among the first singular plant tissues thoroughly characterized at the transcriptomic level (Honys and Twell 2003, Plant Physiol). Arabidopsis pollen developmental

transcriptome has been published over a decade ago revealing the uniqueness of pollen transcriptome and the dynamics of early and late successive global gene expression programs (Honys and Twell 2004, Genome Biol).

In the evaluation period, this first field of interest has been supported by five projects (4x CSF – GA525/09/0994 (no. 45, Honys), GA522/09/0858 (no. 46, Honys), GAP305/12/2611 (no. 50, Honys) and GP13-41444P (no. 53, Dupľáková); 1x MEYS – OC10054 (no. 47, Honys)) and on top of their outcomes it resulted in one defended master thesis (Pičmanová) and two defended doctoral theses (Dupľáková, Reňák). Two more doctoral students (Šolcová, Gibalová) still work on their theses falling into this field.

In the follow-up projects, we focused on the identification of **pollen-expressed transcription factors** (TF) involved in the regulation of male gametophyte development. They were identified by thorough screening of 74 T-DNA insertion lines representing 49 genes of 21 TF families active in either early or late pollen development. 29 screened lines showed strong phenotypic changes (i.e., ≥ 25% aberrant pollen) including four lines that produced a remarkably high proportion (70-100%) of disturbed pollen. Our results served as a basal information resource for future functional characterization of specific TFs in male gametophyte development (Reňák et al. 2012, Sex Plant Reprod, ID 379285; N. Dupľáková – defended doctoral thesis; K. Šolcová – ongoing doctoral study). The article Reňák et al. (2012) was fully prepared and written in our laboratory. This phenotype screen was partly enabled by the optimization of our previously published protocol for large-scale separation of developing spores that is currently being published (Dupľáková, Reňák and Honys – manuscript in revision for the resubmission in Nature Protocols).

The expertise in transcriptomics and molecular techniques was exploited jointly with O. Lapčík (University of Chemistry an Technology, Prague) to study the biosynthesis and distribution of **plant secondary metabolites, isoflavonoids** (Pičmanová et al. 2013, Biol Plant, ID 420942; M. Pičmanová – defended master thesis) that are involved in plant defence mechanisms. The article Pičmanová et al. (2013) was fully prepared and written in our laboratory with the sole exception of HPLS analysis that was done by O. Lapčík's group.

One of the selected TF was early male gametophytic gene **AtREN1**, a close homolog of HSFA5 gene, a member of the **heat shock transcription factor** (HSF) gene family. Although HSFs are involved in multiple aspects of stress response and the generative phase is the most heat stress-sensitive, their role in male gametophyte development is largely unknown. The *atren1* mutation causes multiple defects in male gametophyte development in both structure and function including defective pollen heat stress response, pollen phenotype abnormalities and pollen germination defects associated with the limited transmission via male gametophyte. We localized the AtREN1 protein specifically to the nucleolus that suggests its likely involvement in ribosomal RNA biogenesis therefore linking heat stress response with translation (Reňák et al. 2014, Plant Cell Environ, ID 429991; D. Reňák – defended doctoral thesis). The article was fully prepared and written in our laboratory.

We further functionally analysed the **regulatory network of bZIP transcription factors** in long-term collaboration with D. Twell, University of Leicester, UK. That extended our previous study of AtbZIP34 (Gibalová et al. 2009, Plant Mol Biol) by the inclusion of its interactors AtbZIP18, AtbZIP52 (Gibalová, Honys et al., manuscript in preparation; A. Gibalová – ongoing doctoral study).

The role of auxin in pollen development

Auxin is a key coordinative signal required for many aspects of plant development and its levels are controlled by auxin metabolism and intercellular auxin transport. Within the multilateral network lead by J. Friml (then Ghent University, Belgium, now IST Austria), we found that the non-canonical member of PIN auxin transporter family, **PIN8** was active in Arabidopsis pollen and played a crucial role **in pollen development** and function by **regulating auxin homoeostasis and metabolism**. Unexpectedly, PIN8 co-localized with another pollen-expressed protein, PIN5 to the endoplasmic reticulum (ER) where it acted as an auxin transporter and we shown an antagonistic action of PIN5 and PIN8 in the fine modulation of intracellular auxin levels. Our results revealed a role of the auxin transport in male gametophyte development in which the distinct actions of ER-localized PIN transporters maintained the auxin levels optimal for pollen development and pollen tube growth (Ding et al. 2012, Nature Commun, ID 380680). Within this joint effort, our role was to provide the pollen expertise. We were responsible for numerous analyses of wild type and single (*pin5* and *pin8*) and double (*pin5/pin8*) mutant pollen. These included phenotyping (bright field and fluorescent microscopy), transmission electron microscopy, *in vitro* pollen germination assays, auxin treatment of pollen in a culture and its impact on pollen germination and pollen tube growth. Our results of double mutant functional tests were also the first indication of the possible antagonistic role of PIN8 and PIN5 on the ER. The success of this publication contributed to our decision to continue in this subject area with "Dupľáková, Reňák and Honys manuscript in revision for the resubmission in Nature Protocols" being the first outcome.

2) Tobacco pollen as a bicellular model for –omic studies

The majority of flowering plants produce bicellular pollen. The two cells of the pollen grain are destined for separate fates in the male gametophyte, which provides a unique opportunity to study genetic interactions that govern guided single-cell polar expansion of the growing pollen tube and the coordinated control of germ cell division and sperm cell fate specification.

In the period 2010-2014, this second and more recently launched field of interest has been supported by six projects (3x CSF – GPP501/11/P321 (no. 48, Hafidh), GAP501/11/1462 (no. 49, Honys) and GA14-32292 (no. 56, Hafidh); 3x MEYS – OC08011 (no. 44, Honys), LD13049 (no. 55, Honys) and LD14109 (no. 57, Hafidh)) and on top of their outcomes it resulted in one defended bachelor thesis (Fíla) and one defended master thesis (Fíla). Two more bachelor (Darivčák, Linhart) and three doctoral (Breznenová, Fíla, Bokvaj) students still work on their theses falling into this field.

Tobacco pollen developmental transcriptomics and translatomics

We applied the Agilent 44K tobacco gene chip to conduct the first comprehensive **developmental transcriptomic analysis of the tobacco male gametophyte** representing the first plant species shedding bicellular pollen (Hafidh et al. 2012, BMC Plant Biol, ID 382528; Hafidh et al. 2012, Plant Signal Behav, ID 391243; Bokvaj et al. 2015, Genomics Data – published online in 2014 but appeared in Jan 2015 number; P. Bokvaj – ongoing doctoral study). All three articles were fully prepared and written in our laboratory. These transcriptomic data sets presented a benchmark for future functional studies using developing pollen as a model. In addition, we performed a comparative study of the Arabidopsis root-hair trichoblast transcriptome to evaluate genetic factors and common genes and regulatory pathways involved in polarized cell-tip expansion. Reverse genetic analysis of

selected candidates demonstrated that Cu/Zn superoxide dismutase 1 (CSD1), a WD-40 containing protein (BP130384), and Replication factor C1 (NtRFC1) were among the central regulators of pollen-tube tip growth. Extension of our analysis beyond the second haploid mitosis enabled identification of an opposing-dynamic accumulation of core regulators of cell proliferation and cell fate determinants in accordance with the progression of the germ cell cycle. Our results demonstrated previously unknown functions of certain genes in pollen-tube tip growth. In addition, we highlighted the molecular dynamics of core cell-cycle regulators in the male gametophyte and postulated the first genetic model to account for the differential timing of spermatogenesis among angiosperms and its coordination with female gametogenesis. (Hafidh et al. 2012, BMC Plant Biol, ID 382528). We further showed the stable and even slightly increasing complexity of tobacco male gametophyte transcriptome over long period of progamic phase-24 h of pollen tube growth. We demonstrated the ongoing transcription activity and specific transcript accumulation in post-pollen mitosis II pollen tubes cultivated in vitro. In all, we have identified 320 genes that were newly transcribed as late as at least after 4h of pollen tube cultivation in vitro. This represented the first evidence for such late transcriptional activity in pollen tubes (Hafidh et al. 2012, Plant Signal Behav, ID 391243). Comparison with tobacco pollen tube proteome even revealed that most of these transcripts were not translated (joint effort with Z. Zdráhal, CEITEC MU Brno, manuscript in preparation) that highlighted them as the likely candidates for paternal complement to postfertilization events (K. Breznenová – ongoing doctoral study, unpublished results). As pollen tube growth and competition of pollen tubes in female pistil can be viewed as a race of the fittest, there is an apparent evolutionary trend among higher plants to store large material reserves and nutrients during pollen maturation. This supply ensures that after pollen germination, the pollen tube utilizes its resource predominantly for its rapid elongation in the female pistil. Previous transcriptomic data from Arabidopsis showed massive expression of genes encoding proteins forming both ribosomal subunits that were accumulated in developing pollen, whereas their expression was not detectable in growing pollen tubes (Honys and Twell, 2004). We observed a similar phenomenon in less advanced bicellular tobacco pollen (Bokvaj et al. 2015, Genomics Data).

Recently, we have initiated completely new direction of our research, tobacco **pollen translatomics**. This research was inspired by the discovery and preliminary proteomic analyses of large ribonucleoprotein particles (termed EPP granules) in tobacco male gametophyte that was published just before the start of this evaluation period (Honys et al. 2009, J Proteome Res 8: 2015-2031). It has been well established that both transcription and translation play an important role in global and specific gene expression patterns during pollen maturation. On the contrary, germination of many pollen species has been shown to be largely independent of transcription but vitally dependent on translation of stored mRNAs. We demonstrated that **EPP granules** were formed in immature pollen where they contain translationally silent mRNAs and then served as a **long-term storage of mRNA** transported along with the translational machinery to the tip region where the translation took place (Honys et al. 2009, J Proteome Res 8: 2015-2031). Such an organization is extremely useful in fast tip-growing pollen tube. Moreover, the asymmetric mRNA distribution is the determinant of protein gradient influencing cell polarity, cell fate and overall patterning during development. Following these findings on the unique mechanism of RNP formation in tobacco male gametophyte, we proposed a model outlining the network of posttranscriptional control with a focus on the role of stored RNPs (Hafidh et al. 2011, Adv Exp Med Biol, ID 380676; Hafidh et al. 2013, J Plant Biochem Physiol, ID 440716) and started the functional characterization of RNA-binding proteins (collaboration with C. Bousquet-

Antonelli, INRA Perpignan, France, manuscript in preparation). To experimentally validate this model, we have extended our transcriptomic and proteomic analyses to cover three cytoplasmic subfractions containing mRNAs at different translational status and to demonstrate their developmental dynamics – 1) actively translated transcripts associated with **polysomes** (PS - termed translatome), 2) pollen mRNAs bound to pollen **stored ribonucleoprotein particles** (stored mRNPs/free mRNPs - termed **mRNPome**) and 3) long-term stored transcripts on **EPP granules** (EPPs - termed **sequestrome**. The first comprehensive results clearly demonstrating the importance of translational regulation in the male gametophyte are currently being prepared for the publication - Hafidh, Potěšil, Zdráhal and Honys, manuscript in prep. These results will also be included in two ongoing bachelor theses (P. Darivčák and F. Linhart).

Tobacco pollen developmental phosphoproteomics

Rapid changes of protein phosphorylation play a crucial role in the regulation of many cellular processes. Being post-translationally modified, phosphoproteins are often present in low abundance and tend to co-exist with their unphosphorylated isoforms within the cell. Therefore we first developed the **protein extraction protocol** suitable for subsequent phosphoprotein enrichment from tough tobacco pollen tissue (Fíla et al. 2011, Biol Plant, ID 366403) and selected the appropriate **phosphopeptide enrichment procedure** (MOAC) including the general review of phosphoprotein and phosphopeptide enrichment protocols (Fíla and Honys 2012, Amino Acids, ID 366394; J. Fíla - defended bachelor thesis). We hypothesized that the transition between quiescent mature and the metabolically active germinating pollen grain involved changes in protein phosphorylation. Therefore we have used metal oxide/hydroxide affinity chromatography (MOAC) based on an aluminium hydroxide matrix to generate a population of phosphoprotein candidates from both mature and *in vitro* activated tobacco pollen grains. Both in-gel and gel-free methods allied to MS were applied to identify a set of 139 phosphoprotein candidates, some of them were validated by in vitro phosphorylation including the detection of 52 phosphorylation sites. As a joint effort with Dr. H.-P. Mock's (IPK Gatersleben) and Dr. R. Zahedi's (ISAS Dortmund) groups, we showed for the first time the **dynamics of protein phosphorylation and dephosphorylation** associated with early stages of pollen germination. (Fíla et al. 2012, Proteomics, ID 384621; J. Fíla – defended master thesis). In this work, we designed the experimental design, performed the whole experimental work excluding the actual LC-MS/MS peptide identification and prepared the manuscript.

The published dataset was later used for the comparative study with the Arabidopsis mature pollen phosphoproteome (published by Mayank et al. 2012, Plant J 72: 89-101). The representation of the O-phosphorylated amino acids was evaluated between these two datasets, and the putative pollen-specific or pollen-abundant phosphopeptides were highlighted. Finally, the phosphorylation sites common for both Arabidopsis and tobacco phosphoproteins were listed as well as the phosphorylation motifs identified. (Fíla et al. 2014, Biochem Soc Trans, ID 429936). The article was fully prepared and written in our laboratory. In collaboration with H.-P. Mock's (IPK Gatersleben, Germany) and R. Zahedi's (ISAS Dortmund,Germany) groups, we finely tuned this analysis to three time points - mature pollen, 5-min and 30-min-activated pollen. We identified 471 phosphopeptides (301 phosphoproteins) carrying 432 phosphorylation sites, position of which was exactly matched by mass spectrometry. The majority of differentially phosphorylated proteins fell into GO categories clearly connected to pollen activation with the re-initiation of transcription and translation. The quantitative data highlighted the regulatory trends; we showed that several phosphopeptides representing the

same phosphoprotein underwent different regulation, which pinpointed the **complexity and dynamics of protein phosphorylation** at the initiation of the **progamic phase**. Collectively, we showed the first phosphoproteomics data on activated pollen where the position of the respective phosphorylation sites was clearly demonstrated. (Fíla et al. – manuscript submitted to Mol Cell Proteomics; J. Fíla – ongoing doctoral study)

Tobacco secretomics

Our discovery of transcript storage phenomena during pollen maturation and progamic phase, lead to the hypothesis that some of the stored mRNAs encode for secreted proteins required for male-female signalling during pollen tube guidance. Therefore to understand the spectrum of translational regulation and mRNA storage, we studied **pollen tube secretomics** as "bottom-up" approach to link with our sequestrome transcriptome. It is established that the journey undertaken by the pollen tube in angiosperms to reach the deeply embedded female gametophyte for fertilization involves persistent guidance by the female gametophyte and accurate perception of the signals by the pollen tube. Several ovule-secreted peptides have been identified. Nevertheless, there are no exact findings on how these signals are perceived by the pollen tube. As a novel approach, we have improvised a modified SIV (**semi-*in vivo*) technique**, SIV-PS (SIV pollen tube secretome) in collaboration with M. Johnson, Brown University, USA and R. Palanivelu, Univ. of Arizona, USA. As a joint effort with Z. Zdráhal's group (CEITEC MU, Brno), we performed gel-free LC-MS/MS for highthroughput analysis of pollen-tube-secreted proteins. Our approach has led to the identification of over 341 protein groups on average (801 accessions). Among them are **pollen tube-secreted ligands and receptor proteins** representing potential male components in perceiving ovule-emitted cues for guidance (Hafidh et al. 2014 Biochem Soc Trans, ID 429937). Primarily proteins of ≤30kDa (60%) of which 40% were ≤20kDa dominated the pollen tube secretome. They included Plant defensin subfamily, Cysteine-rich, LORELEI-like GPI-anchored 3 (LLG3), Thionin-like protein, RNAses, lipid transfer proteins (LTPs), pollen Ole-e-allergen, arabinogalactants, pectinases and invertases. The pollen tube secretome comprised vastly of non-classical type of secreted proteins. Intriguingly, we discovered that TCTP1, a **non-classically-secreted protein hijacked the classical secretory pathway** and co-localized with nanovesicles exosome marker Ole-e-1. This follow-up study has uncovered novel pistil-dependent pollen tube-secreted proteins critical for establishing **male-female signalling** interaction map for successful sperm cells delivery and fertilization and as means to overcome interspecies pre-zygotic barriers (Hafidh et al. manuscript ready to be submitted to Nature Communications). The link between pollen tube sequestrome with the secretome is currently evaluated.

3) DNA repair and chromosome maintenance

In the evaluation period, this third field of interest has been supported by five projects (2x CSF – GA13-06595S (no. 51, Angelis) and GP13-06943S (no. 52, Honys); 3x MEYS – 1M0505 (no. 42, Angelis), LC06004 (no. 43, Angelis, Honys) and LD13006 (no. 54, Angelis)) and on top of their outcomes it resulted in one defended bachelor thesis (Náprstková). Two more master (Vágnerová, Náprstková) and two doctoral (Holá, Kozák) students still work on their theses falling into this field.

The Group of DNA Repair is world-wide renowned for developing and use of microscopic based electrophoretic analysis of single cell genomic DNA damage – "**Comet assay**" in plants with modifications enabling **detection of specific DNA lesions** as single (SSB) and double (DSB) strand breaks, DNA-DNA and DNA-protein crosslinks and specific base modifications as oxidative damage or

formation of UV photoproducts CPDs. This methodological advantage of direct measurement of DNA damage (contrary to widely used indirect methods relaying on detection of transient or end products of cell response to damage like histone modifications e.g. γ-H2AX) enabled to established picture of overall two phase DSB repair kinetic with extremely rapid (so far unrecognized) first phase, which depends on Structure Maintenance of Chromosome (SMC) proteins particularly on complex SMC5/6. SMC protein complexes form heteroduplexes that can bind sister chromatid for various purposes as SMC1/3 – chromosome cohesion or SMC2/4 chromosome condensation. Our first observation and description of novel DSB repair pathway in Arabidopsis and the important role of SMC5/6 (Kozák et al. 2009, DNA-Repair (Amsterdam); Kozák – ongoing work on doctoral thesis) was further investigated during 2010-2014 period.

We scouted for new genes thought to be potentially involved in DSB repair to prove their participation by affecting kinetic profile. Firstly in cooperation with G. Böhmdorfer of GMI, Vienna, we studied and described for the first time involvement of **SMCHD (proteins** containing „Hinge" region of SMC proteins) protein GMI1 in recombination and DSB repair (Böhmdorfer et al. 2011, Plant J, ID 365270). In this work, K.J. Angelis and J. Kozák contributed with comet assay data. Only in last years became recognized multiple roles of SMCHD proteins, and particularly of „Hinge" region not only in epigenetic regulation, but also in DNA recombination and repair. In cooperation with J. da Costa-Nunes, Univ. of Lisboa we continued on deciphering the role of **RAD21 kleisins of SMC1/3 complex** and found out the sequential circularization of cohesin SMC1/3 complex firstly by RAD21.1 followed by induced RAD21.3, which return after completion of DSB repair to quiescence RAD21.1 circularization (da Costa-Nunes et al. 2014, BMC Plant Biology, ID 440714). K.J. Angelis and J. Kozák analyzed DSB repair kinetics of single and double mutants with a comet assay and provided the crucial data for explanation of sequential action of both alleles of RAD21 and for eventual switch to another DSB repair pathway in their absence. To ascertain the mechanism of procession of uneven DSB ends we investigated possible role of POLΛ in partial filling of gaps in cooperation with T. Furukawa and A.B. Britt, UC Davis (currently reviewed in Frontiers in Plant Science, section Plant Physiology for publication).

The hurdles of using Arabidopsis model plant for DNA damage and repair study led us to acquire moss **Physcomitrella patens** we **used for the production of recombinant antibodies** (ID 371127). Physcomitrella besides uniquely high homologous recombination rates pose other advantages as haploid gametophyte growing in early stages in filaments (Šmídková et al. 2010, Biol Plant, ASEP ID 370792). This enables by shearing to initiate culture of filament fragments 3-5 cells long, where up to 50% of apical cells are dividing. When compared to older culture one can distinguish processes in dividing vs. differentiated tissue. We described this approach when we in cooperation with A. Cuming, CPS, Univ. of Leeds, F. Nogue, INRA Versailles and D. Scheafer, Univ. de Neuchâtel studied moss mutants of essential DSB repair MRN (MRE11, RAD50 and NBS1) complex (Kamisugi et al. 2012, Nucl Acids Res, ASEP ID 382526). K.J. Angelis, J. Kozák and M. Holá contributed with data on **DSB repair kinetics** and bleomycin-induced mutagenesis. Moreover our group took advantage of haploid state and thus easy selection of mutants to study mutagenesis by positive selection of adenineribosidephosphotransferase (APT) mutants rendering resistance to 2- fluoroadenine (2FA). By combining comet and mutagenesis assays in moss enabled us to show that sensitivity of *ppmre11* and *pprad50* mutants to DSB induction is not due to defect of their capacity to repair them, but rather due to unrestricted high, though error-prone DSB repair leading to **unsupervised mutagenesis** largely representing as deletions of various sizes. This draws a picture of blocked participation of error-free

homologous recombination and shifting equilibrium toward error-prone non-homologous end joining (NHEJ) pathway inducing mutations and inactivation of essential genes, thus expressing sensitive phenotype.

We explored this Physcomitrella-based combined approach to further study the role PpLIG4 (Holá et al. 2013, BioMed Res Int, ASEP ID 423951) and effect of **UV irradiation** (Holá et al. 2014 Plant Physiol Biochem, doi: 10.1016/j.plaphy.2014.12.013) and collected extensive mutagenesis data in various Physcomitrella repair background lines induced by genotoxins potentially representing various environmental stresses (R. Vágnerová – ongoing master study and M. Holá – ongoing doctoral study). This methodology, experience and database background will be explored in upcoming years when we will target environmental stresses like drought and salinity, which are on molecular level conveyed as the burst of oxygen reactive species (ROS) and moss as one of the first plants invading land is the right model.

Finally, in collaboration with J. Fajkus, Z. Zdráhal (CEITEC MU, Brno) and E. Sýkorová (Institute of Biophysics CAS, Brno) we are investigating the role of plant **telomerase and telomerase-associated proteins** in telomeres maintenance in Physcomitrella, Algea, Arabidiopsis and tobacco (A. Náprstková – defended bachelor thesis, R. Vágnerová - ongoing master study). The LS-MS/MS identification of candidate proteins putatively interacting with TAP-tagged TERT (telomerase reverse transcriptase – E. Sýkorová, IBP, Brno and Z. Zdráhal, CEITEC MU, Brno) has lead to the identification of candidate proteins from **ALBA family** that not only showed the likely interaction with TERT but also interesting expression profile especially in the male gametophyte. Their role is being investigated as a main part of the ongoing master study (A. Náprstková). We found in Physcomitrella that telomere phenotypes are absent and DSB repair kinetics is not affected in mutants for DSB factors involved in non-homologous end joining (NHEJ). This is compliant with the overall dominance of homologous recombination over NHEJ pathways in the moss, contrary to the inverse situation in flowering plants (Fojtová et al., Plant Mol Biol 2015 87:591-601) and that algea strains Zygnema sp. 436 and Zygnema circumcatinatum TEL 181 are not responsive to DSB induction and repair at all.

Brief list of national and international collaborators (sorted alphabetically)

Dr. B. Banović, University of Belgrade, Serbia

Dr. G. Böhmdorfer, Gregor Mendel Institute, Vienna, Austria

Dr. C. Bousquet-Antonelli, INRA Perpignan, France

Prof. A.B. Britt and Dr. T. Furukawa, UC Davis, CA, USA

Dr. J. da Costa-Nunes, University of Lisboa, Portugal

Dr. A. Cuming, CPS, University of Leeds, UK

Dr. P. Doerner, University of Edinburgh, UK

Prof. T. Dresselhaus, University of Regensburg, Germany

Prof. J. Fajkus, Assoc. Prof. Z. Zdráhal,

Dr. M. Fojtová, CEITEC MU, Brno, Czech Republic

Dr. N. Firon, Volcano Center, Bet Dagan, Israel

Prof. J. Friml, IST Austria, Austria

Prof. M. Johnson, Brown University, USA

Prof. O. Lapčík, University of Chemistry and Technology, Prague, Czech Republic

Dr. J. Matoušek, Biological Center, České Budějovice, Czech Republic

Dr. H.-P. Mock and Dr. A. Matros, IPK Gatersleben, Germany

Dr. F. Nogue, INRA Versailles, France

Prof. Ravi Palanivelu, University of Arizona, Tucson, AZ, USA

Dr. R. Peyman-Zahedi, ISAS Dortmund, Germany

D. Scheafer, University de Neuchâtel, Switzerland

Prof. E. Schleiff, Goethe University Frankfurt a/Main, Germany

Dr. E. Sýkorová, IBP CAS, Brno, Czech Republic

Prof. D. Twell, University of Leicester, UK

Overall evaluation of the laboratory's acitivities

The Laboratory of Pollen Biology aims to remain a stable research unit of European standard by combining the fundamental research with university education and the active incorporation of students and early-stage scientists. The balanced structure of our young lab combines experienced scientists and young researchers that are increasingly successful in grant contest with postdocs, graduate and undergraduate students that are financed partly from institutional resources and partly from the grants. Our laboratory is well established within the national and international scientific structures as documented by the above list of mutually profitable collaborations. It is also reflected in our publication list that is optically not as extensive as it might have been but displays our strategy to publish the majority of papers with the first and/or corresponding authorship from our lab. Moreover, the recently opened fields of interest will yield in publications in the close future (see the above manuscripts submitted and in preparation). We have contributed to the Institute's financial balance by substantial overheads from our grant resources. Moreover, several lab members have been actively involved in teaching and scientific popularization as documented in the Appendix 3- 10.

Finally, individual members of the laboratory have been active in the demanding Institute management:

• D. Honys - Deputy Director of the Institute (2007-2012)

• D. Honys - member of the Executive Board of the Institute (since 2007)

• J. Fíla - Popularization Secretary of the Institute (from 2014).

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Plant Biotechnologies

INTRODUCTION

During the evaluation period research in our Laboratory has been focused predominantly on the area of plant-xenobiotic interactions, as well as on the area of plant secondary metabolites. It was funded by grant projects no 58-93 (Appendix 3.1).

Our "traditional" approach has been expanded in two directions:

1) more detail study of plant metabolism and plant stress responses, enabled by our success in OPPK grants (Appendix 3.1. No. 67 and 93), which gave us possibility to get the most sophisticated equipment – 2D GC-MS and LC-MS/MS. Additionally, proteomic and transcriptomic methods have become widely used in our Research Unit (RU).

2) successful application of the research results in real scale (see below).

We were able to continue and extend our international cooperations by means of joint projects, mainly with the USA, China (6 Kontakt projects) and other countries. We also succeeded in getting 16 COST projects, which gave us a wide opportunity to cooperate with scientists from European and other COST countries. These cooperations led, among others, to election of Tomas Vanek as a Board member of European Plant Science Organization (EPSO), which further widened our possibilities.

During evaluation period we met some problems, too. The most serious difficulties resulted from movement of the whole RU from one Institute to another before the evaluation period (2008) and once more during the evaluation period in 2011, which have had negative impact on the research itself as well as on number of papers published during the evaluation period.

Another problem is strong dependence of RU on projects, as salaries of the research employees are covered from Institute resources only by 35%, so there are large salary variations among years.

This fact influenced RU research strategy, which is focused broadly on plant responses to stress and plant metabolites, but must be flexible to reflect the available funding.

DESCRIPTION OF SELECTED RESULTS

Plants and Environment

The main focus of the Laboratory research is study of mechanisms of plant-xenobiotic responses, which may enable utilization of plants for **phytoremediation** purposes and optimization of the individual processes.

Toxicity of cadmium, cobalt, copper, zinc, nickel, lead, chromium and arsenic (in a wide concentration scale) was compared in 23 *Linum usitatissimum* cultivars (Soudek et al., Archives of Environmental Contamination and Toxicology 2010, ID 357036). We found that the most toxic element was arsenic. Substantial differences among individual flax cultivars indicated necessity of the selection of a suitable cultivar in case of contaminated soils. In order to find a suitable plant for phytoremediation of soils heavily polluted with Cd, Cr, Cu, Pb or Zn, we compared *Zea mays* and *Helianthus annuus* responses (Soudek et al., Journal of Food Agriculture & Environment 2010, ID 357039). *Helianthus* was found superior in case of Pb and Zn. In further studies, we compared the heavy metal response of *Allium sativum* (Soudek et al., Environmental and Experimental Botany 2011, ID 370674) and *Sorghum bicolor* (Soudek et al., Chemosphere 2014, ID 429931). In the latter case fortification of the antioxidant system with glutathione suppementation significantly increased plant fitness and enhanced the plant accumulation potential. The improvement of the metal accumulation capacities was also achieved by addition of chelating agents (Petrova et al., Journal of Food Agriculture & Environment 2012, ID 380679). The tested chelates enhanced transfer from roots to shoots. Taking into account relatively abundant soil contamination from uranium ore in former mines, along roads and in former processing factories, we tested uranium up-take by *Zea mays* plants (Soudek et al., Journal of Environmental Radioactivity 2011, ID 370666), by hairy root cultures of *Armoracia rusticana* (Soudek et al., Agrochimica 2011, ID 370676), by *Nicotiana tabacum* plants cultivated in hydropony (Soudek et al., Journal of Geochemical Exploration 2014, ID 433558). In the former and the last case, phosphate deficiency substantially enhanced uranium up-take. The reason might be up-regulation of ion transportes. Also tartaric acid and low pH (3.5) had positive effects. Apart of uranium, also radium (Soudek et al., J. Environ. Radioactivity 2010, ID 348487) and thorium (Soudek et al., Chemosphere 2013, ID 399221) accumulation was followed. Presence of citric, tartaric or oxalic acids as well as phosphate deficiency increased thorium accumulation.

Plants can also substantially diminish other types of pollutants, e.g. dust particles, which represent major environmental problem in urban areas (Soudek et al., Journal of Food Agriculture & Environment 2012, ID 38252). Even natural compounds, like biochar (from maize or sludge), may be a potential source of pollution (e.g. heavy metals or polycyclic aromatic hydrocarbons). Detail analysis of both types of biochar revealed that the levels of contaminants (e.g. heavy metals or polycyclic aromatic hydrocarbons) were below the limits and thus did not represent a health risk. Our Laboratory has established methodology of biochar mineralization, and we performed analysis of the samples on AAS, which represented an essential part of the paper of Luo et al. (Environmental Science and Pollution Research 2014, ID 433376).

Apart of heavy metals, serious soil and water contamination is caused also by organic xenobiotics, for example explosives (in former military areas). We characterized the impact of 2,4,6-trinitrotoluen (TNT) on gene expression in *Arabidopsis thaliana* rosettes and roots using microarrays (Landa et al., Functional & Integrative Genomics 2010, ID 350208). The up-regulated genes included nitrate reductase, several glycosyltransferases and ABC transporters. The up-take and transformation of nitroglycerine and ethylene glycol dinitrate from wastewater were optimized for *Juncus inflexus* and *Phragmites australis* plants (Podlipná et al., Journal of Hazardous Materials 2010, ID 350475).

Wide use of pharmaceuticals represents serious, gradually increasing contamination. Following excretion, these substances may persist in the environment and impact non-target organisms. This assumption is especially true for pain releasing drugs (diclofenac, ibuprofen and

acetaminophen). We tested *Armoracia rusticana* hairy root culture, *Linum usitatissimum* cell culture and *Lupinus albus*, *Hordeum vulgaris* and *Phragmites australis* plants (Kotyza et al., International Journal of Phytoremediation 2010, ID 348000). The best *in vitro* results for acetaminophen detoxification were achieved using *Armoracia rusticana* hairy root cultures, while total removal of ibuprofen and diclofenac was achieved using *Linum usitatissimum* suspension culture. In case of hydroponic plants, the best effectiveness of ibuprofen removal was found in case of *Phragmites*. Similar situation was in case of benzimidazole anthelmintics, the drugs against parasitic worms, widely used in human as well as veterinary medicine (Podlipná et al., Bioresource Technology 2013, ID 399919). These compounds were in reed cell cultures metabolized completely. As the metabolites were considerable less active than the original compounds, the biotransformation of the anthelmintics by reed could be useful for decrease of their toxicity in environment.

Taking into account increasing use of **nanoparticles** for multiple purposes and their uncontrolled release into the environment, we evaluated the potential effect of zinc oxide (nZnO), fullerene soot (FS) or titanium dioxide (nTiO2) nanoparticles on gene expression in *Arabidopsis thaliana* roots using microarrays (Landa et al., Journal of Hazardeous Materials 2012, ID 389142). Nanoparticles nZnO and FS caused wide changes of the transcriptome, especially the elevation of abiotic and biotic stress responsive genes. Only mild changes in gene expression were observed upon nTiO2 exposure. The resuts indicate that in case of the use of specific nanoparticles, high attention should be paid also to their impact on the environment. Further research in this area is in progress.

Our extensive knowledge on phytoremediation methodology was summarized in three book chapters (Vaněk et al. In: Application of Phytotechnologies for Cleanup of Industrial, Agricultural, and Wastewater Contamination 2010, ID 348489); Vaněk et al. In: Xenobiotics in the Urban Water Cycle: Mass Flows, Environmental Processes, Mitigation and Treatment Strategies 2010, ID 348491); Vaněk et al. In: Legislation, Technology and Practice of Mine Land Reclamation 2014, ID 443082).

Some of the above mentioned results can be utilized for solving of the real environmental problems, associated with both soil and water contamination. Based on our Laboratory results, we received a grant support for real scale applications from the Ministry of Industry and Trade of the Czech Republic (Wastewaters reclamation in integrated Biotechnology system Appendix 3.1. No. 72) and from the Technology Agency of the Czech Republic (Biotechnology system for agricultural waste-waters cleaning and reuse, Appendix 3.1. No. 69), both in cooperation with other Institutions. The latter one opened the possibility to recycle the agricultural waters and to reuse them, which can partially solve the problem of water shortage in agriculture.

Plant metabolites

Secondary metabolites, including many **pharmaceuticals**, are **of plant origin**. They could become the starting compounds for development of new drugs. Pharmacophore modelling has become an integrated tool in drug discovery. We compared two widely used pharmacophore modelling and screening software programmes and found that they yielded vastly different hit lists, but both predicted active compounds. The results indicate that comprehensive selection of active compounds requires use of more than one programme (Temml et al., Future Medical Chemistry 2014, ID 439549). LRB members, Zsofia Kutil, Premysl Landa and Tomas Vanek, tested compounds selected by Austrian colleagues by two software programmes for cyclooxygenase-1 and -2 inhibition.

We also focused our effort on characterization of natural products with anti-inflammatory properties, which could be used as natural substituents of synthetic pharmaceuticals. Twenty-three quinone compounds of plant origin were tested in vitro for their potential anti-flammatory effects (Landa et al., Natural Product Communications 2013, ID 395042). The screening was complemented with in silico molecular docking to crystal structure of selected lipoxygenase. Cudraflavone B, a prenylated flavonoid from *Morus alba* roots, was identified as a potent inhibitor of several inflammatory mediators (Hošek et al., Journal of Natural Products 2011, ID 367668). The anti-inflammatory effects of four selected legumes, namely *Vigna mungo*, *V. radiata*, *Glycine max* and *Lens culinaris* were reported for the first time (Zia-Ul-Haq et al., Pakistan Journal of Pharmaceutical Sciences 2013, ID 395052). In this paper LRB members Zsofia Kutil and Premysl Landa tested several extracts from legumes prepared by Pakistani colleagues for their potential to inhibit the activity of pro-inflammatory enzyme cyclooxygenase-2.

Anti-proliferative (anti-cancer) and anti-inflammatory effects detected in extracts from *Vaccinium bracteatum* leaves and fruits indicated that this material can be a useful source of biologically active compounds (Landa et al., Pakistan Journal of Pharmaceutical Sciences 2014, ID 433742). The strong antiproliferative effect of *Myrica rubra* essential oil was detected. In contrary, viability of isolated hepatocytes (as a model of normal non-cancerous cells) was not affected (Langhansová et al., Industrial Crops and Products 2014, ID 432566). The anti-inflammatory activity of ten anthra-, nine naphtho-, and five benzoquinone compounds of natural origin was compared with the function of five synthetic naphthoquinones (Landa et al., Planta Medica 2012, ID 380682). The study of the antioxidant capacities of the main quinone constituents of *Nigella sativa* seeds revealed that the most potent antioxidant was thymohydroquinone (Tesařová et al., Natural Product Communications 2011, ID 367700). The evaluation of anti-fungal activity of the black cumin seed (*Nigella sativa*) quinones revealed that thymohydroquinone and thymoquinone could be used in the dairy industry as chemical preservatives of natural origin (Halamová et al, Journal of Food Protection 2010, ID 351528). Red wine as a complex mixture was found as a powerful inhibitor of a number of proinflammatory enzymes (Kutil et al., Mediators of Inflammation 2014, ID 429537). Wine also obtains phenolic compounds with potential antimicrobial activity (Pastorková et al., International Journal of Food Microbiology 2013, ID 395455). The activity was possessed especially by pterostilbene, resveratrol and luteolin.

The activities of plant secondary metabolites may be improved by suitable chemical modification. Due to the occurrence of several stereoisomers of active compounds, which may, however, substantially differ in their biological activity, total chemical synthesis may be tedious and time-consuming. In this case **biotransformation** of a specific isomer may be advantageous. We optimized biotransformation of turpentine to different monoterpenes, especially trans-verbenol and trans-pinocarveol, in *Picea abies* cells (Dvořáková et al., Biocatalysis and Biotransformation 2011, ID 365946). Stereospecific biotransformation of (1S)-2-carene and (1S)-3-carene by *Picea abies* suspension was also described (Dvořáková et al., Molecules 2011, ID 369831).

The effectivity of anticancer drugs may be substantially increased by their conjugation with specific targeting compounds. Simultaneously, their undesirable side effects may be diminished. **Synthesis** of the conjugate of cytostatic paclitaxel with an analogue of the gonadotropin-releasing hormone (as a targeting moiety) allowed to increase the antiproliferative effect of paclitaxel substantially (Přibylová et al., International Journal of Pharmaceutics 2011, ID 370667). The non-

hydrolyzable alkylcarbonate analogues of O-acetyl-ADP-ribose were synthesized to obtain effective inhibitors of sirtuins, histone deacetylases, which are required for gene silencing (Dvořáková et al., Organic & Biomolecular Chemistry 2013, ID 399579). The effective synthesis of biologically active compounds requires suitable methodology. Dvořáková et al. (Tetrahedron 2012, ID 382209) studied mechanisms of the migration of methyloxycarbonyl group from secondary to primary hydroxyl in furanosides. Recently, labelling of physiologically active compounds with fluorescent dye proved to be useful in the visualization of the compound localization *in vivo*. Fluorescent labelling of the brassinosteroid receptor enabled to follow dynamics of its subcellular distribution and regulation of brassinosteroid signal transduction (Irani et al., Nature Chemical Biology 2012, ID 378975). For this study, fluorescent labelling of castasterone with Alexa Fluor 647 was designed and the labelled brassinosteroid was synthesized by Dr. Sisa. This compound enabled the first visualisation of brassinosteroid-receptor complex in plants.

In the frame of project "LD14127 Synthesis of strigolactone derivatives" (Appendix 3.1. No. 90) 9 analogues of strigolactones were prepared by M. Dvořáková and some of them will be patented prior publication.

Some of the above mentioned resuts can be utilized as the first step for preparation of new pharmaceuticals. As an example, synthesis of paclitaxel analogues can be mentioned. New series of conjugates was synthetized in the period 2013-2014. At present, they are undergoing the tests of antiproliferative activity and will be patented later on.

The other example can be new anti-inflammatory compounds derived from plant products. Some of them were synthetized in the period of 2013-2014 and are tested now.

SOCIETAL IMPACTS

Our comprehensive results in the area of utilization plants for environment protection (phytoremedition) gave us possibility to extend our efforts to the area of semi-real as well as real conditions, in order to contribute to the solving of environmental problems not only in the Czech Republic.

In the frame of project "Biological methods of waste-waters decontamination" (Cooperation of AS CR with Pardubice region, no registration No.), we were able to demonstrate the ability of phytoremediation technology to clean explosive containing waste waters from Explosia company, in economically effective way.

Technology Agency funded two our projects containing practical tasks. The former one has been focused on the evaluation of the fate of plastic materials defined as biodegradable in real conditions Appendix 3.5 5 (Appendix 3.1. No. 70). The first results confirmed, unfortunately, many doubts concerning these materials, which are in fact biodegraded very slowly and release some toxic chemicals into the environment. Our results will be utilized by the Ministry of Environment for development of environmental policy as well as by the company ECO-COM, which is responsible for the collection and separation of waste materials in the Czech Republic.

In the frame of the second project, "Bioclean" (Appendix 3.1. No. 69), we have demonstrated the ability of constructed wetlands to clean agricultural waste waters and remove not only "standard" contamination but also the residues of veterinary and other pharmaceuticals. We proved the

possibility to utilize this full scale system not only for decontamination, but for recycling of water, which can be used directly at farm for irrigation, with high economic impact.

More than **40 students** have been educated in our unit, at Bachelor, Master and Doctoral levels, within the frame of our research in the area of plant protection as well as plant bioactive compound synthesis.

We have had considerable impact to the environmental policy due to T. Vanek membership in the Scientific Committee of the Ministry of Environment. Further, he utilized his scientific expertise as an advisor of the Minister of Agriculture of the Czech Republic (in the period 2011- 2013). Being a vicechair of the Committee for Environment and Agriculture of Central Bohemia Region, T. Vanek has had a chance to influence environmental policy also at this level similarly as P.Soudek, member of the Environmental commission of the City council Kladno.

Last but not least, T. Vanek contributes, as a member of the EPSO Board, to the science policy at EU level, too.

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Growth Regulators and Isotope Laboratory

Laboratory of Growth Regulators (LGR) has been created in 1996 as a joint facility of the Faculty of Science, Palacký University and the Institute of Experimental Botany, Academy of Sciences of the Czech Republic. It was growing steadily the last 15 years up to almost 80 workers (scientists, technical assistants, technicians, Ph.D. students). It was becoming too big and difficult to manage. From this reason, LGR was split 2 parts and thus daughter Department of Chemical Biology and Genetics of the Centre of the Region Haná for Biotechnological and Agricultural Research was founded (Dr. Karel Doležal as a Head) – see http://www.cr-hana.eu/en/struktura-centra/cela-struktura/oddeleni-chemickebiologie-a-genetiky/. Researchers at the LGR deal mainly with cytokinins, but recently also with other groups of plant growth regulators. One globally renowned contribution of the LGR in this field is the expansion of a number of cytokinins, especially the aromatic cytokinins and their olomoucine-derived derivatives. These compounds are used in cancer treatment. Olomoucine was the first in a line of antitumor agents derived from cytokinins. Development of other, more effective inhibitors of cyclindependent kinases, key enzymes of cell division cycle like bohemine, roscovitine, olomoucine II and others followed. Roscovitine was licensed by Cyclacel Pharmaceuticals, Inc. (for more information see actual Press Release) and under its commercial name, Seliciclib® (www.cyclacel.com) is completing phase II clinical trials for cancer treatment in Europe and in the USA. However, the development of anti-cancer drugs is not the only field that the LGR deals with. It is also successful in the agricultural research area. For example, we discovered how to increase the amount of endogenous cytokinins using an inhibitor of cytokinin oxidase/dehydrogenase called INCIDE, which supports growth. Owing to this discovery, we were able to increase the yield of a number of agricultural crops and plant stress resistance. Recently, we developed a product which makes the skin youthful aids in the treatment of skin diseases. Cytokinins, which also retard ageing in humans were used in this development. Two international patents were issued for these discoveries. These were licensed to the American company Pyratine Plc in California which incurred the costs for patent protection. The product with the trade name Pyratine is derived from cytokinins (www.pyratine.com). This not only treats skin roughness, wrinkles, and pigmentation, it is also effective for treating various forms of acne. LGR publishes in prestigious international journals and submits a number of applications for international patents annually. LGR focuses on scientific research and teaching in the field of experimental biology, especially in the preparation of new, purine-based growth regulators with potent biological activities, the development of relevant analytical methods, the study of the functions and effects on growth and developmental process in normal and tumor cells, including the development of anti-tumor agents derived from plant hormones. Research on tumor suppressor genes, mechanisms that regulate their expression and the development of mutant organisms with controlled gene expression, are included in our scientific profile. LGR also co-operates in these fields with a number of research groups in the Czech Republic as well as internationally. Our multidisciplinary

research team is composed of experienced plant physiologists and biochemists, as well as organic and analytical chemists.

Research in the **Isotope Laboratory** (IL, Prof. Z. Wimmer as a head) of the IEB AS CR has been focused at the three basic directions:

(1) Investigation of medicinally important plant products with special attention paid to their derivation resulting in semisynthetic compounds with improved physico-chemical characteristics and mostly with higher cytotoxicity. High antimicrobial activity was achieved with several new compounds. Multifarious activity (i.e., pleitropic effect) was found in several highly active compounds.

(2) Development of heterocyclic derivatives of synthetic origin (purine-based compounds), including radiolabeling whenever required.

(3) Preparation of cytokinin derivatives, mostly with radiolabeling.

The research running in the LGR and IL during the period 2010-2014 in the frame of the Research Programme and grant projects can be divided into two main parts reflecting the structure and subject. The original results obtained during the last five years document high scientific quality of the teams both in the national as well as in the international context (see also List of publications and patents). The research is oriented to biomolecules, their structure and analysis, activity, molecular and cellular mechanism of action as well as applications in different fields. The compatibility of the approaches of participating laboratories and even the cooperation of members of the team has been successfully proven by their previous experience, patents and publications. In the following text, a more detailed description of results achieved is divided into two main research areas and these are sub-divided into smaller thematic parts for better orientation. Numbers in parentheses relate to the papers published as listed in 3.8. Our research was supported by grants listed in Enclosure 3.1., No. 94 - 121.

A. NEW PHYTOHORMONE PROBES AND BIOMOLECULES

New phytohormone standards, probes and labelled derivatives

Our laboratories have a long standing experience in organic synthesis, labelling of phytohormones and production of heavy and radioactively labelled phytohormones [14, 15, 17, 23, 32, 44, 48, 87, 89, 98, 101, 139, 145, 146, 165, 168, 201, 214, 216]. For example, we developed a bioactive, fluorescent BR analogue, Alexa Fluor 647- castasterone (AFCS), and visualized the endocytosis of leucine-rich repeat receptor-like kinases in plants, BRASSINOSTEROID INSENSITIVE 1 (BRI1)-AFCS complexes in living *A. thaliana* cells. Our findings provide what is to our knowledge the first visualization of receptor-ligand complexes in plants and reveal clathrin- and ARF-GEF-dependent endocytic regulation of BR signalling from the plasma membrane [86]. We also described a new reversed phase HPLC-MS detection method for quantifying intact cytokinin nucleotides in human K-562 leukaemia cells. Tandem mass spectrometry (MS/MS) was used to identify the intracellular metabolites (cytokinin mono- di- and tri-phosphorylated nucleotides) in riboside-treated cells. Identification of the metabolites was confirmed by synthesis [23]. We also published the first analysis of relationship between chemical structure of cytokinins and their cytotoxic effects against a panel of human cancer cell lines with diverse histopatothological origin. Most cell lines showed greatest sensitivity to *ortho*-topolin riboside. Newly prepared cytokinin nucleotides were usually active in a

similar concentration range to the corresponding ribosides. The study shows that structural requirements for cytotoxic activity of cytokinins against human cancer cell lines differ from their activity in plant bioassays [15]. A capillary zone electrophoresis (CZE) method for separation of adenosine and isopentenyladenosine (cytokinin) nucleotides (prepared for the first time) was developed, optimized and validated.The identities of the reaction products: isopentenyladenosine di- and triphosphate were confirmed by HPLC-QqTOE-MS [146]. We also showed that an ATP-binding cassette transporter in Arabidopsis, AtABCG14, is essential for the acropetal (root to shoot) translocation of the root-synthesized cytokinins. In planta feeding of new C-14 or C-13-labelled tZ suggests that it acts as an efflux pump and its presence in the cells directly correlates with the transport of the fed cytokinin. Therefore, AtABCG14 is a transporter likely involved in the long-distance translocation of cytokinins in planta [201]. We developed a sensitive mass spectrometry-based method to simultaneously profile the majority of known auxin precursors and conjugates/catabolites in small amounts of Arabidopsis tissue. The method also includes a new derivatization technique for quantification of the most labile of the auxin precursors and new labelled IAA derivatives [139].

New compounds modulating phytohormone perception, biosynthesis and degradation

Recently, we have contributed to studies of transgenic plants with cytokinin receptor loss-of-function mutations [38, 131], plants with mutations in cytokinin-synthesizing genes [187, 208], or cytokinindeficient plants with genetically enhanced cytokinin degradation (Werner et al. [41, 42, 52, 68, 171, 126, 147, 181, 190, 198]. These studies showed that such changes lead to distinct alterations in shoot growth parameters, retarded leaf senescence, increased seed size, accelerated germination and enhancement of the root system. Chemical inhibitors of cytokinin perception, biosynthesis and degradation would be thus very potent tools to study further the mechanism of cytokinin action as an alternative to genetic approaches. Furthermore, they would be expected to influence plant growth and development and might thus find interesting applications as growth regulators for modifying traits of crop plants. Several compounds regulating both the perception and metabolism of brassinosteroids and cytokinins were developed during the last five years [45, 79, 86, 96, 150, 170] (for patents see [3, 9, 10, 11, 12, 13, 14, 15], 17, 18, 19, 21, 23, 24, 26, 27 and www.espacenet.com) and their beneficial activity for plant growth and development described. Recently we reported 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55) as the first molecule to antagonize cytokinin activity at the receptor level. The effect of INCYDE at 10 nM on growth, biochemical and photosynthetic efficiency in sodium chloride (NaCl)-stressed tomato plants was investigated [216]. Furthermore, we reported the synthesis and in vitro biological testing of eleven BAP derivatives substituted in the benzyl ring and in the C2, N7 and N9 positions of the purine moiety. 6-(2,5-Dihydroxybenzylamino)purine (LGR-991) was identified as a cytokinin receptor antagonist. At the molecular level LGR-991 blocks the cytokinin receptor CRE1/AHK4 with the same potency as PI-55 [9]. X-ray structure, NMR and stability-in-solution study of 6-(furfurylamino)-9-(tetrahydropyran-2-yl)purine (Pyratine), a new very active cytokinin derivative was determined [14]. We further prepared a series of eight N9-substituted Kinetin derivatives, and characterized them with available physicochemical, biochemical and biological assays [79]. A series of N(6)-[(3-methylbut-2-en-1-yl)amino]purine (iP) derivatives specifically substituted at the N9 atom of purine moiety by tetrahydropyran-2-yl, ethoxyethyl, and C2-C4 alkyl chains terminated by various functional groups were prepared. The reason for this rational design was to reveal the relationship between specific substitution at the N9 atom of purine moiety of iP and cytokinin activity of the prepared compounds [86]. A sensitive and reliable high-performance liquid chromatographic method with tandem mass spectrometric detection has been developed and used for the determination of 2-

methylthio-cytokinin derivatives produced by the phytopathogenic actinomycete *Rhodococcus fascians*. New 2-methylthio- CK probes were prepared for this study [44]. An inhibitory study with numerous urea derivatives was undertaken using the maize cytokinin oxidase/dehydrogenase (ZmCKO1) and the crystal structure of ZmCKO1 in a complex with N-(2-chloro-pyridin-4-yl)-N´-phenylurea (CPPU) was solved. Subsequently, site-directed mutagenesis of L492 and E381 residues involved in the inhibitor binding was performed. All studied compounds were further analysed for cytokinin activity in the Amaranthus bioassay and for binding to the Arabidopsis cytokinin receptors AHK3 and AHK4 [45]. The comprehensive screen through the land plants is presented suggesting that cytokinins (CKs) of *cis*-zeatin (cZ)-type occur generally in plant kingdom. Survey of employed bioassays illustrates ability of high doses of cZ-type CKs to induce various physiological responses and CK signalling. These data argue against the image of cZ-type CKs as the non-active or weakly active natural adjuncts to the *trans*-isomers suggesting their conceivable function as delicate regulators of CK responses in growth-limiting conditions. This work was possible because of new cZ synthesis developed by Dr. J. Hanuš from IL [59]. We also investigated the effect of *meta*-topolin tetrahydropyran-2-yl - a novel derivative of the aromatic cytokinin *meta*-topolin on shoot proliferation, photosynthetic pigment content, phytochemicals and antioxidant activity of two widely used medicinal plants, *Aloe arborescens* and *Harpagophytum procumbens* [206]; tissue-cultured and acclimatized 'Williams' bananas subjected to new cytokinin treatments - 6-(3-methoxybenzylamino)-9- tetrahydropyran-2-ylpurine [207].

Chemical modulators of kinases (cytokinin-derived)

During the last five years we have continued development of increasingly effective cyclin-dependent-kinase (CDK) inhibitors, leading to the discovery of several other potent compounds with various structural motifs [27, 30, 71, 72, 159, 172, 185, 221, 226]. Potential of CDK inhibitors in different therapeutic areas was also reviewed several times [34, 43, 115, 116]. New generations were prepared following well-established methods, including our previously described syntheses of purines, pyrazollo[4,3-d]pyrimidines, 8-azapurines and arylazopyrazoles (for patents see [1, 2, 4, 5, 16, 16, 20, 25] and www.espacenet.com). Selected examples are enclosed: A series of 1,5-diaryl-3-(3,4,5-trihydroxyphenyl)-1H-pyrazolo[4,3- e][1,2,4]triazines [30] and 3-(2-pyridyl)-6-(hetero)aryl-1H-pyrazolo[4,3-c]pyridines [226] was synthesized and their kinase inhibitory activity and cytotoxicity against several cancer cell lines has been evaluated A new potent CDK2 inhibitor with pyrazolo[4,3-d]pyrimidine scaffold has been synthesized, characterized, and evaluated in cellular and biochemical assays. 7-Benzylamino-5(R)-[2- (hydroxymethyl)-propyl]amino-3-isopropyl-1(2)H-pyrazolo[4,3-d]pyrimidine, compound 7, was prepared as a bioisostere of the well-known CDK inhibitor roscovitine. An X-ray crystal structure of compound 7 bound to CDK2 has been determined, revealing a binding mode similar to that of roscovitine [72]. For series of roscovitine derivatives bearing 2-(hydroxyalkylamino) moiety, cytotoxic potency strongly correlated with anti-CDK2 activity. The most potent compounds were investigated further to assess their ability to influence cell cycle progression, p53-regulated transcription and apoptosis. All the observed biological effects were consistent with inhibition of CDKs involved in the regulation of cell cycle and transcription [159]. A series of 2,9-substituted 6-guanidinopurines, structurally related to the CDK inhibitors olomoucine and roscovitine, has been synthesized and characterized. Their increased inhibitory activity and decreased selectivity offer a good starting point for further development of new protein kinase inhibitors [163]. Recently, we synthesized and screened a novel series of 2-substituted-6-biarylmethylamino-9-cyclopentylpurine derivatives for improved CDK inhibitory activity and antiproliferative effects. One of the most potent

compounds, 6b, exhibited strong cytotoxicity in the human melanoma cell line G361 that correlated with robust CDK1 and CDK2 inhibition and caspase activation. In silico modelling of 6b in the active site of CDK2 revealed a high interaction energy, which we believe is due to the 6-heterobiarylmethylamino substitution of the purine moiety [172]. We further focused on the efficacy of the novel compounds BA-12 and BP-14 that antagonize CDK1/2/5/7 and CDK9. Inhibition of those CDKs in human hepatocellular carcinoma cell lines reduced the clonogenicity by arresting cells in S-G(2) and G(2)-M phase of the cell cycle and inducing apoptosis. In contrast, primary human hepatocytes failed to show cytotoxicity and apoptosis. No loss of chemosensitivity was observed in hepatocellular carcinoma cells after long-term exposure to inhibitors. In vivo, treatment of xenografted human hepatocellular carcinomas with BA-12 or BP-14 effectively repressed tumour formation. Moreover, BA-12 or BP-14 significantly diminished diethylnitrosamine (DEN)-induced hepatoma development in mice. These data show that new CDJ inhibitors exhibit superior efficacy compared with currently used chemotherapeutics in hepatocellular carcinomas [179]. We also showed that roscovitine and TRAIL demonstrate synergistic cytotoxicity in hematologic malignant cell lines and primary cells. Pre-treatment of TRAIL-resistant leukemia cells with roscovitine induced enhanced cleavage of death-inducing signalling complex-bound proximal caspases after exposure to TRAIL [166]. We analysed the effect of inhibition of CDKs by olomoucine II on gene expression from viral promoters and compared its effect to widely-used roscovitine. We found that both roscovitine and olomoucine II blocked the phosphorylation of RNA polymerase II C-terminal domain. However the repression of genes regulated by viral promoters was strongly dependent on gene localization - expression only when the viral promoter was not integrated into chromosomal DNA [202]. We further reported that new CDK9 3,5-diaminopyrazole inhibitor CAN508 inhibits endothelial cell migration and tube formation. In addition, it reduces phosphorylation of the C-terminus of RNA polymerase II and inhibits mRNA synthesis in endothelial cells. It has high selectivity towards the positive transcriptional regulator P-TEF. Moreover, CAN508 reduces expression of vascular endothelial growth factor by several human cancer cell lines [76]. We also described a novel member a library of arylazo-3,5-diaminopyrazole family, AAP1742, that reduces the viability of multiple myeloma cell lines in low micromolar concentrations. Consistent with inhibition of CDK9, AAP1742 decreases the phosphorylation of RNA polymerase II and inhibits mRNA synthesis of anti-apoptotic proteins Mcl-1, Bcl-2, and XIAP, followed by apoptosis in the RPMI-8226 cell line in a dose- and a time-dependent manner [221]. Recently, we showed that roscovitine exerts potent antiangiogenic effects and elucidated Cdk5 as a new player in angiogenesis. We also preported the antiangiogenic profile of 15 derivatives of roscovitine in vitro and in vivo and provide structure activity relationships of the roscovitine analogues [77]. We also demonstrated that trisubstituted pyrazolo[4,3-d]pyrimidines constitute a novel class of compounds which potently inhibit angiogenesis [156]. We pharmacologically characterized N-5-(2-aminocyclohexyl)-N-7-benzyl-3-isopropyl-1(2)H-pyrazolo[4,3- d]pyrimidine-5,7-di-amine (LGR1406), a novel derivative of the CDK inhibitor roscovitine (ROSC), in PDGF-BB-activated VSMC. Abnormal vascular smooth muscle cell (VSMC) proliferation contributes to the pathogenesis of restenosis. Thus, drugs interfering with cell cycle progression in VSMC are promising candidates for an antirestenotic therapy [5]. We also investigated the effects of downregulation of Cdk2 activity by olomoucine II in 2 mESC lines. Olomoucine II treatment significantly increased the G1 phase cell numbers, decreased the S phase cell numbers, and inhibited DNA replication in mESCs. In nocodazole-synchronized mESCs, we show that specific down-regulation of Cdk2 activity prolongs G1 phase progression. In addition, down-regulation of Cdk2 activity in mESCs established a somatic cell-like cell cycle and induced expression of differentiation markers [37]. We also report results of screening of new anti-leishmanial drugs among 2,6-disubstituted purines and

corresponding 3,7-disubstituted pyrazolo[4,3-d]pyrimidines. Since some compounds reduced viability of axenic amastigotes of *Leishmania donovani*, we screened them for interaction with recombinant leishmanial cdc-2 related protein kinase (CRK3/CYC6). Some compounds (9A, 12A and 13A) show activity against amastigotes with EC(50) in a range 1.5-12.4 mikroM[73]. To determine which CDKs were involved in regulating neutrophil lifespan we first examined CDK expression in human neutrophils and found that only CDK5, CDK7 and CDK9 were expressed in these cells. Treatment of neutrophils with a potent CDK9 inhibitor increased apoptosis and caused a rapid decline in the level of the antiapoptotic protein Mcl-1, whilst Bcl2A was unaffected. We propose that CDK9 activity is a key regulator of neutrophil lifespan, preventing apoptosis by maintaining levels of short lived anti-apoptotic proteins such as Mcl-1. Furthermore, CDK9 represents a novel therapeutic target in such diseases [133].

Natural phytochemicals as potential drug candidates

We have five small research projects on development of drug candidate with unknown molecular mechanism of action. These usually exist because of our expertise in cellular and molecular testing on new drug candidates.

1. Triterpenoid compounds with anticancer activity - Betulin and saponins based on betulin scaffold possess interesting biological properties which have been extensively studied and reviewed in the last years. A concise synthesis of lupane triterpenes with an elongated carbon chain at the C-28 position, as well as saponins containing D-mannose, L-arabinose, and L-rhamnose moieties at the C-3 position was described. Several triterpenes and the corresponding saponins exhibited an interesting cytotoxic activity profile against human cancer cell lines. The therapeutical index of active triterpenes is very high, since almost none of them were cytotoxic for normal BJ fibroblasts [199]. A practical method for the preparation of benzoyl protected allyl and benzyl alpha-Didopyranosides, and D-idopyranosyl trichloroacetimidate, from 1,2,3,4,6-penta-O-acetyl-alpha-Didopyranose, was described. All new compounds were evaluated in vitro for their cytotoxic Appendix 3.5 6 activities. Novel saponins exhibited interesting cytotoxic activity in the micromolar range against human cancer cell lines [218].

2. New Anticancer Saponins derived from OSW-1 - OSW-1 isolated from the bulbs of *Ornithogalum saundersiae* has a low toxicity for normal cells but inhibits the growth of a variety of malignant tumor cells and is 10-100 times more potent than clinically applied anticancer agents. Analogues of OSW-1 were prepared from the readily available steroidal 16 beta,17 alpha,22-triol. The new 22- deoxo-23-oxa analogues of OSW-1 were screened against eight cancer cell lines and normal human fibroblasts. The analogues proved to be slightly less active than OSW-1 but also less toxic to normal cells. They induce concentration- and time-dependent apoptosis of mammalian cancer cells with caspase-3 activation [78].

3. Steroid plant growth regulators (brassinosteroids and ecdysteroids) - We have developed a number of new brassinosteroid (BR) analogues over the last 20 years which have been tested in different plant bioassays. Six new castasterone analogues with alpha-azido acid ester groups in position 17beta were synthesized. The biological activities were evaluated in two bioassays: a rice lamina inclination test and bean second internode bioassays. The activities of the new analogues differ in these two bioassays [6]. We also developed a bioactive, fluorescent BR analog, Alexa Fluor 647-castasterone and visualized the endocytosis of BRI1-AFCS complexes in living *Arabidopsis thaliana* cells [101]. Thirteen monohydroxylated brassinosteroids analogues were synthesized and tested for their

biological activity in plant and animal systems. The cytotoxic activity of the products was studied using human normal and cancer cell lines with 28- homocastasterone (28-homoCS) as a positive control, their brassinolide type activity was established using the bean second-internode test with 24-epibrassinolide (24-epiBL) as standard [194]. Sixteen platinum(II) complexes of estrone and estradiol were synthesized in this work to evaluate their cytotoxic activity against several cancer cell lines including estrogen dependent and independent ones. Cytotoxicity assays showed that most of the complexes prepared are active against the cancer cell lines used and BJ fibroblasts [137]. Recently, we published the first evidence that some natural BRs induce cell growth-inhibitory responses in several human cancer cell lines without affecting normal non-tumor cell growth (BJ fibroblasts). The antiproliferative activity of the natural BRs 28-homoCS and 24-epiBL in human hormone-sensitive and -insensitive (MCF-7 and MDA-MB-468, respectively) breast cancer cell lines was analysed. The results showed that BRs can affect specific components of the cell cycling machinery with profound consequent effects on cell cycle regulation and also on the induction of apoptosis in the cancer cells [48]. The aim of this study was to examine the mechanism of the anti-proliferative activity of natural BRs 28- homoCS and 24-epiBL in hormone-sensitive and -insensitive (LNCaP and DU-145, respectively) human prostate cancer cell lines. The studied BRs seem to exert potent growth inhibitory and proapoptotic effects and could be therefore highly valuable new candidates for prostate anticancer drugs [142]. Antiangiogenic activity of BRs) and their derivative cholestanon was investigated in human umbilical vein endothelial cells (HUVEC) and in human microvascular endothelial cells (HMEC-1). Synthetic analogue cholestanon inhibited angiogenesis in vitro more effectively than natural BRs. Synthetic BRs cholestanon showed agonistic effects on estrogen-receptor-alpha, estrogen-receptor-beta and androgen receptor. Of the natural BRs, 24-epiBL was found to be a weak antagonist of estrogen-receptor-alpha (ER alpha). Our results provide the first evidence that large group of BRs can inhibit in vitro angiogenesis of primary endothelial cells [148]. A series of new pro-juvenoids (juvenogens, insect hormonogenic compounds, pro-drug-like agents) was synthesized using isomeric synthetic juvenoids (insect juvenile hormone analogues) and steroid molecules as patterns modifying parts of the complex hormonogenic molecules. These projuvenoids were subjected to the topical screening tests and to the drinking assays on the red firebug (*Pyrrhocoris apterus*) [21]. Metabolism of ecdysteroids, insect moulting hormones, was also described including that of active 20-hydroxyecdysone, showing a low activity, and inactive 20- phosphate. In addition to ecdysteroid biosynthesis from phytosterols via cholesterol, insect larvae can obtain ecdysteroids from food as nonpolar and inactive esters with fatty acids. Main ecdysteroid screening tests are described in detail, in relation to digestion, the influence of intestinal symbiotic microbiota, detoxification of xenobiotics and subsequent inhibition of pathogenic microorganisms [19].

4. Phytosterols and triterpenoids - Phytosterols and cholesterol were subjects of structural modifications with the objective to get novel compounds displaying either cytotoxicity and antimicrobial activity or ability to self-assemble into chiral supramolecular systems. An efficient synthesis of novel stigmasterol-amino acid (glycine, L-leucine and L-phenylalanine) conjugates as stimuli responsive gelators was reported [58, 63]. Studies of toxicity, antioxidant activity and bioavailability of unique potent anti-atherosclerotic succinobucol-steroid conjugates were also realised. The conjugates consist of, on one side, the therapeutically important drug succinobucol ([4-{2,6-di-tert-butyl-4-[(1-{[3-tert-butyl-4-hydroxy-5-(propan-2-yl)phenyl]sulfanyl}ethyl)sulfanyl]pheno-xy}-4-oxo-butanoic acid]) possessing an antioxidant and anti-inflammatory activity, and on the other side, plant stanol/sterols (stigmastanol, β-sitosterol and stigmasterol) possessing an ability to lower

the blood cholesterol level. A cholesterol-succinobucol prodrug was also prepared in order to enhance the absorption of succinobucol through the intestinal membrane into the organism and to target the drug into the place of lipid metabolism. The single-crystal structures of polymorphs were determined by X-ray single-crystal diffraction [64, 103, 106]. The current interest of the IL team has been also focused on investigation of novel amides of sterols and triterpenoid acids with potential cytotoxicity. The synthetic protocol was designed in as simple as possible way, and divided into several general methodologies applicable to the compounds synthesized. The cytotoxicity was tested on cells derived from human T-lymphoblastic leukemia, breast adenocarcinoma and cervical cancer, and compared with tests on normal human fibroblasts. ADME parameters as well as selected physico-chemical parameters were either measured or calculated to support experimentally obtained results [111, 167].

5. Chemopreventive phytochemicals, low molecular weight antioxidants - New UHPLC-MS/MS method for determination of phenolic acids [128] was subsequently applied for solution of many physiological problems [7,46,53,69,83,97,103,120,125,176]. Phenolic acids including the hydroxybenzoic and hydroxycinnamic acid derivatives in *M. plumbea* were quantified by LC-MS while the antioxidant activity was evaluated using oxygen radical absorbance capacity (ORAC). Most phytochemicals (for example, gallic acid, ferulic acid protocatechuic acid and caffeic acid) were highest in plants micropropagated on 0.25 microM *meta*-topolin riboside [176, 232]. Similarities in phenolic profiles were identified confirming the chemical signatures that characterize *Pelargonium sidoides* plants. Extracts of greenhouse-acclimatized and wild plants exhibited comparable antimicrobial and antioxidant properties [175]. Antioxidant capacity and various classes of phenolic antioxidants were also quantified in leaf and bark methanol extracts of a medicinal plant, Rhamnus intermedia Steud. et Hochst. [125]. The medlar fruit (*Mespilus germanica* L) has been gaining commercial importance. In this study, we have investigated how the degree of ripeness affects the concentrations and proportions of phenolic acids [69]. The total phenolic content (Folin-Ciocalteu method), free radical scavenging ability expressed as DPPH value, ferric reducing antioxidant capacity (FRAP), and oxygen radical absorbance capacity (ORAC) were also determined in water extracts of leaves from Rosaceae family plants (*Fragaria vesca* L., *Rubus fructicosus* L., and *Rubus idaeus* L.) [66]. Antioxidant and antimicrobial activities as well as the quantity of phenolic substances were determined in aqueous extracts of leaves, stems and flowers of *Moltkia petraea* (Tratt.) Griseb. and *Teucrium arduini* L from different mountainous localities in Croatia [7, 46]. Polyphenol oxidases (PPO) functionality was demonstrated for recombinant PPO6. P. patens was analysed for phenolic compounds and six substances were detected intracellularly by LC-MS analysis: 4-hydroxybenzoic acid, p-cumaric acid, protocatechuic acid, salicylic acid, caffeic acid, and an ester of caffeic acid. Targeted PPO1 knockout (d|ppo1) plants were generated and plants lacking PPO1 exhibited only similar to 30% of the wild-type [120].

B. NEW PHYTOHORMONE BIOANALYTICAL METHODS AND THEIR APPLICATIONS

In order to understand better the network regulation of hormone action, there is need of measuring multiple hormone concentrations simultaneously, i.e. characterize the 'hormone-metabolome'. The most of plant hormones occur in plant tissues at extremely low concentrations (in general pmol/g of fresh weight) which makes qualitative and quantitative analysis difficult and therefore very sensitive analytical tools are required. Moreover, with regard to high complexity of matrix, the need of thorough isolation and high enrichment of these substances as the analytes is absolutely essential prior to the detection by standard analytical techniques. Last but not least, the throughput is also important factor for fully realizing the technology of 'hormonomics', in which the

hormone content of a large number of small amounts of plant samples has to be measured. The technologies are however not well developed. The analysis of plant hormones is challenging not only because these compounds are present in trace amounts but also because many other substances in plant extracts interfere with the analysis, such as pigments, lipids, phenolics and proteins. Moreover, the enzymatic induction of metabolic change or chemical degradation of the compounds investigated can occur, it is usually important to keep plant material cold during extraction process to avoid these processes. Currently, the best suitable and most used analytical technology for phytohormone analysis is based on liquid chromatography-tandem mass spectrometry ((U)HPLC-MS/MS) as evidenced in our reviews [117, 152,220]. Since 2008, this methodology was gradually developed in LGR for plant hormone analyses (cytokinins, auxins, JAs, ABAs, gibberellins, brassinosteroids, phenolics, intact cytokinin nucleotides in human K-562 leukemia cells, 2-methylthio-cytokinin derivatives produced by the phytopathogenic actinomycete *Rhodococcus fascians*, roscovitine oxidation products, isoflavonoids and other phenylpropanoids) [89, 128, 168, 214]. Furthermore, the analysis of cytokinin nucleotides by capillary zone electrophoresis with diode array and mass spectrometric detection as well as LC-NMR was also introduced [146, 192]. Plant products can efficiently be extracted from the plant tissue by supercritical carbon dioxide technology. We have applied this technology to investigate plant phytosterols and phytoecdysteroids. Supercritical carbon dioxide also offers a possibility to mediate enzymic reactions. Examples of this research topic appear in our papers [1, 4]. The ability of LC-NMR to detect simultaneously free and conjugated phytosterols in natural extracts was tested. The results of qualitative and quantitative analyses were in a good agreement with the literature data [192].

We were able to develop a new separation technology UHPLC. The UHPLC uses the columns packed with sub-2μm particles which results in higher peak capacity, greater resolution, and increased sensitivity compared to HPLC. Thereupon, the analytical parameters of mass spectrometry measurements are significantly enhanced after the implementation of UHPLC. While high potential of UHPLC-MS in pharmaceutical, environmental, cosmetic, explosive, proteomic, and metabolomic analysis has been demonstrated in the last years, only a few publications up to now described use of this analytical technique for qualitative and quantitative analysis of plant extracts [23, 44, 139, 145,186]. Mass spectrometry has developed towards increasingly higher sensitivity and selectivity in the analyses, which now makes it possible to perform tissue and cell specific quantification of phytohormones and different phytohormone metabolites - simultaneous profile of the majority of known auxin precursors and conjugates/catabolites (auxin metabolome) in small amounts of Arabidopsis tissue [139, 186]. For minute tissue samples, miniaturization of the extraction and purification steps can improve the sensitivity of analytical method, since it can minimize analyte losses due to adsorption to surfaces and/or increase analyte recovery in the solid phase extraction (SPE) step [145].

The application of new technologies led to several important discoveries. For example:

1) The phytopathogenic actinomycete *Rhodococcus fascians* D188 secretes six cytokinin bases that synergistically redirect the developmental program of the plant to stimulate proliferation of young shoot tissue, thus establishing a leafy gall as a niche. A yeast-based cytokinin bioassay combined with cytokinin profiling of bacterial mutants revealed that the fas operon is essential for the enhanced production of isopentenyladenine, *trans*-zeatin, *cis*-zeatin, and the 2-methylthio derivatives of the zeatins [17]. Typical symptoms of *Verticillium longisporum*-induced disease are stunted growth and

leaf chlorosis. Expression analyses of the senescence marker genes SENESCENCE-ASSOCIATED GENE12, SENESCENCE-ASSOCIATED GENE13, and WRKY53 revealed that the observed chlorosis is a consequence of premature senescence triggered by *Verticillium* infection. Our analyses show that, concomitant with the development of chlorosis, levels of tZ decrease in infected plants. Stabilization of Arabidopsis cytokinin levels by both pharmacological and genetic approaches inhibited *Verticillium* proliferation and coincides with reduced disease symptom development [170]. Cytokinins (CK) play an important role in the formation of nitrogen-fixing root nodules. CK profiling showed that the most abundant CK secreted by *Bradyrhizobium* sp. strain ORS285 are the 2- methylthio derivatives of tZ and iP. In their pure form, these CK can activate legume CK receptors in vitro, and their exogenous addition induced nodule-like structures on host plants. Deletion of the miaA gene showed that transfer RNA degradation is the source of CK production in *Bradyrhizobium* sp. In nodulation studies performed with *A. indica* and *A. afraspera*, the miaA mutant had a 1-day delay in nodulation and nitrogen fixation, bigger but less abundant nodules. These data showed that CK produced by *Bradyrhizobium* sp. strain ORS285 are not the key signal triggering nodule formation during the Nod-independent symbiosis but they contribute positively to nodule development in *Aeschynomene* plants [174].

2) Endogenous cytokinins (CKs), auxins, and abscisic acid (ABA) were identified and quantified in 11 red algae collected from the Brazilian coast. These results confirm that plant hormones were common constituents in red seaweeds, with this being the first report of the occurrence of ABA in Rhodophyta [31]. Next, the endogenous auxins and cytokinins were quantitated in 24 axenic microalgal strains from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Charophyceae. IAA and IAM were present in all microalgal strains; nineteen cytokinins were identified in the microalgal strains. The general trend was that cZ cytokinins were the predominant, iP present in moderate concentrations, low levels of tZ and almost no DHZ-type. In 15 strains, the auxin content was 2- to 4-fold higher than the cytokinin content [164]. Endogenous gibberellins and brassinosteroids were also quantified. Between 18 and 20 gibberellins were quantified in all strains. GA profiles were similar in all strains and the active GA detected in the highest concentration was GA(6), the predominant intermediates were GA(15) and GA(53) and the main biosynthetic end products were GAB(13) and GA(51). Brassinolide and castasterone were determined in all the strains only [173]. The effect of light on growth and endogenous phytohormone concentrations in *Chlorella minutissima* MACC 360 was also investigated [196]. It was also our aim of to quantify plant growth regulators ABA, GAs and brassinosteroids present in *E. maxima* and Kelpak as a biostimulant used in agriculture [203]. In the oleaginous eustigmatophyte *Nannochloropsis oceanica* IMET1, UHPLC-MS/MS also detected a wide array of plant hormones. The presence of such functional flowering plant-like phytohormone signalling systems in *Nannochloropsis* sp. suggests a much earlier origin of phytohormone biosynthesis and degradation than previously believed [223].

3) Furthermore, cytokinin metabolism and function was analysed in different plant tissue cultures. In the three types of embryogenic and non-embryogenic calli all cytokinins were found in each type. These results suggest that the difference in somatic embryo formation capacity observed between embryogenic and non-embryogenic calli is related to their endogenous cytokinin contents [33]. Changes in cytokinin (CK) profiles and their physiological implications in micropropagated *Harpagophytum procumbens* [(Burch.) DC. ex Meisn.] tissues in relation to shoot-tip necrosis (STN) and CK treatments were studied. The data show that metabolites like 9-glucosides of BA have a detrimental effect in plant tissue culture. We also assess the use of topolins in PTC with emphasis on their metabolism, structure-activity relations and effect on morphogenesis in vitro [90, 96, 175, 176,], on

micropropagation of 'Williams' bananas [135, 140, 144, 178, 207], on the micropropagation efficiency and shoot quality of smoke bush (*Cotinus coggygria* S cop.) 'Royal Purple' [150], on the in vitro multiplication and senescence of wych elm (*Ulmus glabra* Huds.) [161], on micropropagation of *Aloe arborescens* and *Harpagophytum procumbens* [206], on micropropagation of *Hypoxis hemerocallidea* Fisch [224], and on phytochemical levels and antioxidant potential in greenhouse grown Merwilla [232]. The endogenous cytokinin and auxin levels were also analysed during adventitious caulogenesis in *Pinus pinea* cotyledons [149], during in vitro organogenesis from vegetative buds of *Pinus radiata* adult trees [162], in in vitro cultures and inflorescences from normal and mantled oil palm (*Elaeis guineensis* Jacq.) [184], after exogenously applied cytokinins in micropropagated *Merwilla plumbea* [217].

4) In Arabidopsis and most plant species, dormancy absolutely requires an unidentified seed coat germination-repressive activity and constitutively higher abscisic acid (ABA) levels upon seed imbibition. We developed a "seed coat bedding" assay monitoring the growth of dissected embryos cultured on a layer of seed coats, allowing combinatorial experiments using dormant, nondormant, and various genetically modified seed coat and embryonic materials. This assay, combined with direct ABA measurements, revealed that, upon imbibition, dormant coats, unlike nondormant coats, actively produce and release ABA to repress embryo germination, whatever the embryo origin [36]. Using a seed coat bedding assay, we further showed that canopy light specifically inactivates phyB activity in the endosperm to override phyA-dependent signalling in the embryo. This interference involves abscisic acid (ABA) release from the endosperm and distinct spatial activities of phytochrome signaling components. Under the canopy, endospermic ABA opposes phyA signalling through the transcription factor (TF) ABI5, which shares with the TF PIF1 several target genes that negatively regulate germination in the embryo; ABI5 enhances the expression PIF1, SOMNUS, GAI, and RGA genes, but also of ABA and GA metabolic genes [119]. Using *Lepidium sativum* as a model target species, experiments were conducted to investigate how environmental cues modulate MyA's interference with key processes of seed germination. Testa permeability and early water uptake by imbibition is enhanced by myrigalone A (MyA: inhibits seed germination and seedling growth). During late germination, MyA inhibits also endosperm weakening and embryo growth – it was evident from the light-modulated severity of the MyA-mediated inhibition of apoplastic superoxide accumulation. It was speculated that MyA is a soil seed bank-destroying allelochemical [122]. *Tagetes minuta* L. achenes are thermoinhibited at temperatures above 35 ◦C and have accelerated radicle emergence (germination) when subsequently transferred to an optimal temperature (25 ◦C) - endogenous cytokinins and cytokinin oxidase/dehydrogenase (CKX) activity were compared [104]. The aims of subsequent study were to monitor endogenous cytokinin levels during germination and early seedling establishment in oats, maize, and lucerne 108].

5) In Arabidopsis, AHK2 and AHK3 were found to be primarily involved in mediating cold to express Atype ARRs despite CK deficiency - cold might induce ARR expression via the AHK2 and AHK3 proteins. The overexpression of the cold-inducible ARR7 in Arabidopsis resulted in a hypersensitivity response to freezing temperatures under cold acclimated conditions. The ahk2 ahk3 and arr7 mutants showed hypersensitive response to abscisic acid (ABA) for germination. These results suggest that AHK2 and AHK3 and the cold-inducible A-type ARRs play a negative regulatory role in cold stress signalling via inhibition of ABA response, occurring independently of the cold acclimation pathway [38]. In subsequent report, the question has been addressed whether members of the small family of Arabidopsis CK receptors (AHK2, AHK3, CRE1/AHK4) are required for BA-induced programmed cell

death (PCD). Cell growth and proliferation of all receptor mutant and wild-type cell cultures were similar, showing that the CK receptors are not required for these processes in cultured cells. The analysis of CK metabolites instead revealed differences between wild-type and receptor mutant lines, and indicated that all three receptors are redundantly involved in the regulation of the steady-state levels of CKs. The results also showed that CRE1/AHK4, the strongest expressed CK receptor gene of this family in cultured cells, is required for PCD, thus linking this process to the known CK signalling pathway [131].

6) The directional auxin distribution within tissues depends on PIN transporters that are polarly localized on the plasma membrane. We identified an evolutionarily conserved phosphorylation site within the central hydrophilic loop of PIN proteins that is important for the apical and basal polar PIN localizations. Inactivation of the phosphorylation site in PIN1(Ala) resulted in a predominantly basal targeting and increased the auxin flow to the root tip. In contrast, the outcome of the phosphomimic PIN1(Asp) manipulation was a constitutive, PINOID-independent apical targeting of PIN1 and an increased auxin flow in the opposite direction [47]. Furthermore, we identified a novel putative auxin transport facilitator family, called PIN-LIKES (PILS). PILS proteins regulate intracellular auxin accumulation at the endoplasmic reticulum and thus auxin availability for nuclear auxin signalling [107]. We also reported the discovery and the functional characterization of the first vacuolar auxin transporter. WALLS ARE THIN1 (WAT1), a plant-specific protein that dictates secondary cell wall thickness of wood fibres, facilitates auxin export from isolated Arabidopsis vacuoles in yeast and in *Xenopus* oocytes. We unambiguously identified IAA and related metabolites in isolated Arabidopsis vacuoles, suggesting a key role for the vacuole in intracellular auxin homoeostasis [182]. In maize, at least five auxin-binding proteins (ABPs) have been identified, yet their functions remain unclear. This study reported the use of maize abp1, abp4, and abp1abp4 mutants to investigate the role of ABPs during maize growth and development. We concluded that ABP1 and ABP4 participate in the growth of maize seedlings, mediate seedling responses to auxin, and interact with light signalling pathway(s) [141]. We also found that knockout of ABP1 or ABP4 results in essentially reduced expression of PHYB gene in dark-grown mesocotyl but not in the expression of PHYA gene and in auxin-induced suppression of PHYA transcript accumulation. The results support the existence of cross-talk between auxin and light signalling and indicate for the first time that ABP1, ABP4 and PHYB genes could share common signalling pathway(s) [143]. Further study was performed to investigate the possible role of ABP1 and ABP4 in Ca2+/auxin-regulated growth in maize (*Zea mays* L.). We provided evidence for a cross talk between ABP4, exogenous auxin, Ca2+, and ZCAX3 during growth of etiolated maize mesocotyl [235]. A combination of pharmacological, genetic, and biochemical approaches indicate that TAA1/TARs and YUCs function in a common linear biosynthetic pathway that is genetically distinct from the CYP79B2/B3 route. In the redefined TAA1- YUC auxin biosynthetic pathway, TAA1/TARs are required for the production of indole-3-pyruvic acid (IPyA) from Trp, whereas YUCs are likely to function downstream. These results strongly suggest that the enzymatic reactions involved in IAA production via IPyA are different than those previously postulated [92]. We also identified an Arabidopsis pyridoxal-phosphate-dependent aminotransferase, VAS1, whose loss-of-function simultaneously increases amounts of the phytohormone auxin and the ethylene precursor 1-aminocyclopropane-1-carboxylate [160]. In the other article, we have demonstrated that 2-oxindole-3-acetic acid (oxIAA) is a major primary IAA catabolite formed in *A. thaliana* root tissues. There is cell type-specific regulation of oxIAA levels in the Arabidopsis root apex. We propose that oxIAA is an important element in the regulation of output from auxin gradients and, therefore, in the regulation

of auxin homeostasis and response mechanisms [186]. Furthermore, we have shown that the auxin response factors ARF6 and ARF8, targets of the microRNA miR167, are positive regulators of adventitious rooting, whereas ARF17, a target of miR160, is a negative regulator. This complex network of transcription factors regulates the expression of three auxininducible Gretchen Hagen3 (GH3) genes, GH3.3, GH3.5, and GH3.6, encoding acyl-acid-amido synthetases. We show that these three GH3 genes are required for fine-tuning adventitious root initiation in the *A. thaliana* hypocotyl, and we demonstrate that they act by modulating jasmonic acid homeostasis [124]. Endogenous auxins were investigated in floral and fruit tissues of the Christmas rose (*Helleborus niger* L.). Free IAA, indole-3-ethanol (IEt), and seven amino acid conjugates were afforded by LC-MS/MS. Among amino acid conjugates, novel IAA conjugates with Val, Gly, and Phe were identified and quantified in the anthers, and in the fruit during development [98].

7) We generated transgenic *A. thaliana* and tobacco (*Nicotiana tabacum* L.) plants with enhanced root-specific degradation of the hormone cytokinin, a negative regulator of root growth. These transgenic plants form a larger root system, whereas growth and development of the shoot are similar. We thus demonstrated that a single dominant gene (cytokinin oxidase/dehydrogenase, CKX) could regulate a complex trait, root growth in a largely organ-autonomous fashion [52]. In the following paper we showed that enzymes CKX3 and CKX5 regulate the activity of the reproductive meristems of *Arabidopsis thaliana*. CKX3 is expressed in the central WUSCHEL (WUS) domain, while CKX5 shows a broader meristematic expression. An increased size of the WUS domain and enhanced primordia formation indicate a dual function for cytokinin in defining the stem cell niche and delaying cellular differentiation [68]. Responses to drought, heat, and combined stress were compared in tobacco (*Nicotiana tabacum* L.) plants ectopically expressing the CKX1 gene of *A. thaliana* L. under the control of either the predominantly root-expressed WRKY6 promoter or the constitutive 35S promoter, and in the wild type. The results indicate that modulation of cytokinin levels may positively affect plant responses to abiotic stress through a variety of physiological mechanisms [171]. We also analysed the CKX7 gene. pCKX7:GUS expression was detected in the vasculature, the transmitting tissue and the mature embryo sac. A CKX7-GFP fusion protein localized to the cytosol, which is unique among all CKX family members. 35S:CKX7-expressing plants developed short, early terminating primary roots with smaller apical meristems, contrasting with plants overexpressing other CKX genes. We hypothesized that the pool of cytosolic cytokinins is particularly relevant in the root procambium where it mediates the differentiation of vascular tissues through CRE1/AHK4 [198]. R50 (sym16) is a pea nodulation mutant that accumulates cytokinin (CK) in its vegetative organs. Total CK content increases as the plant ages because of the low activity of CKX responsible for CK degradation. Thus, although there is a definite CKX post-transcriptional defect in R50 dry seeds, an abnormal CK homeostasis is not the basis of the delay in R50 seedling establishment, which we linked to abnormal amylase activity early in development [126]. We also unravelled the function of the previously described reticulated EMS-mutant dov1 (differential development of vascular associated cells 1). Metabolite profiling unravelled that amino acids that are involved in purine biosynthesis are increased but purine-derived total cytokinins are lowered in dov1. This mutant line has the potential for further investigation of the interaction between metabolism and leaf development [147]. This study looked into the question of whether cytokinins in moss derive from tRNA exclusively. Targeted gene knockout of ipt1 along with localization studies revealed that the chloroplast-bound IPT1 was almost exclusively responsible for the A(37) prenylation of tRNA in Physcomitrella. Cytokinin profiling demonstrated that the total amount of all free cytokinins in tissue was almost unaffected. The data provide evidence for an

additional and unexpected tRNAindependent cytokinin biosynthetic pathway in moss [208]. We were also presenting a comprehensive characterization of the nucleoside N-ribohydrolase (NRH) family in two model plants, *Physcomitrella patens* (PpNRH) and maize (*Zea mays*; ZmNRH), using in vitro and in planta approaches. We identified two NRH subclasses in the plant kingdom, solved their crystal structures and prepared knock-outs of single NRH genes in *P. patens*. All PpNRH knockout plants display elevated levels of certain purine and pyrimidine ribosides and cytokinins that reflect the substrate preferences of the knocked out enzymes [187]. The putative role of cytokinins in reproductive development of oilseed rape (*Brassica napus* L. var. *oleifera*, cv. Górczański) was also investigated in the shoot apices of vegetative and vernalized plants. These results suggest that cytokinins, especially those of the *cis*-zeatin type, are involved in vernalization-induced reproductive development of *B. napus* [99].

8) In contrast to the well-defined polar transport of auxins, the molecular basis of cytokinin transport is poorly understood. We showed that an ATP-binding cassette transporter in Arabidopsis, AtABCG14, is essential for the acropetal (root to shoot) translocation of the root-synthesized cytokinins. AtABCG14 is expressed primarily in the pericycle and stelar cells of roots. Knocking out AtABCG14 strongly impairs the translocation of tZ-type cytokinins from roots to shoots, thereby affecting the plant's growth and development. AtABCG14 localizes to the plasma membrane of transformed cells. In planta feeding of C-14 or C-13-labelled tZ suggests that it acts as an efflux pump and its presence in the cells directly correlates with the transport of the fed cytokinin. Therefore, AtABCG14 is a transporter likely involved in the long-distance translocation of cytokinins in planta [201].

9) Auxin and cytokinin are both critical for division and patterning, but it is unknown how these hormones converge upon tissue development. We identify a genetic network that reinforces an early embryonic bias in auxin distribution to create a local, nonresponding cytokinin source within the root vascular tissue. Experimental and theoretical evidence shows that these cells act as a tissue organizer by positioning the domain of oriented cell divisions. We further demonstrate that the auxincytokinin interaction acts as a spatial incoherent feed-forward loop, which is essential to generate distinct hormonal response zones, thus establishing a stable pattern within a growing vascular tissue [209].

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Laboratory of Signal Transduction, Laboratory of Pathological Plant Physiology and Laboratory of Biologically Active Compounds

The evaluated team consists of three laboratories: the Laboratory of Signal Transduction (LST), Laboratory of Pathological Plant Physiology (LPPP), and Laboratory of Biologically Active Compounds (LBAC). These laboratories have collaborated in the past (mainly LST with LPPP); share laboratory equipment, space (LST with LBAC) and research subjects (responses of plants to biotic and abiotic stresses); and, most importantly, they are willing to converge thematically and personally in future.

Since its establishment the **Laboratory of Signal Transduction** has been focused upon phospholipid signalling. The role of phosphatidylinositol-specific phospholipase C was studied at the beginning, and our main contribution to the field is represented by three articles: Scanlon et al. (1996), Identification and preliminary characterization of a Ca^{2+}-dependent high-affinity binding site for inositol-1,4,5- trisphosphate from *Chenopodium rubrum*, Plant Physiology 110: 867–874; Martinec et al. (2000), Subcellular localization of a high affinity binding site for D-myo-inositol 1,4,5-trisphosphate from *Chenopodium rubrum*, Plant Physiology 124: 475–483; and Krinke et al. (2007) Inositol trisphosphate receptor in higher plants: is it real? Journal of Experimental Botany 58: 361–376. All three articles dealt with inositol-1,4,5 –trisphosphate receptor (IP3R) in plants. Interestingly, the existence and role of IP3R in plants is presently the subject of considerable debate.

In 2002, Jan Martinec published together with Gunther Scherer, leader of a team at the University of Hannover in Germany, an article dealing for the first time with plant phosphatidylcholinehydrolysing phospholipase C, today known as non-specific phospholipase C (NPC) (Scherer et al. (2002), Down-regulation by elicitors of phosphatidylcholine-hydrolyzing phospholipase C and up-regulation of phospholipase A in plant cells. BBRC 293: 766–770). Since that time, the Laboratory of Signal Transduction has been more and more focused upon investigating the role of NPC in plant development and stress responses.

Non-specific phospholipase C (NPC) catalyses the hydrolysis of phosphatidylcholine (PC) to generate phosphocholine and diacylglycerol (DAG). NPC has a long tradition in animal signal transduction (where it is called PC-PLC) to generate DAG as a second messenger in addition to the classical phosphatidylinositol splitting phospholipase C (PI-PLC). Until 2005, however, there was no information as to a role of NPC in plants at gene level. In 2005, Nakamura et al. (J. Biol. Chem. 280) published a paper characterizing the *Arabidopsis* NPC family. In 2006, after several years of unsuccessful effort, we succeeded with the project entitled "Phosphatidylcholine-specific phospholipase C at the crossing point of stress signalling pathways" (project no. 124). From the results of this and ensuing projects (nos. 138–140), we published a series of articles dealing with functional analysis of NPCs.

In three articles, we reported upon the role of NPC in responses of plants to aluminium toxicity. Aluminium ions (Al) have been recognized as constituting a major toxic factor for crop production in

acidic soils, and therefore aluminium's interaction with plants is widely studied. Nevertheless, the exact molecular mechanism and time sequence of individual changes occurring upon Al exposure remains under investigation. In the first of our articles (Pejchar et al., 2010 ID - 349924), we showed biochemically that a significant decrease of DAG in cells treated with $AlCl_3$ was caused by an inhibition of NPC activity. In the next article (Pejchar et al. (2015) Non-specific phospholipase C4 mediates response to aluminum toxicity in Arabidopsis thaliana. Frontiers in Plant Science 6: 66), we focused upon a plasma membrane-bound isoform of NPC, the non-specific phospholipase C4 (NPC4). We examined the impact of Al on the expression, activity, and function of the NPC4. The growth of tobacco pollen tubes rapidly arrested by Al was partially rescued by the overexpression of AtNPC4 while Arabidopsis npc4 knockout lines were found to be more sensitive to Al stress during long-term exposure to Al under conditions of low phosphate. Our observations suggest that NPC4 plays a role in both early and long-term responses to Al stress. In the third article (Pejchar and Martinec, Plant Signaling & Behavior, in press), we hypothesize that the activity of NPC is affected by Al-induced changes in plasma membrane's physical properties.

Salt stress was another stress that was studied in connection with *Arabidopsis* NPCs (Kocourkova et al., 2011 – ID 369946). Expression of NPC4 was highly induced by NaCl. Results from histochemical analysis of P_{NPC4}:GUS plants showed localization of salt-induced expression in root tips. On the biochemical level, increased NPC enzyme activity, as indicated by accumulation of DAG, was observed after salt treatment of *Arabidopsis* seedlings. Phenotype analysis of *NPC4* knockout plants showed increased sensitivity to salinity as compared with wildtype plants. Expression levels of abscisic acid-related genes *ABI1, ABI2, RAB18, PP2CA,* and *SOT12* were substantially reduced in salt-treated *npc4* plants. These observations demonstrated a role for NPC4 in the response of *Arabidopsis* to salt stress.

In the collaboration with the research group from Hannover, we studied a possible role of NPCs in plant development and hormone signalling (Wimalasekera et al., 2010 - ID - 349937). In auxin-treated P_{NPC3}:GUS and P_{NPC4}:GUS seedlings, strong increase of GUS activity was visible in roots, leaves, and shoots and, to a weaker extent, in brassinolide-treated (BL) seedlings. Compared to wild-type, knockouts *npc3* and *npc4* showed shorter primary roots and lower lateral root density at low BL concentrations but increased lateral root densities in response to higher BL concentration. BL-induced expression of *TCH4* and *LRX2*, which are involved in cell expansion, was impaired in BL-treated *npc3* and *npc4*. These observations showed for the first time that NPC3 and NPC4 are important in BLmediated signalling in relation to root growth. We hypothesize that at least one NPC is a plant signalling enzyme in BL signal transduction.

In 2013, we also published a review about non-specific phospholipase C in plants. This review for the first time summarizes information concerning this relatively new plant protein family while focusing upon its sequence analysis, biochemical properties, cellular and tissue distribution, and physiological functions. Possible modes of action are also discussed (Pokotylo et al., 2013 - ID 395057).

All of these articles constitute original work of the Laboratory of Signal Transduction with minor contributions from various collaborators. An exception is Wimalasekera et al. (2010), wherein our contribution consisted in that part of the work dealing with the role of brassinolide signalling/activation of NPC.

It should be noted as well that out of 11 articles dealing with plant NPC presently included in the WOS database, 5 are from the Laboratory of Signal Transduction.

Currently, three more articles dealing with NPC are in preparation. First, we have found that manipulation with *NPC1* expression leads to impaired heat sensitivity in *Arabidopsis*. Second, we have demonstrated that the level of *NPC2* transcripts decreased during PAMP-triggered immunity as well as during effector-triggered immunity. Third, we have analysed the NPC protein family in tobacco. Interestingly, while the tobacco genome does not contain *Arabidopsis* NPC3, NPC4 and NPC5 orthologs, tobacco NPCs (NtNPC1, NtNPC2 and NtNPC6) nevertheless play functionally similar roles as seen in *Arabidopsis*.

In addition to work on NPC, we are participating in many other research projects based on collaboration with other teams, both within the Institute of Experimental Botany and from other institutes and universities in the Czech Republic. Within the Institute of Experimental Botany, we long have collaborated with the Laboratory of Pathological Plant Physiology (Janda et al., 2013 - ID 397251; Matoušková et al., 2014, ID 429540). Further, we collaborate with the Laboratory of Cell Biology (Pleskot et al. 2010, ID 350391; Pečenková et al., 2011 – ID 367701; Pleskot et al., 2012 – ID 390490; Pleskot et al., 2012 - ID 380699; Potocky et al. 2012, ID 0381547; Potocky et al., 2014, ID 429592) and with the Laboratory of Hormonal Regulations in Plants (Jelínková et al., 2010 – ID 343962). We have long-enduring collaboration with the Institute of Chemical Technology Prague (Pleskot et al., 2010 – ID 350391, Kocourkova et al., 2011 – ID 369946, Hynek et al., 2011 – ID 365269; Janda et al., 2013 – ID 397251; Matoušková et al., 2014 - ID 429540) and with Charles University in Prague (Krtkova et al, 2012 – ID 380983). In all such cases, the role of the Laboratory of Signal Transduction was minor and the ideas in those articles originated within the collaborating laboratories.

On an international level, we collaborate with the group from Hannover already mentioned. In the evaluated period, two joint publications were published (Wimalasekera et al., 2010 - ID - 349937; Kocourkova et al., 2011 – ID 369946). Another fruitful collaboration is with Dr. Eric Ruelland from Université Paris Est-Créteil (UPEC), formerly from UPMC Paris (project no. 140). We have published two reviews. One about the role of phosphoglycerolipids in plant hormone signal transduction (Janda et al., 2013 - ID 397251; together with the Laboratory of Pathological Plant Physiology) and another one about NPC (Pokotylo et al., 2013 - ID 395057). A joint experimental article is currently under preparation (regarding NPC1 and heat stress, see above). We have also long-lasting collaboration with Dr. Volodymyr Kravets from the Institute of Bioorganic Chemistry and Petrochemistry, National Academy of Sciences of Ukraine (Kolesnikov et al., 2012 – ID 0380581; Pokotylo et al., 2013 - ID 395057).

The **Laboratory of Pathological Plant Physiology** has been studying plant–microbe interactions for many years. The main interest of the laboratory resides in signalling pathways implicated in defence responses and induced resistance against pathogens. During the evaluated period, the team consisted (on average) of two scientists and three PhD students. The laboratory has a well-defined research direction within which it collaborates with other teams on both national and international levels. Implementing the joint projects with universities has created favourable conditions for incorporating students (Bc., MS. and PhD.) into the team. Thus, they have had good opportunity to benefit from the knowledge and facilities of the laboratory while working on their theses. Besides collaboration with

the academic sphere, the laboratory endeavours to transfer the achieved results to applications via collaboration with institutes of applied research.

The research performed by the laboratory comprises the following three subject areas:

1) <u>Hormonal signalling pathways involved in plant defence against pathogens</u>

Despite huge effort to elucidate hormonal regulations of plant defence in recent years, only limited data have been available to date concerning signalling pathways implicated in resistance to hemibiotrophic pathogens. Our laboratory has contributed significantly to the field, revealing signalling events distinct from biotrophs and necrotrophs (Šašek et al., 2012, - ID 382093). Regarding the interaction of *Brassica napus* with the hemibiotrophic ascomycete *Leptosphaeria maculans*, we clearly showed that resistance to this pathogen is mediated by salicylic acid (SA) signalling in combination with ethylene (ET). This was confirmed both by expression study of SA-associated (*ICS1, WRKY70, PR-1*) and ET-associated (*ASC2a, HEL, CHI*) genes, hormone quantification in infected tissues, as well as pharmacological experiments. We ascribe this unusual cooperation of SA and ET signalling to the hemibiotrophic nature of *L. maculans*. Additional value of this work lies in that it was performed within a natural pathosystem, wherein we demonstrated profound difference between the natural host *B. napus* and the model plant *Arabidopsis* in their response to *L. maculans* infection. This work was done wholly in our laboratory with the exception of electron microscopy.

Another study performed in the laboratory challenged the current understanding of defence signalling during plant interaction with a necrotroph (Nováková et al., 2014 - ID 433554). On the interaction of *Sclerotinia sclerotiorum* with *B. napus*, we demonstrated that hormonal regulation of plant resistance to this necrotroph is more complex than had previously been shown on the model plant *Arabidopsis*. In addition to jasmonic acid (JA) and ethylene, both SA and abscisic acid (ABA) play roles. Moreover, a gene for putative chorismate mutase (*SS1G_14320*) was identified and found to be highly expressed during *S. sclerotiorum* infection. This finding indicates the pathogen's manipulation of the SA level. This work was done wholly in our laboratory, including to formulate hypotheses and experimental work. The other co-authors critically read the manuscript and performed HPLC analysis of hormones.

Another topic of the research was directed to determining the role of reactive oxygen species (ROS) in plant interaction of a hemibiotrophic pathogen *L. maculans* with *B. napus*. ROS serve as a defence compound and important signalling molecule during plant interactions with biotrophs, but their role in the interaction with hemibiotrophs has not yet been fully explained. Our study demonstrates that *L. maculans* behaves like a necrotroph during the early stages of infection, as its virulence is limited in the presence of ROS scavenger. This conclusion also supports the finding that this pathogen resists relatively high levels of hydrogen peroxide *in vitro* (Jindřichová et al., 2011 - ID 429974). The study was conducted entirely in our laboratory in cooperation with a colleague from PPI HAS in Budapest and in consultation with a co-author from UCT Prague.

The research was supported by grant projects (no. 128 and 142) from the Czech Grant Agency.

2) <u>Phospholipid signalling in biotic stress</u>

The research is based upon long-term collaboration with the laboratory of Dr. Eric Ruelland from Université Paris Est-Créteil (UPEC) and prof. Olga Valentová (UCT Prague). Our earlier work had

revealed an interconnection between the SA signalling pathway and a phospholipid signalling system (Krinke et al. (2007) Phosphatidylinositol 4-kinase activation is an early response to salicylic acid in Arabidopsis suspension cells. Plant Physiology 144:1347–1359, and Krinke et al. (2009) Phospholipase D Activation is an Early Component of the Salicylic Acid Signaling Pathway in Arabidopsis Cell Suspensions. Plant Physiology 150:424–436). Recently, we have focused upon the role of phosphatidylinositol 4-kinases (PI4Ks). Our study revealed that PI4Kβ1 and PI4Kβ2 are negative regulators of SA biosynthesis and act upstream of EDS1 (Šašek et al. 2014 - ID 432723, Janda et al., 2014 - ID 441351). The hypothesis was proposed by Dr. Šašek and Dr. Ruelland and the predominant part of the work was done by our laboratory, including to generate *Arabidopsis* triple mutants, expression kinetics, light microscopy observations, resistance tests to Pseudomonas syringae, and ROS quantification. The manuscript was written by Dr. Šašek in cooperation with Dr. Ruelland and critically revised by all co-authors (Šašek et al. 2014 - ID 432723). The team members also participated in a study showing interconnection between the SA pathway, actin cytoskeleton and phospholipid signalling (Matoušková et al. 2014, - ID 429540). This work was done in cooperation with UCT Prague and the second laboratory of our group (the Laboratory of Signal Transduction, IEB ASCR). Team members participated significantly in proposing the hypothesis, quantifying gene expression, and revising the manuscript.

3) <u>Induced resistance to plant pathogens</u>

β-aminobutyric acid (BABA) is generally seen to be a resistance-inducing and priming compound and it is widely utilized experimentally. In our paper (Šašek et al., 2012, - ID 382522), we demonstrated that BABA protected *B. napus* plants against *L. maculans* and, importantly, the BABA had in addition to its priming activity also a direct antifungal activity comparable with that of fungicide. This activity had heretofore been overlooked. The paper also discusses this compound's mode of antifungal action. This finding is of substantial importance to colleagues using BABA in their experiments. With the exception of hormonal analysis, this work was fully performed in our laboratory.

A search for compounds inducing resistance in plants against pathogens has resulted in two publications, prepared in collaboration with UCT Prague. The first demonstrates induction of resistance in *B. napus* against *L. maculans* by elicitors isolated from *L. maculans* mycelium and describes a new trisaccharide fungal pathogen-associated molecular pattern (Kim et al., 2013 - ID 397255). The laboratory's role was in proposing the hypothesis, introducing methods for pathogen cultivation and gene expression analyses, and providing the tests on induced resistance. In the second work, the antimicrobial peptide anoplin revealed its unknown antifungal activity and ability to induce resistance in plants (Jindřichová et al., 2014, - ID 429974). All experimental work was performed in our laboratory while the co-author from UCT Prague participated in discussions and writing the manuscript.

This research was supported by projects from the Czech Grant Agency (No. 133), National Agency for Agricultural Research (No.127 and 129), and Ministry of Education, Youth and Sports (No. 145).

Collaboration with applied research

Long-time collaboration with the Institute of Oilseed Crops (OSEVA Pro. Ltd.), OSEVA Development and Research Ltd., and the Institute of Plant Production Prague has led to three patent

applications which were submitted to the Industrial Property Office of the Czech Republic in 2011 (Nos. PV 2011- 670, PV 2011-774, and PV 2011-842, for which no decisions have yet been issued), one utility model (No. 24517, recognized on 12 November 2012), and one certified methodology (No. SRS 028110/2013, recognized on 27 May 2013). The subjects of these applications are bio-based resistance-inducing compounds/molecules utilizable as active substances in potential crop-protecting preparations. The idea originated within our laboratory, where the main part of the experimental work was performed. The results of our laboratory tests were validated in field experiments by our collaborators. The source material for inducers preparation originated from TBU Zlín (protein inducers) or was prepared in collaboration with UCT Prague (mycelial elicitors).

International collaboration is of a great importance for the laboratory. Research on phospholipid signalling has been proceeding in close collaboration with Dr. Eric Ruelland from Université Paris EstCréteil (UPEC), as shown by joint publications (Šašek et al. 2014 - ID 432723 and Janda et al., 2013 - ID 397251). Another close collaboration has been implemented with the Plant Protection Institute of the Hungarian Academy of Sciences in Budapest (Dr. Joszka Fódor) and Eötvös Loránd University in Budapest, Hungary (Dr. Károly Bóka). Collective work has related to ROS signalling (Jindřichová et al., 2011) and electron microscopy (Šašek et al., 2012, - ID 382093, Šašek et al. 2014 - ID 432723). Work involving *L. maculans* has been carried out in collaboration with the laboratory of Dr. Thiery Rouxel, INRA, Centre de recherche de Versailles-Grignon, France (Nováková et al., 2015 – submitted). New collaborations have been set up on SA signalling and growth trade-offs with Dr. Kenichi Tsuda, which emerged from Martin Janda's 2014 fellowship at Max Planck Institute in Köln, Germany, and on fungal effectors with Dr. Peter Solomon from The Australian National University, Canberra, Australia.

The majority of findings published by the **Laboratory of Biologically Active Compounds** (LBAC) can be assigned to three fields: 1) studies of somatic embryogenesis in conifers; 2) studies of endogenous polyamines, phenolic compounds and phytohormones; and 3) studies of abiotic stresses. Our published papers often address the intersection of these three areas.

In a pair of papers (Schwarzerová et al. 2010 - ID 356952, Vondráková et al. 2014 - ID 432115), we examined the role of actin cytoskeleton in somatic embryo development. The first paper uprooted the dominant opinion that use of the actin-depolymerizing drug latrunculin B conclusively arrests the development of somatic embryos in conifers (Smertenko et al. 2003). We found that latrunculin applied in a particular concentration and at a particular phase of embryo development damaged only the suspensor cells but not the meristematic cells of developing embryos. Moreover, the chemical cleavage of suspensors triggered additional, more synchronized development of embryos. Although the number of fully matured embryos decreased, these embryos were characterized by a higher germination rate and lower incidence of malformations. We describe the functioning of latrunculin (the only anti-actin compound with this effect) as well as a different effect of another actindepolymerizing drug – cytochalasin. The effect of latrunculin is currently being tested in several laboratories gathered under the IUFRO programme. Our findings could be important for automatized commercial production of somatic embryos, where the uniformity and quality of embryos is crucial. In regard to the work leading to publication of Schwarzerová et al. 2010, our team provided the majority of tissue culture experiments, anatomical analyses and cytoskeleton studies, confocal microscopy, and computer image analyses and participated in manuscript preparation. The work reported in Vondráková et al. 2014, meanwhile was fully done by our team.

Additional work examining the role of auxins and their antagonists during the development of somatic embryos in *Abies alba* was described in Vondráková et al. 2011 - ID 367372 and was fully executed by our team. That project proved that a decrease of endogenous indole-3-acetic acid (IAA) level is necessary for the development of fir somatic embryos. The blocking of IAA synthesis did not produce the same effect.

Studies of endogenous polyamines in plants based upon an excellently elaborated method of extraction remains our laboratory's long-term specialization. The role of endogenous polyamines has been studied both during the preparation of embryogenic culture of Norway spruce prior to cryopreservation and during culture regrowth after cryopreservation (Vondráková et al. 2010 - ID 357035; fully the work of the LBAC team). The changes in the endogenous levels of polyamines due to external treatment by putrescine are important for the development of embryos (Vondráková et al., 2015, Plant Growth Regul. 75: 405; fully the work of the LBAC team). Polyamines are active in the defence reaction of cells of spruce embryogenic culture after infection by fungus *Gremmeniella* (Cvikrová at al. 2010 - ID 357022; the work of the LBAC team included analyses of PAL, phenolic acids and polyamines, as well as preparation of the publication). Similarly, two fractions isolated from mycelia of the fungus *Sirococcus strobilinus* evoked a defence reaction comprising changes in the spectrum and concentrations of phenolic compounds and stilbenes (Malá et al. 2011 - ID 367792; all biochemical experiments and preparation of publication were by the LBAC team). Our experiences with the role of polyamines in the somatic embryogenesis of Norway spruce have been reviewed (Malá et al. 2012 - ID 390495; the LBAC team is responsible for a major part of the manuscript).

Aside from conifer somatic embryogenesis, we focus our research on plant stress. The application of cold stress resulted in a decrease in the levels of endogenous phytohormones associated with cell growth and division. During the acclimation phase of the stress response, the plants increased their frost tolerance and began accumulation of dehydrines. Active gibberellins, cytokinins and auxin were elevated, accompanied by a decrease of abscisic acid. Up-regulation of phenolic acids was observed (Vaňková et al. 2014 - ID 429933; the LBAC team was responsible for the phenolic acid studies and part of the phytohormone studies). We also examined the effect of drought stress, heat stress, and a combination of the two on polyamines metabolism in a tobacco plant, characterizing it by an overproduction of proline (Cvikrová et al. 2012 - ID 379082 and Cvikrová et al. 2013 - ID 423949; in the cases of both papers, the LBAC team studied polyamines and the activities of related enzymes and also prepared the publications). The studied abiotic stresses also include salinity. In salt-sensitive and salt-tolerant barley varieties the retention of K^+ , a key determinant of salinity tolerance, was observed to depend upon the endogenous levels of polyamines and reactive oxygen species (ROS) (VelardeBuendia et al. 2012 - ID 387809; the LBAC team was responsible for analyses of polyamines and partly for preparation of the manuscript). Long-term exposure of Norway spruce trees to elevated CO_2 led to increase in the CO_2 assimilation rate and decrease of dark respiration, whereas the structure of needles' mesophyll and the accumulation and localization of phenolic compounds remained unchanged (Lhotáková et al. 2012 - ID 379249: the LBAC team was responsible for analyses of phenolics).

Our collaboration with other teams, both within the Institute of Experimental Botany and from other institutes and universities in the Czech Republic and abroad, is evident from the profile of the published papers. The laboratory frequently participates in projects that are international in scope. Several papers were born in cooperation with scientists in Israel and other states in an international

research programme involving bioactive compound content in fruits (Poovarodom et al. 2010 - ID 356876, Park et al. 2011 - ID 367794; the LBAC team was responsible for analyses of phenolic compounds for both papers). The aforementioned paper Velarde-Buendia et al. 2012 - ID 387809 was prepared under the auspices of the COST Program. Two more papers resulted from bilateral cooperation with Poland (Filek et al. 2010 - ID 357013, Szafranska et al. 2011 - ID 368139; the LBAC team was responsible for analyses polyamines in both papers). Another paper, which originated in Czech–French cooperation under the Barrande programme (Morel et al. 2014 - ID 433191), dealt in a rather complex way with the development of somatic embryos of *Pinus pinaster* under conditions of reduced water availability. This study comprises proteomic, transcriptomic and morphologic analyses, as well as monitoring of endogenous abscisic acid and carbohydrate levels during pine somatic embryo development. The study remains the first paper to describe early molecular mechanisms during pine somatic embryo development and the first paper to combine transcriptomic and proteomic data in the somatic embryogenesis of conifers (the LBAC team was responsible for analyses of ABA and all microscopy, and in part for work involving proteomic data and carbohydrates and preparation of the manuscript).

Two papers on the regulation of photoperiodicity in *Chenopodium* were done in cooperation with the Laboratory of Plant Reproduction (Drabešová et al. 2014 - ID 429593 and Štorchová et al., 2015); the LBAC team was responsible for physiological photoperiodic studies). We also have contributed to other papers, such as a microscopic study reported in Viehmanová et al. 2014 - ID 429935 and a study on accumulation of radionuclides (Soudek et al. 2010 - ID 348487).

This list of publications is far from exhaustive.

During evaluated period 2010-2014 our research was supported by grant projects No. 123, 125, 126, 141, 143 and 144 (Ministry of Education, Youth and Sports) and grant projects No. 131, 132 and 136 (Ministry of Agriculture).

Research Report of the team in the period 2010–2014

Institute	Institute of Experimental Botany of the CAS, v. v. i.

Scientific team	Centre of Plant Structural and Functional Genomics

Research activities of the team followed its long term research program, which focuses on molecular organization and evolution of plant genomes. The emphasis has been on crop plant species with polyploid genomes and, in particular, those which originated by interspecific hybridization. Apart from gaining insights into nuclear genome structure and evolution, an important goal was to produce novel data and biological resources to support the use of molecular and biotechnological tools in plant breeding. The research involved three groups of plants: (a) cereals from the tribe Triticeae (wheat, barley and rye); (b) forage grasses (fescue, ryegrass and their hybrids); and (c) banana. The latter was a continuation of international collaborative projects with IAEA/FAO and more recently Bioversity International. A unique character of the team is the extensive experience and expertise in a broad range of cytogenetic, flow cytometric, molecular biology and genomic methods that permit multidisciplinary and original experimental approaches. Due to its unique position, the team has been a popular partner in international projects and has been working also on species outside the three groups of plants. The research of the team in 2010 - 2014 was supported by grant awards no. 147 - 172.

2.1. Cereal genome analysis

The analysis of many plant genomes, including those of the Triticeae species is complicated by their enormous size and sequence redundancy resulting from high DNA repeat content and presence of homoeologus genomes in polyploids. To overcome these difficulties, the team pioneered the chromosome genomics strategy, which relies on dissection of nuclear genomes to chromosomes or chromosome arms by flow cytometric sorting and preparation of chromosomal DNA suitable for downstream genomics applications. During the past five years, the team employed the strategy in own research projects as well as in collaborative projects with foreign research teams. They also continued in improving and expanding the range of tools so that the strategy can be easily integrated in any genome mapping and sequencing program.

The work involved refinement of a protocol for construction of large insert BAC libraries from flow-sorted chromosomes, and the team remains the only one in the world that can construct this type of DNA libraries. These unique resources are making the biggest impact in the development of ready to sequence physical map of bread wheat. The team is one of the key members of the International Wheat Genome Sequencing Consortium (IWGSC) and after a huge effort, they constructed BAC libraries from all chromosomes of bread wheat (Šafář et al. 2010; ASEP ID 349181). The entire work was done by the team members (projects no. 147 and 154). The availability of chromosome BAC libraries permitted estimation of the fidelity of BAC contig assembly using genomic libraries (Luo et al. 2010; ASEP ID 347998). This was a collaborative project with prof. Jan Dvořák (UC Davis) in which the team provided a set of chromosome BAC libraries and participated in data analysis and publication of

the results. The availability of chromosome BAC libraries from homoeologous chromosomes enabled characterization of changes in genic sequences during wheat genome evolution, including the rates of non-collinear gene insertion and gene duplication (Bartoš et al. 2012; ASEP ID 382219). In this project, the team did all the work, except of DNA sequencing (projects no. 157 and 172).

The suitability of chromosome genomics for producing reference sequence of the wheat genome was definitely confirmed in a collaborative project in which the first reference sequence of (the largest) wheat chromosome (3B) was produced after sequencing BAC clones from its physical maps. This result was published in Science (Choulet et al. 2014; ASEP ID 433903) and provided so far the most detailed information on genome structure of bread wheat. Also in this project, the team contributed key biological materials, including BAC library and DNA from flow-sorted chromosome 3B, participated in data analysis and publication of the results (projects no. 165 and 171). To date, members of IWGSC constructed physical maps from 16 out of 21 wheat chromosomes, including the map of chromosome 6A (Poursarebani et al. 2014; ASEP ID 432116). To generate this physical map, the team contributed by preparing unique biological materials, participated in data analysis and publication of the results. During the process of constructing physical maps, BAC contigs are ordered and oriented predominantly using genetic markers. However, genetic linkage maps suffer from poor resolution in (peri)centromeric regions. To tackle this problem, the team developed a FISH technique to localize cDNA probes shorter than 3.5 kb on plant mitotic metaphase and prometaphase chromosomes, and demonstrated that it could be a valuable tool to order physical map and provide a complementary approach to genetic mapping for chromosome regions with limited or no recombination (Karafiátová et al. 2013; ASEP ID 424633). The team conceived the study, performed all experiments and data analysis and prepared manuscript for publication (project no. 172).

Despite some limitations, genetic linkage maps remain one of the main tools in gene mapping and positional gene cloning projects. These projects benefit from high density maps, and an attractive approach to construct such maps is to develop new markers specifically from target genome regions. In collaborative projects, the team demonstrated that this can be done using DNA from flow-sorted chromosomes (Shatalina et al. 2013; ASEP ID 393905). For example, they showed that the Diversity Arrays Technology (DArT) can be coupled with chromosome sorting to increase the density of genetic maps for specific chromosomes or chromosome arms of wheat. Since a small amount of chromosomal DNA (5 ng) is needed to develop DArT markers, this approach can be readily applied to any crop, for which chromosome sorting is available (Wenzl et al. 2010; ASEP ID 349830). The team conceived the study, prepared chromosomal DNA for DArT analysis and participated in data analysis and publication of the results (project no. 147).

Sequencing DNA amplified from flow-sorted chromosomes by next generation sequencing (NGS) technologies is another powerful application of chromosome genomics developed by the team. Although not suitable for producing gold standard reference sequence assemblies, it finds numerous and important applications. The main advantage is low cost and the speed by which a draft chromosome/genome sequence can be produced. Thus, sequencing DNA of flow-sorted chromosomes of barley allowed assembling a majority of its genes and establishing their putative linear order along all chromosomes. This was the first blueprint of a diploid Triticeae genome and provided a framework for the study of Triticeae genome evolution and the development of novel strategies in cereal breeding (Mayer et al. 2011; ASEP ID 365200). The team contributed to the concept of the study, prepared the unique biological materials and participated in data analysis and publication of the results. In a similarly

novel study, a virtual linear gene order models were established for all chromosomes of rye. A genome-wide high-density comparative analysis of synteny between rye, barley and model grass genomes indicated that introgressive hybridizations and/or a series of wholegenome or chromosome duplications played a role in rye speciation and genome evolution (Martis et al. 2013; ASEP ID 423097). The team contributed to the concept of the study, prepared the unique biological materials and participated in data analysis and publication of the results (projects no. 165 and 172).

In collaboration with other groups, the team demonstrated the utility of chromosome sequencing for the analysis of complex genome of bread wheat in several studies (Berkman et al. 2011, ASEP ID 365378; Berkman et al. 2012, ASEP ID 380686; Wicker et al. 2011, ASEP ID 365266; Vitulo et al. 2011, ASEP ID 366036; Fluch et al. 2012, ASEP ID 380689; Lucas et al. 2012, ASEP ID 380697; Lucas et al. 2014, ASEP ID 441332; Akhunov et al. 2013, ASEP ID 394040; Ma et al. 2013, ASEP ID 423953; Tanaka et al. 2014, ASEP ID 432722). For example, sequencing both arms of chromosome 4A (Hernandez et al. 2012, ASEP ID 380583) facilitated construction of an ordered gene map of the chromosome, embracing over 85% of its genes. The work provided new insights into the evolutionary dynamics between homoeologous chromosomes and syntenic chromosomal regions and enabled precise localization of translocation and inversion breakpoints on the chromosome. Following these pioneering studies, IWGSC decided to sequence all chromosomes of bread wheat using NGS technology. This large-scale exercise was successfully completed in 2014 and the results were published in Science (Mayer et al. 2014, ASEP ID 433366). The work provided novel insights into the genome biology of a polyploid species and the results obtained will facilitate faster gene isolation, genetic marker development, and precise breeding of the important crop. In all projects mentioned in this paragraph, the team contributed to the concept of the studies, prepared unique biological materials and participated in data analysis and publication of results (projects no. 147; 152; 153; 158; 162; 165; 171 and 172).

Given the success of chromosome genomics in obtaining draft genome sequences of cultivated Triticae, the team decided to expand this work to wild relatives of bread wheat (see also the section 3 below). As the first step, they developed a protocol for chromosome sorting in a set of species from genus *Aegilops* (Molnár et al. 2011, ASEP ID 368586). The team conceived the study, performed all experiments except of FISH analyses, and participated in data analysis and publication of the results (projects no. 147; 152 and 172).

The chromosome-centric approaches developed by the team provide and attractive opportunity to study molecular organization and evolution of specialized chromosomes, such as B chromosomes. Their analysis using whole genomic DNA is hampered by the presence of sequences from all remaining chromosomes. Analysing DNA from the chromosome of interest provides and important advantage, including the reduction of sample complexity. The team employed this approach to study the evolution of B chromosome of rye. After sequencing DNA amplified from flow-sorted A and B chromosomes, the origin of Bs could be traced to parts of the A genome. The origin of rye B chromosome could be dated and a comprehensive model was proposed of the stepwise evolution of Bs, which included insertions of organellar DNA (Martis et al. 2012, ASEP ID 381546). In this work the team contributed to the concept of the study, prepared the unique biological materials and participated in data analysis and publication of the results (project no. 165).

2.2. Genome analysis in forage and amenity grasses

The research in forage grasses (*Festuca* spp., *Lolium* spp. and their hybrids), focused on the analysis of their genome structure and evolution at cytogenetic and DNA level. The experiments prior to 2010 established molecular karyotypes of fescue and ryegrass using FISH and determined genomic constitution in a majority of commercially available hybrid Festulolium cultivars using GISH. With the aim to increase the resolution of genome analysis and achieve higher throughput, the team developed DArT markers specific for fescue and ryegrass. Validation of the DArTFest array confirmed its suitability to determine genome constitution in *Festuca* x *Lolium* hybrids at high resolution and its ability to discriminate between Festulolium cultivars. The results also confirmed the potential of the array to follow changes in genome composition of *Festuca* x *Lolium* hybrids during successive generations (Kopecký et al. 2011, ASEP ID 364120). The team conceived the study, performed all experiments except of DArT genotyping, analysed the experimental data and wrote the manuscript (project no. 151).

The DArTFest array was also used to saturate genetic maps of *F. pratensis* and *L. multiflorum* and identify markers associated with traits of interest. Three genomic loci associated with freezing tolerance co-localized with chromosome segments and QTLs previously implicated in freezing tolerance. Moreover, sequencing the markers enabled comparison of genome structure of both species with the genomes of rice and Brachypodium and revealed their syntenic relations (Bartoš et al. 2011, ASEP ID 364761). These results again confirmed the potential of the DArTFest array in genetic studies of the Festuca-Lolium complex. The annotated DArTFest array resources could accelerate further studies and improvement of desired traits in Festuca-Lolium species. The team conceived the study, performed all experiments except of phenotyping and DArT genotyping, analysed the experimental data and wrote the manuscript (projects no. 151 and 172).

Given the lack of detailed information on genome structure of *F. pratensis*, the team applied their chromosome genomics approach to generate a draft genome sequence assembly. As the first step, they flow sorted chromosome 4F and sequenced it by NGS technology. This provided the first insight into the composition of the fescue genome, permitted estimation of gene content, enabled the construction of virtual gene order of the chromosome, and facilitated detailed comparative analysis with the sequenced genomes of rice, *Brachypodium*, sorghum and barley. The analysis of chromosome sequences enabled identification of new tandem repeats, which were mapped using FISH and were found suitable as cytogenetic markers for karyotyping *F. pratensis*, *Lolium* species and their hybrids. This was the first report on dissection of a complex and large forage grass genome using chromosome sorting (Kopecký et al. 2013, ASEP ID 422267). The team conceived the study, performed all experiments, analysed the experimental data and wrote the manuscript (projects no. 159 and 172).

2.3. Analysis of banana genomes

A majority of banana (*Musa* spp.) cultivars are parthenocarpic triploid clones, which originated from natural intra- and interspecific hybridizations between various subspecies of at least to different *Musa* species. However, their exact origin is not known. The research on *Musa* focused on two areas: genetic diversity and phylogeny, and genome structure. The team has been appointed by Bioversity International to serve as Musa Genotyping Centre and genotyping and phylogenetic analyses are part of this mandate (Christelová et al. 2011a, ASEP ID 365578). As the classification of species from the Musaceae (banana) family and their phylogenetic inter-relationships remain controversial, the team

studied evolutionary relationships using 13 species and DNA sequences obtained from a set of unlinked nuclear genes. The study contributed significantly to the classification of the Musaceae family species. The first estimates for the divergence times of the four sections of genus *Musa* were obtained and the study provided a substantial insight into the course of speciation within the Musaceae. An understanding of the main phylogenetic relationships between banana species provided data to fine-tune the taxonomy of Musaceae (Christelová et al. 2011b, ASEP ID 365203). The team conceived the study, performed all experiments, analysed the experimental data and wrote the manuscript (project no. 150).

Internal transcribed spacer (ITS) loci show high level of interspecific divergence and have been used frequently in genetic diversity and phylogenetic studies in various taxa. The team obtained the first detailed information on ITS sequence diversity in the genus *Musa* and characterized the structure and diversity of the ITS region in 87 representatives of the family Musaceae. Phylogenetic reconstruction based ITS sequences showed that the genus is divided into two distinct clades. A need to identify putative pseudogenic ITS sequences, which may have negative effect on phylogenetic reconstruction at lower taxonomic levels was shown. Independent evolution of parental rDNA in hybrids enabled determination of genomic constitution of hybrids using ITS sequences (Hřibová et al. 2011, ASEP ID 365197). The team conceived the study, performed all experiments, analysed the experimental data and wrote the manuscript (projects no. 150 and 156).

In order to provide more insights into the *Musa* genome structure and evolution, the team characterized genomic organization of two main banana DNA satellites together with other DNA sequences in nineteen accessions of *Musa*, including inter-specific hybrids. Molecular analysis of DNA satellites revealed their sequence conservation within and between the accessions. The pattern of their genomic distribution makes them suitable as cytogenetic markers. The study expanded the number of individual chromosomes which can be identified cytogenetically and provided tools to support determination of genomic constitution in interspecific hybrids (Čížková et al. 2013, ASEP ID 394043). The team conceived the study, performed all experiments, analysed the experimental data and wrote the manuscript (projects no. 156; 161 and 172).

In order to provide the knowledge needed to apply molecular and genomics tools in improvement of banana and contribute to the understanding of *Musa* evolution, the team participated in a large-scale sequencing project, which produced a draft sequence of the 523- megabase genome of *M. acuminata*. The banana genome sequence was the first of its kind for a monocotyledon outside Poales and represented an essential bridge for comparative genome analysis in plants. The study revealed three rounds of whole-genome duplications in the *Musa* lineage, which were followed by gene loss and chromosome rearrangements, resulting in little synteny conservation between lineages. The results of the work, which also led to discovery of conserved noncoding sequences predating monocotyledon–eudicotyledon divergence, were published in Nature (D'Hont et al. 2012, ASEP ID 381157). The team contributed to the concept of the study, they were responsible for the analysis of repeated part of the genome, participated in data analysis and publication of the results.

2.4. Legume genome analysis

The team has also made significant contributions to sequencing nuclear genomes of some other species, with the biggest efforts concentrating on legumes, and chickpea in particular. In this species, they used DNA of flow-sorted chromosomes to assign all genetic linkage groups to

chromosomes and thus completed the efforts on integration of genetic and physical maps of this crop (Zatloukalová et al. 2011, ASEP ID 365945). The team conceived the study, performed all experiments, data analysis and prepared manuscript for publication (project no. 147). The team has also developed a powerful approach to validate genome sequence assemblies obtained after whole genome shotgun sequencing (typically using NGS technologies). In two whole genome chickpea assemblies (kabuli and desi types of chickpea) the chromosome-centric approach highlighted short defined regions that were misassembled in the kabuli genome and identified large-scale misassembly in the desi genome (Ruperao et al. 2014, ASEP ID 432721). The results of the study showed that the integration of chromosome genomics tools within genome sequencing projects has a great potential as the whole genome approaches are prone to errors and misassemblies. The team contributed to the concept of the study, prepared the unique biological materials and participated in data analysis and publication of the results (projects no. 165 and 171).