



**ICGEB** International Centre for Genetic  
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## ICGEB RESEARCH GRANTS PROGRAMME

RESEARCH GRANTS  
COMPLETED  
IN 2015

## CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2015



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ARGENTINA	Marcelo J. YANOVSKY	Genetic and genomic analysis of the role of alternative splicing in the regulation of circadian rhythms and flowering time
BANGLADESH	Mohammad Nurul ISLAM	Development of tomato leaf curl disease resistant tomato lines using antiviral strategy
BULGARIA	Anastas G. GOSPODINOV	Mammalian INO80-class chromatin remodelers in genome integrity maintenance
CHILE	Miguel L. ALLENDE	Assessing the influence of oxidative stress and aging on tissue regeneration
CHINA	Shaogang QU	Molecular determinants of the interaction of the glutamate transporters with cation and the transporters' conformational transitions during the substrate transport
COLOMBIA	Thaura GHNEIM HERRERA	Biological nitrogen fixation in rice ( <i>Oryza sativa</i> L.): Targeting American wild species ( <i>Oryza glumaepatula</i> and <i>Oryza latifolia</i> ) as new sources of endophytic diazotrophic bacteria populations with high N <sub>2</sub> -fixing capacity
COSTA RICA	Esteban CHAVES OLARTE	Dynamics of the activation of the two component system BvrRS and the type four secretion system VirB during the initial stages of the intracellular replication of <i>B. abortus</i>
CROATIA	Hrvoje FULGOSI	Dissecting complex regulators of photosynthetic energy conversion
HUNGARY	Ervin BALÁZS	Molecular mode of action of smoke-derived germination cues
MEXICO	Gustavo PEDRAZA ALVA	Characterisation of the transduction pathways activated by <i>M. tuberculosis</i> leading to inefficient immune response
POLAND	Jacek JAWORSKI	mTOR-driven phosphorylation of ZBP1 and Ago2 in neuronal development
RUSSIA	Vladimir A. MITKEVICH	Binase as a potential anticancer agent
SERBIA	Natasa BOZIC	Production and application of alpha-amylases from soil <i>Bacillus</i> strains
SLOVENIA	Tea LANISNIK RIZNER	The role of progesterone metabolism in endometriosis
SOUTH AFRICA	Bongani M. MAYOSI	Genotyping and autonomic analysis of founder families with cardiomyopathy
TUNISIA	Saber MASMOUDI	Microarray-based system for the detection of North African mutations
TURKEY	Birsen CEVHER KESKIN	Microarray analysis and functional characterization of drought stress related genes in wheat

## ARGENTINA

**Title:** Genetic and genomic analysis of the role of alternative splicing in the regulation of circadian rhythms and flowering time

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**Abstract:** Circadian clocks allow organisms from cyanobacteria to humans to anticipate daily and seasonal changes in their environment, and adjust physiological and developmental processes accordingly. Transcriptional feedback loops are important for clock function, but co- and post-transcriptional mechanisms are also emerging as key regulatory steps. In particular, recent work conducted in our laboratory indicates that alternative splicing is an important mechanism controlling clock function in plants and flies. Alternative splicing is prevalent in many organisms, but the mechanisms regulating this process are far from being well understood. Whilst traditional studies have focused mainly on the combinatorial effect of positive and negative auxiliary splicing factors on the regulation of alternative splicing, recent studies suggest a key role for additional mechanisms such as changes in the levels/activity of core spliceosomal components. A long-term goal of this project is to better understand general rules governing the process of alternative splicing. Plants are an excellent model system for this task since many splicing factors/regulators that are essential for viability in a number of eukaryotic organisms are not required for viability in plants. The circadian clock is also an excellent model system for the study of splicing since it depends on a complex set of interactions between transcriptional and post-transcriptional regulatory mechanisms that are highly dosage sensitive. We have preliminary data indicating that core-spliceosomal components, and auxiliary splicing factors such as SR and hnRNP proteins, are regulated at the gene expression level by the circadian clock, and we have also evidence that mutations in some spliceosomal components/regulators affect circadian rhythms in Arabidopsis. Thus, our system constitutes an ideal biological context in which to address how highly dynamic and recurrent changes in the concentration of splicing regulators affect the control of alternative splicing events associated with the proper operation of daily and seasonal regulatory networks.

**Objectives:**

A long-term goal of this project is to better understand the general rules governing the process of alternative splicing and, for that, we will characterise in detail the interactions between light, circadian and splicing regulatory networks, using genetic and genomic approaches. The first specific aim of the project is to perform a genome-wide characterisation of alternative splicing events that are controlled by environmental conditions that regulate clock function and/or are regulated by the circadian clock. We will then use genetic and molecular approaches to characterise the physiological role of the alternative splicing regulated events identified. The second specific aim of this project is to characterise mechanisms underlying the regulation of alternative splicing by the circadian clock as well as by environmental signals that regulate its pace. To achieve this aim, we will analyse defects in alternative splicing present in a subset of mutants that are affected in genes encoding spliceosome components or regulators, or splicing factors that are regulated by the clock at the transcriptional level, for which we have preliminary data indicating that they display alterations in circadian rhythms and/or flowering time. The combination of both approaches will allow us to identify general mechanisms regulating alternative splicing, particularly the role that dynamic changes in the levels of a subset of splicing components play in the control of alternative splicing.

**Results Obtained:**

During this project we conducted an extensive characterisation of the changes in alternative splicing regulated by light and/or temperature changes that control the function of the plant circadian clock. The work conducted resulted in the generation of approximately 400 cDNA libraries, all of which were sequenced using Illumina high-throughput sequencing technology. In total, we have generated more than 4 Tb of RNA-seq data. These datasets include samples from Arabidopsis plants collected under different light and temperature regimes (ranging from 12 to 27 degrees centigrade). It also includes samples from Arabidopsis plants exposed to a light pulse in the middle of the night that simulates seasonal changes in photoperiod, as well as samples from Arabidopsis plants exposed to cold or heat. In addition, we also obtained RNA-seq data using samples from Arabidopsis mutants affected in genes encoding splicing factors as well as genes encoding proteins involved in the regulation of spliceosome assembly such as *PRMT5*, *pICLn* and *GEMIN2*. We also characterised alternative splicing in mutants affecting genes that encode core-spliceosomal components such as LSm4, LSm5 and SFP30, which we show affect the clock and/or the regulation of flowering time, or are regulated by the clock at the transcriptional level. We have

analysed and validated a significant amount of this huge dataset. This analysis was conducted using a bioinformatic pipeline that was specifically developed by our group for this purpose. We found that there is a strong interplay between the signalling networks controlling alternative splicing and those regulating the circadian clock, flowering time as well as acclimation to abiotic stress. In particular, we found that a brief light pulse regulates the splicing of several clock genes as well as splicing factors. Furthermore, we found that a brief pulse of red light controls the alternative splicing of a splicing factor even in a quintuple phytochrome mutant, confirming previous evidence generated by the Kornblihtt lab that light controls alternative splicing in light grown plants acting through the photosynthetic apparatus and independently of photosensory photoreceptors. We also found that the spliceosome assembly factor, GEMIN2, is part of a buffering mechanism that attenuates the effect of temperature changes on a large subset of alternative splicing events, including the alternative splicing of core clock genes. Indeed, in the absence of a functional copy of the GEMIN2 gene, temperature changes cause large alterations in alternative splicing patterns, which result in physiological alterations, including defects in temperature compensation of circadian rhythms. All the knowledge generated has strongly contributed to improve our understanding of the mechanisms regulating alternative splicing in plants, as well as the functional consequences of such regulatory mechanisms. We are sure that these results will allow us, in the near future, to develop novel crops with enhanced productivity by increasing their tolerance to abiotic stress and/or by increasing their synchronicity with favourable environmental conditions.

**Publications:**

- Perez-Santángelo, S., Schlaen, R.G., Yanovsky, M.J.** Genomic analysis reveals novel connections between alternative splicing and circadian regulatory networks. 2013. *Brief. Funct. Genomics* **12(1)**, 13-24
- Rugnone, M.L., Faigón Soverna, A., Sanchez, S.E., Schlaen, R.G., Hernando, C.E., Seymour, D.K., Mancini, E., Chernomoretz, A., Weigel, D., Más, P., Yanovsky, M.J.** LNK genes integrate light and clock signalling networks at the core of the *Arabidopsis* oscillator. 2013. *Proc. Natl. Acad. Sci. USA* **110**, 12120-12125
- Beckwith, E.J., Yanovsky, M.J.** Circadian regulation of gene expression: at the crossroads of transcriptional and post-transcriptional regulatory networks. 2014. *Curr. Opin. Genet. Dev.* **27**, 35-42
- Perez-Santángelo, S., Mancini, E., Francey, L.J., Schlaen, R.G., Chernomoretz, A., Hogenesch, J.B., Yanovsky, M.J.** Role for LSM genes in the regulation of circadian rhythms. 2014. *Proc. Natl. Acad. Sci. USA* **111**, 15166-15171
- Hernando, C.E., Sanchez, S.E., Mancini, E., Yanovsky, M.J.** Genome wide comparative analysis of the effects of PRMT5 and PRMT4/CARM1 arginine methyltransferases on the *Arabidopsis thaliana* transcriptome. 2015. *BMC Genomics* **16**, 192
- Romanowski, A., Yanovsky, M.J.** Circadian rhythms and post-transcriptional regulation in higher plants. 2015. *Front. Plant Sci.* **6**, 437
- Schlaen, R.G., Mancini, E., Sanchez, S.E., Perez Santángelo, S.P., Rugnone, M.L., Simpson, C.G., Brown, J.W.S., Zhang, X., Chernomoretz, A., Yanovsky, M.J.** GEMIN2 attenuates the effects of temperature on alternative splicing and circadian rhythms in *Arabidopsis thaliana*. 2015. *Proc. Natl. Acad. Sci. USA* **112**, 9382-9387

## BANGLADESH

**Title:** Development of Tomato Leaf Curl disease resistant tomato lines using antiviral strategy

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**Abstract:** Tomato Leaf Curl Virus (ToLCV) is a serious threat to tomato production and responsible for the maximum yield loss of tomato in Bangladesh. Yield potential of tomato can be improved through the development of ToLCV resistant tomato lines. In the present investigation, development of ToLCV resistant tomato lines was attempted using an RNAi-based antiviral vector constructed with the goal of generating ToLCV resistance by targeting part of the silencing suppressor gene (AC4) and Coat protein (CP) gene. Genetic diversity of the local tomato cultivars was assessed to find out specific plant genotype to use as source of explants. BARI Tomato 8 and 14 were selected as sources of explants during the establishment of transformation compatible regeneration protocol. Best response towards multiple shoot regeneration from cotyledonary leaf explants of both varieties observed on MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l IAA. Half strength of MS medium supplemented with 0.2 mg/l IAA was found to be the best for root induction. The cotyledonary leaf explants of BARI tomato-8 and BARI tomato-14 were employed for genetic transformation experiments using *Agrobacterium* strain LBA4404 harbouring plasmid pBI121 carrying  $\beta$ -glucuronidase (GUS) reporter gene and nptII gene. Highest rate of transformation was noticed using bacterial OD600 - 0.2, 20 min. infection and two days co-cultivation period in randomly selected explants through transient GUS assay. Molecular confirmation of the plants developed through this protocol validated the efficiency of the protocol. DNA sequences of ToLCV genomes cloned from four different regions showed huge variation among the ToLCV genomes. ORFs were identified and used for primer designing to clone AC4 and CP gene part. AC4(650bp) and CP(260bp) part of ToLCV genome were isolated through PCR and cloned into TOPO Blunt-II end vector. Sequences of AC4 and CP fragment were confirmed through NCBI BLAST search. A 260bp CP fragment was cloned by replacing the GUS gene using BamHI and SacI restriction recognition sites to develop asCP/pBI121 construct and the AC4 fragment was sub-cloned using BamHI restriction recognition site resulting asAC4-CP/pBI121 construct. The anti-sense orientations of AC4 and CP fragments in compared to the CaMV35S promoter were confirmed by restriction digestion, PCR assay and sequencing of final clone of the antiviral vector. The *Agrobacterium tumefaciens* strain-LBA4404 was transformed with the cloned construct (asAC4-CP/pBI121) to exploit the vector for transforming local tomato cultivars.

### Objectives:

- (i) Establishment of suitable protocol for developing transgenic tomato lines;
- (ii) Isolation and cloning of ToLCV coat protein (CP) gene and AC4 gene in pBI121 vector in an anti-sense orientation resulting asAC4-CP/pBI121;
- (iii) Transformation of suitable explants with asAC4-CP/pBI121 construct expressing ToLCV CP and AC4 (viral silencing suppressor) gene in an antisense orientation for developing transgenic tomato lines conferring ToLCD resistance;
- (iv) Resistance/tolerance analysis of transgenic plants will be conducted in confined green house infested with white flies.

### Results Obtained:

- (i.a) Analysis of genetic diversity in eleven tomato (*Lycopersicon esculentum* Mill.) varieties using RAPD Markers;
- (i.b) Establishment of suitable protocol for developing transgenic tomato lines;
- (ii) Isolation and cloning of ToLCV coat protein (CP) gene and AC4 gene in pBI121 vector in an anti-sense orientation resulting asAC4-CP/pBI121.

### Results Unforeseen in the Original Project:

- (i) Transformation of suitable explants with asAC4-CP/pBI121 construct expressing ToLCV CP and AC4 (viral silencing suppressor) gene in an antisense orientation for developing transgenic tomato lines conferring ToLCD resistance (ongoing);
- (ii) Resistance/tolerance analysis of transgenic plants will be conducted in confined green house infested with white flies.

### Publications:

**Dai, X.Y., Gu, X.C., Luo, M., Zheng, X.F.** Protein expression, crystallisation and preliminary X-ray crystallographic studies on HSCARG from *Homo sapiens*. 2006. *Protein Pept. Lett.* **13**, 955-957

**Tabassum, N., Sony, S.K., Bhajan S.K., Islam, M.N.** Analysis of genetic diversity in eleven tomato (*Lycopersicon esculentum* Mill.) varieties using RAPD Markers. 2013. *Plant Tissue Cult. Biotech.* **23(1)**, 49-57

**Das, P., Ansari, A., Islam M.N., Sarker R.H.** Genetic transformation of a local tomato (*Solanum*

*lycopersicum* L.) variety of Bangladesh. 2015. Plant Tissue Cult. Biotech. **25(1)**, 87-97

## BULGARIA

**Title:** Mammalian INO80-class chromatin remodelers in genome integrity maintenance

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**Abstract:** This project aimed to evaluate, in parallel, the roles of mammalian INO80-class chromatin remodelers in 3 aspects of genome integrity maintenance: double strand break (DSB) repair, resistance to replication stress and interstrand crosslink (ICL) repair.

We investigated the role of SRCAP chromatin remodeler in DSB repair. Comet assay analysis and reporter constructs indicated that both HR repair and NHEJ were affected in SRCAP deficient cells. RPA and Rad51 recruitment to nuclear foci after gamma irradiation was impaired in these cells, suggesting that SRCAP is involved in DSB processing prior to HR repair.

A parallel line of investigation was the study of the mammalian INO80 remodeler in replication and replication stress recovery. We found that INO80 was needed for efficient replication elongation, while it was not required for initiation. Using bulk and fibre labelling of DNA, we found that cells deficient for INO80 subunits had impaired replication restart and accumulated collapsed forks. These data indicate that under conditions of replication stress mammalian INO80 protects stalled forks from collapsing and allows their subsequent restart.

The next line of investigation was to study the role of INO80-class chromatin remodelers in interstrand crosslink repair. We found that cells depleted of Ino80 are hypersensitive to crosslinking agents, had impaired FANCD2 monoubiquitination and recruitment, as well as defective DNA damage signalling and we are now looking into the cause of these phenotypes.

**Objectives:**

- (i) To identify the mechanisms by which mammalian INO80-class chromatin remodelers promote double strand break repair;
- (ii) To identify the mechanisms by which mammalian INO80-class chromatin remodelers participate in the replication stress response;
- (i) To study the role of INO80-class chromatin remodelling in interstrand crosslink repair.

**Results Obtained:**

To understand the role of SRCAP remodeler in double strand break (DSB) repair, we tested the efficiency of the process in cells depleted of SRCAP using comet assay and GFP-reporter constructs for homologous recombination (HR) repair and non-homologous end joining (NHEJ). We found that the two DSB repair pathways are affected in knock-down cells. We found that both RPA and Rad51 recruitment are deficient when SRCAP is depleted, indicating a defect in 5'-3' resection step during HR repair. To assess the effect on NHEJ, we followed the recruitment of Lig4-GFP in living cells and found that it is not impaired suggesting that the alternative NHEJ pathway may be affected. These data, together with our previous results regarding the involvement of INO80 remodeler in DSB repair indicate that INO80-class remodelers are mostly required for 5'-3' resection of DSB ends prior to HR repair. We are now trying to understand the contribution INO80 and SRCAP complexes to end resection by measuring the kinetics of the process in living cells.

A parallel line of investigation was the study of the mammalian INO80 chromatin remodelers in replication and replication stress recovery. We have systematically investigated the involvement of the catalytic subunit of the human INO80 complex during unchallenged replication and under replication stress by following the effects of its depletion on cell survival, S-phase checkpoint activation, the fate of individual replication forks, and the consequences of fork collapse. We found that INO80 was specifically needed for efficient replication elongation, while it was not required for initiation of replication. In the absence of the Ino80 protein, cells became hypersensitive to hydroxyurea and displayed hyperactive ATR-Chk1 signalling. Using bulk and fiber labelling of DNA, we found that cells deficient for Ino80 and Arp8 had impaired replication restart after treatment with replication inhibitors and accumulated double-strand breaks as evidenced by the formation of  $\gamma$ -H2AX and Rad51 foci. These data indicate that under conditions of replication stress mammalian INO80 protects stalled forks from collapsing and allows their subsequent restart. A follow up of this work focuses on understanding the precise molecular events that underlie the function of the remodeler and obtained data indicate that it functions in avoiding conflicts between replication and transcription.

The next line of investigation was to study the role of INO80-class chromatin remodelers in interstrand crosslink repair. We found that cells depleted of Ino80 subunit are hypersensitive to mitomycin C in clonogenic survival assay. Since all compounds that cause DNA interstrand crosslinks produce DNA adducts as well, we focused on a process exclusively linked to ICL repair - the monoubiquitination of FANCD2 by the FA core complex and its recruitment to sites of damage.

Monoubiquitinated FANCD2 acts as a landing pad for recruiting the multiple nucleases that are thought to participate in lesion incision. Immunofluorescence staining and live cell imaging indicated that FANCD2 recruitment to lesions is impaired in Ino80-depleted cells after induction of crosslinks. Consistently, Western blot analysis revealed that the rate of monoubiquitination of FANCD2 was reduced in Ino80-deficient cells, as the ratio between the ubiquitinated and non-ubiquitinated forms of FANCD2 increased with slower kinetics. Both ATR and ATM-dependent DNA damage signalling were affected in Ino80-deficient cells. In line with these observations, we found that Rad51 foci formation is impaired in Ino80-deficient cells treated with mitomycin C. We are investigating if slower replication fork movement in Ino80-depleted cells may be the root cause of reduced FANCD2 recruitment and defective ICL repair. In regard of SRCAP involvement in ICL repair, though depleted cells were more sensitive to ICLs, we failed to detect differences in FANCD2 recruitment. Sensitivity of these cells to mitomycin C may be due to a requirement for SRCAP in other processes such as nucleotide excision repair.

**Results Unforeseen in the Original Project:**

At the beginning of this project we did not anticipate the requirement for INO80 remodeler during unchallenged replication, as well as its possible role in preventing replication-transcription conflicts. We also did not expect that SRCAP and INO80 remodeler would both affect DSB end resection.

**Publications:**

**Botev, P., Russev, G., Anachkova, B., Gospodinov, A.** Structural aspects of double strand break repair. 2013. *Biotech. Biotech. Equip.* **27**, 3485-3492

**Gospodinov, A., Hezceg, Z.** Chromatin structure in double strand break repair. 2013. *DNA Repair* **12**, 800-810

**Gospodinov, A., Herceg, Z.** Shaping chromatin for repair. 2013. *Mutat. Res. Rev.* **752**, 45-60

**Vassileva, I., Yanakieva I., Peycheva M., Gospodinov A., Anachkova B.** The mammalian INO80 chromatin remodelling complex is required for replication stress recovery. 2014. *Nucleic Acids Res.* **42**, 9074-9086

## CHILE

**Title:** Assessing the influence of oxidative stress and aging on tissue regeneration

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**Abstract:** Because only the presence of blood vessels can ensure the perfusion of tissues with nutrients and immune cells, vascularisation is a key process in wound healing and tissue regeneration. Under several pathophysiological conditions, vascularisation capacity is decreased and limited regeneration of tissues is observed. As a consequence, low vascularisation represents one of the major problems in the field of regenerative medicine. Accordingly, several approaches that may induce vascularisation are currently under investigation. Although aging and oxidative stress are described as the main conditions related to impaired regeneration of tissues, their influence in tissue vascularisation is not clearly established. Moreover the cellular and molecular mechanisms involved in these processes are not well elucidated. In this work, we studied the influence of oxidative stress on the regeneration capacity of vascular structures in a well-established *in vivo* model, the zebrafish (*Danio rerio*). Transgenic lines expressing the Green Fluorescent Protein (GFP) allowed for effective monitoring of cell and tissue behaviors in the live animal. We used transgenic fish expressing GFP in the vasculature (*fli1::GFP*) and in cells of the innate immune system (e.g., *lysC::GFP*), which allowed us to evaluate the relative contributions of vasculogenesis and inflammation to regeneration and how these were affected by age and oxidative status. We used two models for this work. First, we used adult fish, which will undergo tail fin clips, a harmless procedure that is followed by robust and rapid regeneration. On the other, we used larvae, in which mechanosensory cells were chemically or physically ablated, events that are also followed by regenerative processes that restore the lost cells. In the case of tail fin clips, we compared the regeneration efficiency between fish of different ages (juvenile, adult and senile) and we monitored the progress of vascularisation in the growing fin among these animals. In addition, adult fish and larvae were treated with different oxidative stressors, including cigarette smoke extracts, to compare the extent of regeneration and vascularisation to control animals. We tested diverse conditioning protocols that might accelerate and improve tissue regeneration and that can potentially be translated into clinical settings.

### Objectives:

The principal aim of this project was to establish the zebrafish as an effective model for the study of the effects of oxidative stress on vascular regeneration.

### Results Obtained:

We were able to establish robust regeneration models in both adult and larval animals. In larvae, we used a method described by us to ablate cells, tissues and organs. The use of transgenic lines greatly facilitated the visualisation and evaluation of cell behaviours in vascular tissue and immune cells during regeneration. As a way to induce an oxidative stress state, we developed a cigarette smoke extraction protocol to which the fish were exposed. Regeneration was significantly impaired in treated fish compared with control animals. We also examined the expression of genes induced by a strong oxidant (copper). Finally, we conceived a strategy to supplement tissues with oxygen during regeneration after damage. It consists of implanting photosynthetic algae into the animal tissues to produce oxygen during the critical stage of neovascularisation. Algae can also be genetically engineered to produce growth factors during the process and can be conveniently eliminated after use by simply failing to illuminate the tissue under treatment. We have published proof of concept experiments carried out in fish and mice with the intention of eventually transferring this technology to the clinic.

### Publications:

**Quezada, M., Alvarez, M., Peña, O.A., Henríquez, S., d'Alençon, C.A., Lange, S., Oliva, B., Owen, G.I., Allende, M.L.** Antiangiogenic, antimigratory and antiinflammatory effects of 2-Methoxyestradiol in zebrafish larvae. 2012. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **157**, 141-149

**Armas, P., Margarit, E., Mouguelar, V.S., Allende M.L., Calcaterra, N.B.** Beyond the binding site: *in vivo* identification of *tbx2*, *smarca5* and *wnt5b* as molecular targets of CNBP during embryonic development. 2013. *PLoS One* **8**, e63234

**Ceci, M.L., Mardones-Krsulovic, C., Sánchez, M., Valdivia, L., Allende, M.L.** Axon-Schwann cell interactions during peripheral nerve regeneration in zebrafish larvae. 2014. *Neural Dev.* **9**, 22

**Schenck, T.L., Hopfner, U., Chávez, M.N., Machens, H.G., Somlai-Schweiger, I., Giunta, R.E., Bohne, A.V., Nickelsen, J., Allende, M.L., Egaña, J.T.** Photosynthetic biomaterials: a pathway towards autotrophic tissue engineering. 2015. *Acta Biomater.* **15**, 39-47

- Alvarez, M., Reynaert, N., Chávez, M.N., Aedo, G., Araya, F., Hopfner, U., Fernández, J., Allende, M.L., Egaña, J.T.** Generation of viable plant-vertebrate chimeras. 2015. PLoS One **10(6)**, e0130295
- Moya-Díaz, J., Peña, O., Sánchez, M., Ureta, D., Reynaert, N., Anguita-Salinas, C., Marín, G., Allende, M.L.** Electroablation: a method for axotomy and localised tissue injury. 2015. BMC Dev. Biol. (in press)
- Carrillo, S.A., Anguita-Salinas, C., Peña, O.A., Morales, R.A., Muñoz-Sánchez, S., Muñoz-Montecinos, C., Paredes-Zúñiga, S., Tapia, K., Allende, M.L.** Macrophage recruitment contributes to regeneration of mechanosensory hair cells in the zebrafish lateral line. 2015. (submitted)
- Chávez, M.N., Aedo, G., Fierro, F.A., Allende, M.L., Egaña, J.T.** Zebrafish as an emerging model organism to study angiogenesis in development and regeneration. 2015. Front. Cell. Dev. Biol. (invited review submitted)
- Chávez, M.N., Schenk, T.L., Hopfner, U., Centeno, C., Machens, H.-G., Somlai-Schweiger, I., Heinkenwalder, M., Bono, M.R., Schwarz, C., Allende, M.L., Nickelsen, J., Egaña, J.T.** Towards autotrophic tissue engineering: Photosynthetic gene therapy for tissue regeneration. 2015. Biomaterials (submitted)
- Gallardo, V.E., Peña, O., Elkaloun, A., Burgess, S.M., Allende, M.L.** Activation of immune and tissue repair pathways in zebrafish larvae acutely exposed to copper. 2015. (submitted)

## CHINA

**Title:** Molecular determinants of the interaction of the glutamate transporters with cation and the transporters' conformational transitions during the substrate transport

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**Abstract:** Glutamate transporters are key elements in the termination of the synaptic actions of neurotransmitters. Moreover, they serve to keep the extracellular glutamate concentrations below neurotoxic levels. In this project we elucidated the function of TM2, TM4, TM5, HP1 and HP2 during the substrate transport. We found that the accessibility of some positions of the external part of the TM5 is conformationally sensitive during the transport cycle. Our results indicate that some residues of TM5 take part in the transport pathway during the transport cycle. Our study also suggests that the spatial relationship between TM2 and HP1 is altered and there is a complex relative motion between TM5 and HP2 during the transport cycle. We found that the transporter may undergo complex relative movement between TM4 and HP1/HP2 during the transport cycle. The glutamate and potassium can induce a relative motion between TM2 and TM4. The position of TM2 is considered to have no change during transport cycle for the reason that TM2 is highly conserved between transporter subtypes, so we speculated that this conformational shift occurred mainly due to the movement of TM4. TM4 may moves inward with the transport core relative to the rest of the transporter or rotates toward the Ile-93 and Ile-97 of TM2 in the inward-facing state of transporter.

**Objectives:**

The function and movement relationship of TM2, TM4, TM5, HP1 and HP2 of the glutamate transporters during the substrate transport

**Results Obtained:**

Glutamate transporters play an important role in keeping the synaptic glutamate concentration below neurotoxic levels by translocating this neurotransmitter into the cell. We used cysteine mutagenesis with treatments with membrane-impermeable sulfhydryl reagent MTSET to investigate the change of accessibility of TM5. Cysteine mutants were introduced from position 291 to 300 of the cysteine-less version of EAAT1. We checked the activity and kinetic parameters of the mutants before and after treatments with MTSET, furthermore we analysed the effect of the substrate and blocker on the inhibition of the cysteine mutants by MTSET. Inhibition of transport by MTSET was observed in the mutants L296C, I297C and G299C, while the activity of K300C got higher after exposure to MTSET.  $V_{max}$  of L296C and G299C got lower while that of K300C got higher after treated by MTSET. The L296C, G299C, K300C single cysteine mutants showed a conformationally sensitive reactivity pattern. The sensitivity of L296C to MTSET was potentiated by glutamate and TBOA, but the sensitivity of G299C to MTSET was potentiated only by TBOA. All these facts suggest that the accessibility of some positions of the external part of the TM5 is conformationally sensitive during the transport cycle. Our results indicate that some residues of TM5 take part in the transport pathway during the transport cycle.

We used chemical cross-linking of introduced cysteine pair (V96C and S366C) in a cysteine-less version of EAAT1 to assess the proximity of TM2 and HP1. Inhibition of transport by Copper(II)(1,10-Phenanthroline)<sub>3</sub> (CuPh) and cadmium ion (Cd<sup>2+</sup>) were observed in the V96C/S366C mutant. Glutamate or potassium significantly protected against the inhibition of transport activity of V96C/S366C by CuPh, while TBOA potentiated the inhibition of transport activity of V96C/S366C by CuPh. We checked the kinetic parameters of V96C/S366C treated with or without CuPh in the presence of NaCl, NaCl + L-glutamate, NaCl + TBOA and KCl respectively. The sensitivity of V96C and S366C to membrane-impermeable sulfhydryl reagent MTSET was attenuated by glutamate or potassium. TBOA had no effect on the sensitivity of V96C and S366C to MTSET. These data suggest that the spatial relationship between Val-96 of TM2 and Ser-366 of HP1 is altered in the transport cycle.

We introduced cysteine residue pairs in HP2 and TM5 of cysteine-less-GLT-1 by using site-directed mutagenesis in order to assess the proximity of HP2 and TM5. A significant decrease in substrate uptake was seen in the I283C/S443C and S287C/S443C mutants when the oxidative cross-linking agent CuPh was used. The inhibitory effect of CuPh on the transport activity of the S287/S443C mutant was increased after the application of glutamate or potassium. In contrast, an apparent protection of the transport activity of the I283C/S443C mutant was observed after glutamate or potassium addition. The membrane-impermeable sulfhydryl reagent MTSET was used to detect the aqueous permeability of each single mutant. The aqueous permeability of the I283C mutant was identical to that of the S443C mutant. The sensitivity of I283C and S443C to MTSET was

attenuated by glutamate and potassium. All these data indicate that there is a complex relative motion between TM5 and HP2 during the transport cycle.

Using CuPh for crosslinking cysteine pairs, we found strong inhibition of transport when A243C (TM4) was combined with S366C (HP1), I453C (HP2) or T456C (HP2). These findings were reinforced by the impact of cadmium on transport activity, and both approaches consistently showed that proximity was exclusively intra-monomeric. Under conditions that promote the inward-facing state, inhibition by CuPh in A243C/S366C was reduced, while the opposite was seen when the outward-facing one was stabilised suggesting that the two positions are farther apart in the former conformation than in the latter. Surprisingly, maximal crosslinking of A243C with I453C or T456C was not observed under conditions that promote the inward-facing state. Altogether, our data suggest that the transporter may undergo complex relative movement between these positions on TM4 and HP1/HP2 during the transport cycle.

We investigate the positional relationship between TM2 and TM4 during the substrate translocation. Two cysteine pairs between TM2 and TM4 of cysteine-less-GLT-1 were constructed by using site-directed mutagenesis. A significant decrease of transport activity was seen in the I93C/V241C and I97C/V241C mutants by using CuPh. The inhibitory effects of CuPh on the transport activity of I93C/V241C and I97C/V241C mutants were increased after the application of glutamate or potassium. The MTSET-induced transport activity inhibition of I93C and I97C had no obvious change at different transport phase while the sensitivity of V241C to MTSET was attenuated by glutamate, potassium and TBOA. These data suggest that the TM4b~4c loop has an effect on the substrate transport in eukaryotic glutamate transporter. The TM4b~4c loop plays an importantly assistant role in the conformational changes of transport channel because it can greatly support the conformational shift of HP1、HP2、TM7 and promote the substrate release into the cell. At the same time, some amino acid residues of TM4b~4c loop may be involved in the constitution of the transport channel.

#### **Results Unforeseen in the Original Project:**

The crystal structure of Gl<sub>t</sub><sub>ph</sub> provide some good reference templates for the research of structure and function of EAATs. In our research work sometimes we found that two amino acid residues in different sections of Gl<sub>t</sub><sub>ph</sub> are very close, but the corresponding amino acid residues of EAATs are far away.

#### **Publications:**

**Zhang, X., Qu, S.** The accessibility in the external part of the TM5 of the glutamate transporter EAAT1 is conformationally sensitive during the transport cycle. 2012. PLoS ONE, **7**, e30961

**Rong, Rong, X., Zomot, Elia., Zhang, X., Qu, S.** Investigating substrate-induced motion between the scaffold and transport domains in the glutamate transporter EAAT1. 2014. Mol. Pharmacol. **86**, 657-664

**Zhang, Y., Zhang, X., Qu, S.** Cysteine mutagenesis reveals alternate proximity between transmembrane domain 2 and hairpin loop 1 of the glutamate transporter EAAT1. 2014. Amino Acids **46**, 1697-1705

**X., Zhang, X., Qu, S.** A complex relative motion between HP2 and TM5 in the glutamate transporter GLT-1. 2015. Int. J. Biochem. Cell. Biol. **60**, 1-7

## COLOMBIA

**Title:** Biological nitrogen fixation in rice (*Oryza sativa* L.): Targeting American wild species (*Oryza glumaepatula* and *Oryza latifolia*) as new sources of endophytic diazotrophic bacteria populations with high N<sub>2</sub>-fixing capacity

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**Abstract:** In this study we demonstrated the presence of endophytic diazotrophs bacteria in seeds and vegetative tissues from *Oryza latifolia* and *Oryza glumaepatula* and established the existence of richer bacterial communities in accessions of these wild species compared to commercial cultivars of *Oryza sativa*. A total of 48 distinct bacterial strains were isolated from the cultured fractions as follows *O. latifolia* (11) and *O. glumaepatula* (29), and from *O. sativa* (8). A full characterisation of the cultured bacteria was completed including molecular, biochemical and morphological tests. A total of 40 cultured strains showed the ability to fix nitrogen in *in vitro* bioassays and express the *nifH* genes while 9 strains produced IAA at high concentrations and significantly promoted shoot and root growth of wild and commercial accessions. Analysis of the uncultured fraction of bacterial communities using denaturing gradient gel electrophoresis and metagenomic showed that leaves hosted more complex communities of endophytic bacteria than shoot and roots and that diversity changed in response to the plant phenological stage. Comparison of the profiles obtained for two successive generations indicated that the composition and dynamic of the community is persistent. *In vivo* experiments confirmed the ability of selected strains, transformed with autofluorescent proteins, to cross-colonise different rice genotypes, highlighting their potential for contributing to the plant nitrogen balance. Whole genome sequencing of three of the isolated strains indicates they represent novel species within the genera *Rhizobium*, *Chryseomicrobium* and *Chryseobacterium*.

### Objectives:

- (i) To evaluate the presence endophytic diazotrophic bacteria populations in the tissues of the wild rice species of *O. glumaepatula* and *O. latifolia* collected from natural and cultivated regions in Venezuelan Llanos;
- (ii) To identify culturable and non-culturable components of the bacterial communities by the combination of microbiological and molecular techniques (PCR-DGGE and gene sequencing);
- (iii) To characterise the structure of endophytic bacterial communities established in the tissues of the rice species *O. glumaepatula*, *O. latifolia* and *O. sativa* using PCR-DGGE based in the amplification of 16S rRNA and *nifH* gene fragments;
- (iv) To compare the composition and structure of endophytic bacterial communities from different tissues (seed, stem, leaves and roots) of *O. glumaepatula*, *O. latifolia* and *O. sativa* using PCR-DGGE based in the amplification of 16S rRNA and *nifH* gene fragments;
- (v) To compare the composition and structure of endophytic bacterial communities established in the tissues of *O. glumaepatula*, *O. latifolia* and *O. sativa* through different developmental stages (seedlings and mature plantas) using PCR-DGGE based in the amplification of 16S rRNA and *nifH* gene fragments;
- (vi) To establish the growth promoting effect of auxin producing bacteria in rice plants;
- (vii) To establish the ability of identified culturable diazotrophs and growth promoting bacteria to colonise commercial rice cultivars;
- (viii) To evaluate the capacity of identified diazotrophs for N<sub>2</sub> fixation by acetylene reduction assays and detection of *nifH* gene expression by reverse transcriptase PCR (RT-PCR);
- (ix) To establish the *in vivo* contribution of identified culturable diazotrophs to the nitrogen balance in rice plants.

### Results Obtained:

We demonstrated the presence of endophytic diazotrophs bacteria populations in seeds and vegetative tissues from both *Oryza latifolia* and *Oryza glumaepatula*; and established the existence of richer bacterial communities in these wild species compared to commercial cultivars of *Oryza sativa*. Our evaluations were focused on nine (9) *O. glumaepatula* genotypes and six (6) *O. latifolia* genotypes, representing each of the sampled populations in the field (Venezuelan Llanos). These genetic materials were compared to *O. sativa* cultivars, either locals (2 cultivars: Fedearroz 50 and D-sativa) or international reference materials (2 cultivars: IR64 and Nipponbare). A total of 48 distinct bacterial strains were isolated from the cultured bacteria fractions as follows *O. latifolia* (11 strains) and *O. glumaepatula* (29 strains), and from *O. sativa* (8 strains). From these, 40 strains showed the ability to fix nitrogen in *in vitro* bioassays (growth on JNFB and NFB media) and expressed the *nifH* genes. A full characterisation of the cultured fraction was completed including

molecular (16S rRNA, *rpoB* and *nifH*), biochemical (C-sources, pectinase and cellulose production, antibiotic resistance, auxin and siderophores production and antagonism tests) and morphological tests. A greater number of strains were isolated from seeds (40) than from leaves and stems (8) for all rice species. Sequencing of 16S rRNA gene using primer pairs 27F/1492R and 518F/800R identified 29 different bacterial species, belonging to genera *Kocuria*, *Pantoea*, *Bacillus*, *Ralstonia*, *Agrobacterium*, *Microbacterium*, *Arthrobacter*, *Rhizobium*, *Staphylococcus*, *Erwinia*, *Pseudomonas*, *Curtobacterium*, *Enterobacter*, *Novosphingobium*, *Sphingomonas* and *Chryseobacterium*, all of them with reported biological nitrogen fixation in different crop species. The analysis of cultured fractions was complemented with denaturing gradient gel electrophoresis (DGGE) and metagenomic analysis of the uncultured members of the bacterial communities. DGGE based of the V5 region of the 16S rRNA gene was used to assess community diversity and its changes throughout plant organs. Band patterns analysis revealed a higher number of bands in samples from leaf tissues compared to stems and roots indicating that leaves hosted more complex communities of endophytic bacteria. Differences in community composition were also observed in relation to the phenological stage of the plant. Tissues collected from the same plants at two different phenological stages showed that in leaves, the number of bands decreased from juvenile to flowering plants; therefore in this tissue the bacterial community richness decreased throughout time. In shoots an opposite pattern was observed with an increase in the number of bands from juvenile to mature plants. Comparison of the profiles obtained for two successive generations indicated that the composition and dynamic of the community is persistent, that is, similar differences were observed when comparing the same tissues (shoot and leaves) at the same phenological stage (juvenile and flowering) through two consecutive generations. DGGE profiles were overall similar differing in up to 6 bands between generations. The values of Shannon-Wiener index, which is widely used as an indicator of biodiversity, indicated that the endophytic communities from leaves are more diverse at the juvenile stage, and that overall, leaves hosted more diverse communities than shoots. For metagenomic analysis we decided to prepare a combined DNA sample (seed, leaves and stems) for a pilot evaluation of community composition and diversity before performing detailed analysis of community changes by organs and plant ages. Library construction for V1-V3 regions of the 16S rRNA gene and 454 GS-FLX sequencing were provided by Macrogen. For phylogenetic inference of bacteria, 16S rRNA sequences were analysed against curated NCBI and IMG 16S rRNA databases, using ncbi-blast 2.2.30. A total of 327 clones were identified, representing the classes'  $\gamma$ -proteobacteria (48.6%),  $\alpha$ -proteobacteria (34.3%), actinobacteria (12.8%), clostridia (3.4%), bacteroidetes (0.3%), methanobacteria (0.3%) and  $\beta$ -proteobacteria (0.3%). A total of 26 genera were identified: *Acinetobacter*, *Alkanindiges*, *Arthrobacter*, *Azomonas*, *Bacteroides*, *Beggiatoa*, *Bifidobacterium*, *Butyrivibrio*, *Enhydrobacter*, *Eubacterium*, *Jahnella*, *Leifsonia*, *Methylosphaera*, *Microbacterium*, *Micrococcus*, *Nesterenkonia*, *Novosphingobium*, *Polymorphobacter*, *Prolinoborus*, *Pseudomonas*, *Rheinheimera*, *Sandaracinobacter*, *Sphingobium*, *Sphingomonas*, *Sphingorhabdus*, *Zavarzinia*. Some of these genera (*Acinetobacter*, *Arthrobacter*, *Pseudomonas* and *Sphingobium*) have been already described as endophytes with nitrogen fixing capacity. Among the cultured bacteria set isolated from wild rice species *O. latifolia* and *O. glumaepatula*, we identified 9 species (5 *Pantoea* sp., 2 *Rhizobium* sp., 1 *Pseudomonas* sp., and 1 *Chryseobacterium* sp.) able to produce IAA *in vitro*. High IAA concentrations were detected using a colorimetric assay based in the reaction with Salkowsky reagent. LC-MS analysis unequivocally confirmed the production of IAA by these bacterial species. *In vitro* experiments were designed to test whether and at what extend the IAA produced by the different bacteria promoted the growth of rice seedlings. In order to avoid masking growth effects exerted by the bacteria potential to fix N<sub>2</sub> or to produce siderophores, rice seedlings were treated with supernatants from bacterial growth medium, previously assayed for IAA presence. Seven out of the nine bacteria produced IAA in sufficient amounts to significantly promote shoot and root growth of commercial rice cultivars. Induced growth correlated well with the level of IAA produced by each bacteria species. *Pantoea* and *Rhizobium* were the species with the highest IAA production. A short-term experiment confirmed the ability of selected strains transformed with autofluorescent proteins to cross-colonise different rice genotypes. Preliminary results obtained with SOG26, a strain identified as *Neo* (*Rhizobium*) indicate that it is able to endophytically colonise the *O. glumaepatula* accession from which it was originally isolated but also a commercial cultivar of *O. sativa*. SOG26- *Neorhizobium* was isolated from seed of *O. glumaepatula*; in the current colonisation bioassay we showed it is able to endophytically colonise the roots of *O. glumaepatula* and those of an elite *O. sativa* variety widely cultivated in Colombia. As mentioned above, we have isolated 40 bacterial strains which potentially fix N<sub>2</sub>, as indicated by growth on JNFB media and amplification of *nifH* genes, but their fixation rate need are still being confirmed by APA and *in planta* fixation analysis.

### Results Unforeseen in the Original Project:

Whole genome sequencing (WGS) of putative novel species of bacteria. We chose to perform WGS for unequivocal identification of 3 bacterial strains potentially representing novel species (ID TOG1, SOG18 and SOG26). Our first phylogenetic analysis based in the 16S rRNA gene indicated these bacteria belonged to *Chryseobacterium* (TOG1), *Pantoea* (SOG18) and *Rhizobium* (SOG26) but

they shared < 97% similarity with known bacteria species from these genera. WGS results confirmed the classification within *Chryseobacterium* and *(Neo) Rhizobium* genera for TOG1 and SOG26 respectively, while SOG18 was identified as *Chryseomicrobium* SP. For the genus *Chryseomicrobium* there are no reports of complete genome sequences, thus our results will represent the first published genome for this genus. The strain TOG1, isolated from shoot tissues of *Oryza glumaepatula*, was classified within the *Chryseobacterium* genus (Flavobacteriaceae). ANI indexes showed evolutionary genetic distances over 0.94, the limit for species demarcation, thus TOG1 might represent a new species. For SOG26 WGS analysis supported its classification within the *(Neo) Rhizobium* genus. The strain clustered close to *R. huautlense*. Analysis of its genome structure is currently underway, simultaneously with its genome annotation. Multilocus sequence analysis will be performed to assign a final identity.

Generation of a novel molecular tool. An important component of our molecular work has been the design of *rpob* gene primers that can be used to amplify a fragment of 800-900 pb within the same gene region for different bacterial taxa commonly reported as plant endophytes (Bacillaceae, Burkholderiales, Enterobacteriaceae, Microbacteriaceae, Micrococcaceae, Paenibacillus, Pseudomonadaceae, Rhizobiales, Sphingomonadales, Straphylococcaceae). This is remarkable as *rpob* has been reported to be more robust than *16S rRNA* to discriminate among species within a genus and even among variants of a species, but its use has been hindered by the highly genus specific primers reported.

## COSTA RICA

**Title:** Dynamics of the activation of the two component system BvrRS and the type four secretion system VirB during the initial stages of the intracellular replication of *B. abortus*

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**Abstract:** Bacteria of the genus *Brucella* are zoonotic pathogens that affect domestic mammalian species and have an economic impact. The pathogenesis of *Brucella* lies on its ability to survive within eukaryotic host cells. Two systems are essential for intracellular replication, the two component system (TCS) BvrRS and the type IV secretion system (T4SS) VirB. We have shown that the TCS BvrRS transcriptionally regulates the T4SS VirB and its positive regulator VjbR. Thus, we hypothesised that this TCS senses the transition from the extracellular milieu to the intracellular host cell in order to trigger the expression of VirB and then allow *Brucella* microorganism to reach the Endoplasmic Reticulum. To prove this hypothesis we developed a method to purify intracellular bacteria and a strategy to measure activation of the TCS BvrRS through the determination of levels of BvrR phosphorylation. These methods allowed us to determine that indeed the TCS BvrRS is activated intracellularly in a variety of cell lines and that this activation leads to an increase in the expression of VjbR and the T4SS VirB. Furthermore, we were able to mimic this intracellular virulence circuit *in vitro* by incubating the bacteria in low nutrient medium under acidic pH. This *in vitro* activation was able to circumvent the inhibition of intracellular replication exerted by bafilomycin, demonstrating the relevance of this molecular signaling mechanism in the pathogenesis of brucellosis.

**Objectives:**

- (i) To demonstrate and measure the degree of activation of the TCS BvrRS during the first hours of the infection of host cells;
- (ii) To directly demonstrate and measure the degree of activation of the T4SS VirB during the first hours of the infection of host cells;
- (iii) To demonstrate that upon entrance to eukaryotic host cells, the activation of the TCS BvrRS directly leads to the activation of the VirB promoter.

**Results Obtained:**

We were able to set up a methodology to measure the activation of the TCS BvrRS using an electrophoretic methodology based on the interaction of phosphorylated residues with acrylamide phostag. Using this methodology we were able to demonstrate that BvrR is activated early during the intracellular infection. In addition, mimicking intracellular conditions as nutrient deprivation and acidification we were able to induce the *in vitro* activation of BvrR. Finally, *in vitro* activation of BvrR was able to overcome the bafilomycin-inhibition of intracellular replication demonstrating that this is a biological relevant event in the pathogenesis induced by *B. abortus*. We standardised a methodology for the purification of intracellular bacteria using different sucrose gradients and ultracentrifugation. Using this methodology we were able to determine that the expression of the T4SS VirB increases early during the infection. Furthermore, we expanded our research and proved that the transcriptional regulator VjbR is also induced intracellularly. Both VirB8 and VjbR were also induced *in vitro* under the same conditions that activate BvrR indicating that a virulence circuit exists upon entrance of *B. abortus* to eukaryotic cells in which the TCS BvrRS senses the transition from the extracellular milieu to the intracellular niche. This activation would then lead to VjbR transcription and the cooperation between VjbR and BvrR by binding to the VirB promoter would induce the expression and assembly of this secretion machinery. All these events in concert allow *B. abortus* to evade killing by the lysosomal machinery and reach its final destination within the cell, the endoplasmic reticulum. Finally, we were able to demonstrate that BvrR directly binds to the VjbR promoter and the VirB promoter by mobility shifts assays (EMSAs). This direct proof of regulation together with the above-mentioned data on the activation of the expression of VirB early during the early stages of the intracellular life of *B. abortus* indicate that the TCS BvrRS is activated in order to promote the assembly of the T4SS machinery.

**Results Unforeseen in the Original Project:**

We were able to determine that the ExoS protein from *Sinorhizobium meliloti* is able to complement a *bvrS*-negative *B. abortus* mutant. The complemented mutant was able to survive intracellularly, recovered the ability to chronically-infect mice, was resistant to cationic peptides and the properties of its outer membrane were reestablished. This indicates that alpha-proteobacteria use similar molecular strategies to establish close relationships with distant eukaryotic cells like plant and mammalian cells. In addition, we determined that upon phosphorylation, BvrR dimerizes and that this dimer is the functional form of the activated transcriptional regulator that interacts with the promoters of the regulated genes.

**Publications:**

**Chaves-Olarte, E., Altamirano-Silva, P., Guzmán-Verri, C., Moreno, E.** Purification of intracellular bacteria: isolation of viable *Brucella abortus* from host cells. 2014. In: Host-Bacteria Interactions: Methods in Molecular Biology. D. O'Callaghan and A.C. Vergunst (eds.), **1197**, 245-260

**Chaves-Olarte, E., Herrera-Rodríguez, F., Lizano, E., Cheng, H.P., Walker, G., Cloeckert, A., Frisán, T., Moreno, E., Guzmán-Verri, C.** An environmental bacterial sensor protein from the rhizosphere functions in a mammal intracellular niche. 2015. (in preparation)

**Meza-Torres, J., Altamirano-Silva, P., Chacón-Díaz, C., Moreno, E., Guzmán-Verri, C., Chaves-Olarte, E.** *Brucella abortus* two component system BvrR/BvrS senses transition from an extracellular to an intracellular milieu to trigger virulence mechanisms required for replication. 2015. (in preparation)

## CROATIA

**Title:** Dissecting complex regulators of photosynthetic energy conversion

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**Abstract:** Previously, we have identified a thylakoid membrane protein responsible for tethering of ferredoxin:NADP<sup>+</sup> oxidoreductase (FNR), the enzyme responsible for the final reaction of NADPH synthesis in oxygenic photosynthesis. Using gene inactivation approach in the model plant *Arabidopsis*, we have shown that the binding of FNR to this thylakoid rhodanese-like protein (TROL) is necessary for sustaining optimal rates of photosynthetic electron flow. We have also proposed that TROL is an important checkpoint for redox-regulation of photosynthetic energy distribution.

In the scope of this project, we tested the hypothesis that TROL-FNR interaction influences electron partitioning beyond PSI. We further explored the molecular background of the TROL-FNR interaction, with special emphasis on enhancement of NPQ and plant anti-oxidative responses. In depth characterisation of TROL domains and its interacting partners, by means of elaborate molecular techniques, might contribute to a general understanding of proteins and mechanisms involved in plant responses to stress conditions. Linking observed molecular alterations with changes in plant metabolism should reveal the existence of so-far-overlooked pathways connecting photosynthesis with other organellar and cellular stress avoidance responses.

### Objectives:

The project was divided into eleven objectives: (i) verification of the deletion/exchange mutant complementation plants bearing different forms of TROL precursor, (ii) determination of TROL topology in thylakoid membranes, (iii) determination of factors influencing dual localisation of TROL, (iv) detailed analyses of the ITEP-FNR interaction(s), (v) possible identification of other proteins interacting with TROL, (vi) analyses of possible interaction of rhodanese domain (RHO domain) with plastoquinone, (vii) exploration of the influence of TROL on photosynthetic performance and enhancement of non-photochemical quenching (NPQ), (viii) investigations of the influence of TROL depletion on redox parameters of photosystem I (PSI), (ix) assessment of the redox status of *trol* chloroplasts, as well as selected plant stress responses, (x) verification of nuclear gene expression alterations caused by TROL inactivation, and (xi) characterisation of the At4g01150 gene product.

### Results Obtained:

Imports of all *in vitro* site-directed mutants of the TROL presequence have been imported into isolated intact pea chloroplasts. A single amino acid exchange in the conserved sequence surrounding experimentally determined N-terminus of TROL renders preprotein unprocessed, following the imports in the presence of ATP and subsequent protease treatment. Apparently, the protein integrates only into the inner envelope. ITEP sequence was *in vitro* mutagenised in the residues that were assumed responsible for high-affinity binding of FNR. The constructs were successfully inserted into bait vector pBD, while *Arabidopsis* FNR was cloned in pAD vector. Both constructs were co-transformed into yeast cells. Yeast two-hybrid screen has been performed and the initial results indicate that the exchanges of certain residues in ITEP can either induce or reduce FNR binding. The pH- and light- quantity- dependences of TROL-FNR interaction were also tested in a comprehensive array of Western analyses. Further, collaboration was established with a group performing molecular dynamics simulations. The molecular dynamics simulations indicate that TROL-FNR interaction is complex, and most likely not only pH dependent.

TROL-containing supramolecular complexes were analysed by using dual approach. Complexes resolved on blue-native gel electrophoresis were subjected to tandem mass spectrometry, alternatively, mildly solubilised thylakoid membranes were resolved on sucrose density gradients. All fractions were probed with a set of antibodies directed toward representative components of major photosynthetic complexes. Initial MS analyses were performed and the results are being evaluated. For tandem affinity purification (TAP), *trol* gene was 3'-end tagged with HA and FLAG epitopes. The construct in Gateway binary vector was transformed into *trol* plants and the lines were screened and grown to T4 generation. Western analyses indicate that the affinity labelled TROL can efficiently be detected in whole protein extracts by using HA antibody.

Regarding the modulation of NPQ and other parameters of photosynthetic performance, especially those involving PSI, changes have been observed in almost all tested parameters. The extensive data sets are being evaluated.

We have further demonstrated that the absence of TROL triggers efficient O<sub>2</sub><sup>-</sup> scavenging mechanisms. We propose that the dynamic binding and release of FNR from TROL represents novel and efficient mechanism that maintains linear electron transfer, before pseudo-cyclic flow is

activated. In this respect we have proposed three scenarios: first, it is possible that scavengers utilised by the free FNR contribute to the generation of other radical forms; second, Fd efficiently passes electrons to alternative pathways when FNR is not sequestered to the TROL, which contributes to over-reduction of other pathways; third, electrons leak to various sinks upstream of the PSI, as the absence of FNR sequestration site causes slower rates of NADP<sup>+</sup> reduction and results in over-reduction of linear electron transfer.

**Results unforeseen in the original project:**

We establish that without TROL, light-dependent O<sub>2</sub><sup>•-</sup> generation is reduced, while the generation of other reactive oxygen species is enhanced. Most significantly, O<sub>2</sub><sup>•-</sup> formation induced by herbicide methyl-viologen (paraquat) is greatly diminished in chloroplasts lacking TROL, when plants are pre-acclimated to dark and growth light. We propose that other Fd-dependent pathways downstream of PSI and different from the linear electron transport become dominant by the dynamic detachment of FNR from TROL, thus suggesting novel mechanism of photosynthesis regulation. Alternatively, efficient scavenging of O<sub>2</sub><sup>•-</sup> by FNR-dependent pathways can be envisaged.

**Publications:**

**Vojta, L., Carić, D., Antunović Dunić, J., Lepeduš, H., Kveder, M., Cesar, V., Fulgosi, H.** TROL-FNR interaction reveals alternative pathways of electron partitioning in photosynthesis. 2015. *Sci. Rep.* **5**, 10085

**Tomašić Paić, A., Fulgosi, H.** Chloroplast immunophilins. 2015. *Protoplasma* DOI 10.1007/s00709-015-0828-z

## HUNGARY

**Title:** Molecular mode of action of smoke-derived germination cues

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**Abstract:** Plants interaction with their environment substantially consists of sensing the environmental cues surrounding the plant and responding to the stimuli. In this project we focused on novel emerging signal molecules derived from smoke released by burning vegetation. The active compound KAR1 represents a germination cue, which stimulates the germination and seedling vigour of numerous species including crops, while the inhibitory compound TMB blocks germination. The recent characterisation of the inhibitory cue TMB showed that this compound determines the activity of the promontory compounds and can be used in simplified model systems to simulate the effect of crude smoke. In this project we made efforts to unravel the molecular mode of action of KAR1/TMB interplay, and to establish a model of their action during seed germination using *Arabidopsis* and lettuce as a model plant. Conducting the in-depth molecular biology studies using custom-made microarray, functionalisation tests with the candidate gene *DLK2*, and a structure-activity relationship assay of TMB will definitely add a further dimension to the emerging picture on the effect of smoke compounds and strigolactones on seed germination, seedling development and environmental sensing.

**Objectives:**

Smoke released by natural fires is a major environmental cue in fire-prone habitats and a wide-range of species show enhanced germination responses after exposure to aerosol smoke or smoke-water (for review please see Light et al., 2009). Being a very old evolutionary development, the responsiveness to fire cues remained active even in several species from non-fire prone regions and major crops also. Besides, smoke can also positively affect the post-germination growth, which results in increased seedling vigour. Efforts to identify the active compound from smoke-water resulted in the characterisation of 3-methyl-2H-furo[2,3-c]pyran-2-one (KAR1). Currently, at least six analogues of butenolide (referred as KAR1-KAR6) can be found in smoke and interestingly enough, an inhibitory cue was recently isolated. It was showed that a related butenolide, 3,4,5-trimethylfuran-2(5H)-one is responsible for the inhibitory effects of smoke-water, and showed that it significantly reduces the effect of KAR1 (promoter) when the seeds were treated simultaneously. The positive and negative germination cues represent a diverse suite of chemical signals provided by the environment to signal germination. It is of great interest that the tri-substituted butenolide ring is a common structural feature of these butenolide compounds as well as strigolactones, the carotenoid-derived molecules were shown to be involved in the forming of mycorrhizae, inhibit branching, regulate stature, root growth and architecture, light responses and germination.

There is currently little knowledge on the molecular background of smoke- and KAR1-stimulated germination and the observed increase in seedling vigour. The studies published to date have typically been physiological in nature, investigating similarities between the effects of smoke and other plant growth regulators, such as gibberellins and strigolactones. Deeper insight into the molecular background of smoke action and pilot studies have been published more recently. The exact mechanism and the chain of events during KAR1 and TMB exposition, and the genes orchestrating the effect of both cues were still elusive. However, the recent breakthroughs in butenolide research have revealed a very close parallel between strigolactone and karrikin signalling, presenting enhanced opportunities for elucidating the mode of action of these compounds.

**Results Obtained:**

It was previously reported that smoke released from burning vegetation has a 'dual regulatory' effect on germination, since high concentrations of smoke were shown to inhibit germination, whereas lower concentrations had a promotory effect. The assumption that inhibitory cues may also present in the smoke, was recently supported by the isolation of a related butenolide, 3,4,5-trimethylfuran-2(5H)-one (TMB), that results in delayed and inhibited germination of lettuce achenes. The study indicated that this compound is responsible for the inhibitory effect of smoke-water, and showed that it significantly reduces the effect of KAR1 (promoter) when the seeds were treated simultaneously. Given that TMB and KAR1 share significant structural similarity while their application results in fully contrasting physiological responses, it was intriguing to consider that the two compounds antagonise each other activity. We set up detailed pharmacological and transcriptome analysis studies to shed light on the molecular mechanisms lie behind the effect of these sister molecules. We found that the two cues trigger contrasting expression pattern: KAR1 activates the light-signalling pathway, while TMB shuts it down by promoting ABA-related events.

An intricate pharmacological study supported with a microarray analysis revealed that the two compounds do not compete for the same binding site. The obvious structural and functional similarity between karrikins and strigolactones turned our attention toward these carotenoid-derived molecules, which were shown to be involved in the forming of mycorrhizae, inhibit branching, regulate stature, root growth and architecture, light responses and germination. The recent breakthroughs in SL research have revealed a very close parallel between SL and karrikin signalling and a specific seed-related signalling linked to karrikins. KAR1 treatment in Arabidopsis and lettuce specifically induces the expression of DLK2, the third member of the  $\alpha/\beta$  hydrolase family responsible for strigolactone perception (KAI2 and D14). DLK2 is structurally very similar to the other DWARF proteins, but no functions have been assigned yet. Using *dlk2* mutant and DLK2 overexpressing Arabidopsis lines, simple and double mutant lines with the related  $\alpha/\beta$  hydrolases (*kai2*, *d14*, *dlk2/d14*, *dlk2/kai2*, *d14/kai2*) and triple mutants (*dlk2/d14/kai2*), together with a detailed binding/hydrolase assay, a gene expression study and a protein degradation assay, we identified the role of DLK2 in plant development and unravelled the interaction network between the three hydrolases. Now it seems that DLK2 regulates strigolactone signalling through a push-and pull feedback system.

**Publications:**

**Soós, V., Sebestyén, E., Posta, M., Light, M.E., Kohout, L., Van Staden, J., Balázs, E.** Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce achenes. 2012. *New Phytol.* **196(4)**, 1060-1073

**Ghebrehiwot, H.M., Kulkarni, M.G., Szalai, G., Soós, V., Balázs, E., Van Staden, J.** Karrikinolide residues in grassland soils following fire: Implications on germination activity. 2013. *South African Journal of Botany*, **88**, 419-424

## MEXICO

**Title:** Characterisation of the transduction pathways activated by *M. tuberculosis* leading to inefficient immune response

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**Abstract:** Given that tuberculosis represents a transcendental public health problem, in recent years funding for research aiming the development of new treatments has increased in developed countries. However, to translate this investment into tuberculosis control, it is necessary to overcome the lack of basic knowledge about the molecular mechanisms that dictate the interaction between the bacillus and the macrophage. Understanding how *Mycobacterium tuberculosis* (Mtb) takes control of the macrophage molecular machinery for its own benefit is crucial to develop innovative strategy for tuberculosis control. Without this knowledge, most probably the next generation of drugs to treat tuberculosis, as the present ones, will be aimed to block a basic function of the bacillus thus, leading inevitably to the appearance of new drug resistant strains. In contrast, defining the signal transduction pathway that Mtb activates in the macrophage to invade, survive, proliferate and modulate immune response will allow to design specific drug aimed to target crucial components of the signaling pathways required for these important biological functions and thus eventually control infection by *M. tuberculosis* disease.

It is well known that INF $\gamma$  produced by T lymphocytes plays a key role against Mtb infection. However, Mtb promotes the expression of cytokines like IL-10 and TGF $\beta$  that antagonise INF $\gamma$  production and functions, thus altering the normal immune response allowing successful infection. The signal transduction pathways activated by Mtb leading to IL-10 and TGF $\beta$  expression in macrophages, are poorly understood. Therefore, here we propose that defining the transduction pathways activated by Mtb in the macrophage to negatively modulate the immune response will provide new therapeutic targets for the rational design of specific drugs aimed to restore the immunological response against Mtb.

Dectin-1 activates a signaling pathway involving the tyrosine kinase Syk leading to IL-10 expression in macrophages. This, together with the fact that dectin-1 in conjunction with TLR2 is involved in the production of cytokines induced by Mtb, suggests the involvement of this pathway in Mtb-mediated IL-10 expression. In agreement with data showing that in response to zymosan Syk regulates PKC $\delta$ , and that the PKC pathway is critical for Mtb infection, we determined whether in macrophages, Syk regulates PKC activity in response to *Mycobacterium bovis* (*M. bovis*) infection. Our results indicate that Syk is upstream of PKC in the pathway leading to cytokines gene expression in *M. bovis*-infected macrophages. However, we observed that inhibiting PKC activity resulted in reduced Syk tyrosine phosphorylation. The fact that the PLC $\gamma$  inhibitor had no effect on Syk tyrosine phosphorylation indicate that the PKC isoform that regulate Syk is calcium and diacylglycerol independent and that most probably belong to the atypical PKC subfamily ( $\zeta$ ,  $\lambda$ ). According with this, PKC inhibition also resulted in reduced Syk-dependent tyrosine phosphorylation in *M. bovis* infected macrophages. So far, there is no report indicating Syk direct- or indirect-regulation by PKC. Together these results suggest a positive feed back loop in Syk and PKC activation induced by *M. bovis* infected macrophages and that Syk activity is required for normal PKC activity.

The functional relevance of the crosstalk between Syk and PKC in *Mycobacterium* infected macrophages is highlighted by the fact that blocking either pathways results in a substantial reduction in NF $\kappa$ B and AP-1 transcriptional activity as well as in STAT3 phosphorylation. Interestingly, we also showed that although NF $\kappa$ B and AP-1 activation resulting from the interaction of the Mtb chaperone Cpn60 with CD43 in macrophages is required for TNF $\alpha$  production, this was independent of PKC activation. Together these results suggest that blocking the Syk/PKC pathway might result in the inhibition of IL-10 and TGF $\beta$  expression without affecting that of TNF $\alpha$ , which is critical to control Mtb infection. Thus, our data support the idea that these two signaling pathways might constitute therapeutic targets to reestablish the immune response against *M. tuberculosis*.

**Publications:**

**Pedraza-Alva, G., Pérez-Martínez, L., Valdez-Hernandez, L., Meza-Sosa, K.F., Ando-Kuri, M.** Negative regulation of the inflammasome: keeping inflammation under control. 2015. *Immunol. Rev.* **265**, 231-257

**Torres-Huerta, A., Villaseñor, T., Flores-Alcantar, A., Parada, C., Alemán-Navarro, E., Espitia, C., Pedraza-Alva, G., Rosenstein, Y.** Interaction of the CD43 sialomucin with the

*Mycobacterium tuberculosis* Cpn60.2 chaperonin leads to tumor necrosis factor alpha production. 2017. Infect. Immun. **85(3)**, e00915-16

**Villaseñor, T., Madrid-Paulino, E., Maldonado-Bravo, R., Urbán-Aragón, A., Pérez-Martínez, L., Pedraza-Alva, G.** Activation of the Wnt Pathway by *Mycobacterium tuberculosis*: A Wnt-Wnt Situation. 2017. Front. Immunol. **8**, 50

**Villaseñor, T., Madrid-Paulino, E., Maldonado-Bravo, R., Pérez-Martínez, L., Pedraza-Alva, G.** *Mycobacterium bovis* BCG promotes IL-10 expression by establishing a SYK/PKC $\alpha$ / $\beta$  positive autoregulatory loop that sustains STAT3 activation. 2019. Pathog. Dis. **77(3)**, pii: ftz032

**POLAND**

**Title:** mTOR-driven phosphorylation of IMP1 and Ago2 in neuronal development

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**Abstract:** Local protein synthesis occurs in subdomains of a cell, distant from the major sites of protein production. Accurate spatial distribution of mRNA to dendrites regulates proper dendritic arbor development and dendritic spine formation. Recently, several groups presented evidence that mTOR couples extracellular cues to local translational machinery during neuronal development and plasticity. However, besides translation initiation control, an important aspect for local translation is regulation of translational competence of mRNA to be translated. Such competence can be regulated by either RNA-binding proteins or microRNAs. This grant aimed to verify hypothesis that mTOR can control local translation by means of regulation of function of selected RNPs (IMP1) and RISC complex components (Ago2). As a result of the proposed research we show that IMP1 but not Ago2 is a substrate for mTOR. mTORC2 phosphorylates IMP1 on Ser181 in neurons and this phosphorylation is additionally modulated by Src kinase and RNA-binding to IMP1. The phosphorylation does not regulate IMP1 RNA-binding capability, its docking to RNPs or its stability. However, it seems to control either mobility of IMP1 or its inclusion to specific types of granules. Finally, we show that phosphorylation of S181 of IMP1 is critical for dendritogenesis.

**Objectives:**

Thus far, mTOR was postulated to control translation initiation and elongation. Our preliminary results suggested that it can also influence translational competence of mRNA, acting on RNA-binding protein IMP1 and Ago2, a RISC complex protein. This grant aimed to verify hypothesis that mTOR can control local translation by means of regulation of function of IMP1 and Ago2. Two major objectives were established: (i) to verify the role of mTOR kinase in regulation of functional status of IMP1 and Ago2 in neurons and by this means mTOR role in control of local status of mRNA translational competence and (ii) to establish the biological meaning of mTOR-dependent regulation of local status of mRNA translational competence for neuronal development.

**Results Obtained:**

In *in vitro* kinase assay, we found that IMP1, but not Ago2, serves as a substrate for mTOR kinase and using bioinformatic approach we selected S181 and T249 as potentially phosphorylated residues. In course of our project, other team reported that S181 is phosphorylated by mTOR complex 2. Therefore we focused on this particular residue. Using antibody against P-S181 IMP1 we showed that in cortical neurons long-term (48 h) treatment with ATP-competitive inhibitors of mTOR or knockdown of Rictor (mTORC2 component) resulted in decrease of P-IMP(S181) (Fig. 2). On the other hand, activation of mTOR by ~20-30 min treatment with BDNF increased P-IMP1 (S181). We also showed that similar to non-neuronal cells phosphorylation of S181 of IMP1 can be modulated by Src-driven phosphorylation of IMP1Y398 and subsequent release of RNA. Next we tested, which properties of IMP1 can be influenced by phosphorylation of S181 in neurons or neuron-like cells (N2a). There were no differences between wild type and mutated IMP1 with regard to: (i) binding canonical target of IMP1-  $\beta$ -actin, (ii) binding of IMP1 to RACK1 (protein shown recently to be critical for IMP1-mRNA docking to RNP granules), (iii) IMP1 stability and (iv) IMP1 sumoylation. However, our experiments revealed that in hippocampal neurons phosphorylation of S181 of IMP1 is needed for proper dendritic distribution of IMP1 and its canonical target -  $\beta$ -actin mRNA. In case of both, IMP1 itself and  $\beta$ -actin mRNA, lack of IMP1 S181 phosphorylation resulted in lack of characteristic enrichment of IMP1 and  $\beta$ -actin mRNA in dendrite branch points and in their accumulation in proximal dendrites. Additionally live imaging of IMP1 and its mutants in neurons showed that IMP1 phospho-deficient mutant is more mobile regardless of the type of movement (oscillatory movements vs. long-distance movements), while WT seems to be more stationary. Finally we showed that phosphorylation of S181 of IMP1 is necessary for proper development of dendritic arbor of developing hippocampal neurons cultured *in vitro*.

**Results Unforeseen in the Original Project:**

In the original project we assumed that mTOR will affect mRNA binding by IMP1. However evidence obtained in frame of this project argues against this hypothesis, at least in case of its canonical target -  $\beta$ -actin mRNA. On the other hand, unexpectedly we discovered that phosphorylation of S181 of IMP1 regulates mobility of IMP1 in dendrites. This result suggests that depending on phosphorylation status IMP1 might be either recruited to different types of granules or differentially interacts with motor proteins.

**Publications:**

**Urbanska, A.S., Janusz, A., Urbanska, M., Hawthorne, A., Basell, G.J., Jaworski, J.** Role of IMP1 serine 181 phosphorylation in IMP1 mobility and development of dendritic tree of hippocampal neurons. 2015. (in preparation)

## RUSSIA

**Title:** Binase as a potential anticancer agent

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**Abstract:** Some RNases selectively attack malignant cells, triggering apoptotic response, and therefore are considered as alternative chemotherapeutic drugs. We turn our attention to *Bacillus intermedius* RNase (binase) as a potential platform for anticancer therapy. In the frame of the project we have found that the expression of an activated oncogene determines the sensitivity of cells to binase. It was shown that the mutation of the residues Glu43 and Phe81 playing a key role in the formation of the dimeric structure of binase results in that the mutant form loses the ability to form dimers. The mutant form showed higher enzymatic and cytotoxic activity. We have shown that intraperitoneal injection of binase at a dose range 0.1-5 mg/kg results in retardation of primary tumour growth up to 45% in Lewis lung carcinoma (LLC) and inhibits metastasis up to 50% in LLC and up to 70% in B-16 melanoma. Binase does not exhibit overall toxic effect and displays a general systemic and immunomodulatory effects. It was demonstrated that the cytotoxic effect of binase is realized via the induction of the intrinsic and extrinsic apoptotic pathways. Thus binase is a potential anticancer therapeutics inducing apoptosis in cancer cells.

**Objectives:**

- (i) To evaluate the input of activated oncogenes-transcription core factors AML1-ETO, RUNX1 and receptor tyrosine kinases KIT, FLT3, which are frequently detected in myelogeneous leukemia, in the sensitivity to binase of cancer and transgenic model cells, expressing these oncogenes;
- (ii) To increase the cytotoxicity of binase by modifying the enzyme molecule using site-specific mutagenesis;
- (iii) To determine the anticancer and antimetastatic activity of binase in a model system using mice with transplanted solid tumours – Lewis lung carcinoma and Hepatoma A-1;
- (iv) To determine the key elements of the signalling network, taking part in the mechanisms of binase cytotoxic actions, including the changes in the gene expression levels and second messenger contents.

**Results Obtained:**

(i) We studied the effects of binase on murine myeloid progenitor cells FDC-P1, transduced FDC-P1 cells ectopically expressing mutated human *KIT* N822K oncogene and/or human *AML1-ETO* oncogene, and human leukemia Kasumi-1 cells expressing both of these oncogenes. Besides that, we compared effects of binase on transgenic B-cells BAF3/*FLT3*-ITD and original BAF3 cells. Expression of both *KIT* and *AML1-ETO* oncogenes makes FDC-P1 cells sensitive to the toxic effects of binase. Kasumi-1 cells were the most responsive to the toxic actions of binase among the cell lines used in our work with an  $IC_{50}$  value of 0.56  $\mu$ M. Either blocking the functional activity of the *KIT* protein with imatinib or knocking-down oncogene expression using lentiviral vectors producing shRNA against *AML1-ETO* or *KIT* eliminated the sensitivity of Kasumi-1 cells to binase toxic action and promoted their survival, even in the absence of *KIT*-dependent proliferation and antiapoptotic pathways. Our data suggest that the cooperative effect of the expression of mutated *KIT* and *AML1-ETO* oncogenes is crucial for selective toxic action of binase on malignant cells. In addition we show that BAF3/*FLT3*-ITD cells are much more sensitive to the binase cytotoxic effects than the original BAF3 cells. Increased binase cytotoxicity toward the cells, expressing *FLT3*-ITD oncogene, suggests that, as in the case of FDC-P1 cells, transduced by *KIT* oncogene, the expression of an activated oncogene determines the sensitivity of cells to binase. These findings can facilitate clinical applications of binase providing a useful screen based on the presence of the corresponding target oncogenes in malignant cells.

(ii) It was hypothesized that the mutant form (Glu43Ala/Phe81Ala) of binase would not form dimers, what should lead to increase in its enzymatic activity and cytotoxicity. In our study we prepared the Glu43Ala/Phe81Ala mutant of binase, determined its structure by X-ray diffraction, and studied catalytic and cytotoxic properties. It was shown that the mutation of the residues Glu43 and Phe81 playing a key role in the formation of the dimeric structure of binase results in that the mutant form loses the ability to form dimers. The mutant form showed 1.7 times higher enzymatic activity toward poly(I) compared to the wild-type enzyme, and the cytotoxic activity in acute myeloid leukemia Kasumi-1 cells of the mutant is 23 % higher.

(iii) The antitumour potential of binase was evaluated *in vivo* and the mechanism of cytotoxic effect of binase on tumour cells was comprehensively studied *in vitro*. We investigated tumouricidal activity of binase using three murine tumour models of Lewis lung carcinoma (LLC), lymphosarcoma RLS<sub>40</sub> and melanoma B-16. We show for the first time that intraperitoneal injection

of binase at a dose range of 0.1-5 mg/kg results in retardation of primary tumour growth up to 45% in LLC and RLS<sub>40</sub> and inhibits metastasis up to 50% in LLC and RLS<sub>40</sub> and up to 70% in B-16 melanoma. Binase does not exhibit overall toxic effect and displays a general systemic and immunomodulatory effects. Thus binase is a potential anticancer therapeutics inducing apoptosis in cancer cells.

(iv) We have shown that binase induces extrinsic and intrinsic apoptotic pathways in leukemic Kasumi-1 cells. It was demonstrated dissipation of the mitochondrial membrane potential, opening of mitochondrial permeability transition pores, activation of caspases, increase of intracellular Ca<sup>2+</sup> and decrease of reactive oxygen species levels. We found that expression of 62 apoptotic genes is up-regulated, including 16 genes that are highly up-regulated, and only one gene was found to be down-regulated. The highest, 16-fold increase of the expression level was observed for TNF gene. Highly up-regulated genes also include the non-canonical NF-κB signalling pathway and inflammatory caspases 1,4. These results suggest that binase induces evolutionary acquired cellular response to a microbial agent and triggers unusual apoptosis pathway.

**Publications:**

**Mitkevich, V.A., Petrushanko, I.Yu., Spirin, P.V., Fedorova, T.V., Prassolov, V.S., Ilinskaya, O.N., Makarov, A.A.** Sensitivity of acute myeloid leukemia Kasumi-1 cells to binase toxic action depends on the expression of *KIT* and *AML1-ETO* oncogenes. 2011. *Cell Cycle* **10**, 4090-4097

**Mironova, N.L., Petrushanko, I.Y., Patutina, O.A., Sen'kova, A.V., Simonenko, O.V., Mitkevich, V.A., Markov, O.V., Zenkova, M.A., Makarov, A.A.** Ribonuclease binase inhibits primary tumour growth and metastases via apoptosis induction in tumour cells. 2013. *Cell Cycle* **12**, 2120-2131

**Mitkevich, V.A., Kretova, O.V., Petrushanko, I.Yu., Burnysheva, K.M., Sosin, D.V., Simonenko, O.V., Ilinskaya, O.N., Tchurikov, N.A., Makarov, A.A.** Ribonuclease binase apoptotic signature in leukemic Kasumi-1 cells. 2013. *Biochimie* **95**, 1344-1349

**Mitkevich, V.A., Schulga, A.A., Trofimov, A.A., Dorovatovskii, P.V., Goncharuk, D.A., Tkach, E.N., Makarov, A.A., Polyakov, K.M.** Structure and functional studies of ribonuclease binase Glu43Ala/Phe81Ala mutant. 2013. *Acta Crystallogr. D. Biol. Crystallogr.* **D69**, 991-996

**Mitkevich, V.A., Burnysheva, K.M., Ilinskaya, O.N., Pace, C.N., Makarov, A.A.** Cytotoxicity of RNase Sa to the acute myeloid leukemia Kasumi-1 cells depends on the net charge. 2014. *Oncoscience* **1**, 738-744

**Sen'kova, A.V., Mironova, N.L., Patutina, O.A., Mitkevich, V.A., Markov, O.V., Petrushanko, I.Y., Burnysheva, K.M., Zenkova, M.A., Makarov, A.A.** Ribonuclease binase decreases destructive changes of the liver and restores its regeneration potential in mouse lung carcinoma model. 2014. *Biochimie* **101**, 256-259

## SERBIA

**Title:** Production and application of  $\alpha$ -amylases from soil *Bacillus* strains

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**Abstract:** Starch represents an inexpensive source for the production of glucose, fructose and maltose syrups and for obtaining the products of their fermentation, including biofuels. The importance of the enzymatic liquefaction of granular starch below the temperature of gelatinisation has been well recognised, mainly due to energy savings and the effective utilisation of biomass, which reduces the overall cost of starch processing. The availability of raw-starch-digesting amylases (RSDA) that are able to catalyse hydrolysis of raw starch below gelatinisation limits is a key factor for developing processes to better utilise natural resources and optimise energy consumption. Since wheat, corn and potato are the most important sources of starch in EU, enzymes able to digest these types of raw starches efficiently are economically attractive. Enzyme discovery through mining of natural sources has already increased the spectrum of useful catalytic properties. However, few of the identified natural enzymes have been applied in design and development of the process. The best candidate identification would depend on superior kinetic capabilities of the enzyme: intrinsic activity, stability, inhibition, thermal stability, pH stability, etc. The identification of the most promising candidate enzymes from the set of soil *Bacillus* sp. candidates and the identification of their controlling genes will ultimately result in their transfer to and expression in a suitable fermentative or expression system, for application in the food industry and for obtaining the products such as fuel ethanol.

**Objectives:**

(i) Isolation and characterisation of *Bacillus* strains (creation of bank of strains available to scientific community) suitable for application in the food and bioethanol industries with the use of raw materials available and produced in Serbia and creation of technology directly applicable to our environment;

(ii) To scale-up production of amylase and to obtain enough quantities of enzyme for biochemical characterisation such as pH optimum, effect of temperature on activity and stability, the effect of metal ions, absorptivity, etc.;

(iii) Recombinant production of highly efficient RSDA with desired properties in *Escherichia coli*. *E. coli* expression systems in many cases are advantageous to overexpress *Bacillus* hydrolytic enzymes, especially for protein secretion. The secretion system of *E. coli* has been extensively studied and much is known about the molecular mechanism of protein secretion;

(iv) Following recombinant amylase production the goal is to optimise the production of amylase by scaling up the production to laboratory fermenter in the batch and fed-batch cultures and to optimise downstream processing in order to get economical procedure for amylase purification;

(v) Application of recombinant raw starch digesting amylase in real industrial conditions (raw starch materials) for successful direct hydrolysis of raw starches below gelatinisation temperature.

**Results Obtained:**

Bank of about 160 wild type amylase producing *Bacillus* strains was created and several strains, members of *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. methylotrophicus* and *B. vallismortis* subspecies, were analysed for possibilities of application in hydrolysis of raw starch originating from main agronomic crops in Serbia and Europe. In addition effects of different cultivating conditions (solid state fermentations – media, temperature, humidity etc. and liquid culture fermentations – media, temperature, oxygen level etc.) were analysed in order to optimise fermentations of isolated strains and several native enzymes were isolated and characterised. Purification and characterisation of RSDA showed that not all of the media and temperatures tested induced RSDA. From an economic point of view it might be significant to obtain pure isoenzyme for potential use in the raw starch hydrolysis, since it was 5 times more efficient in raw corn starch hydrolysis than the crude amylase preparation.

*Bacillus* strains with unique characteristic among *Bacillus* amylase were identified. Characterisation of enzyme properties revealed that for example strain identified as *B. amyloliquefaciens* produced amylase with relatively high optimum temperature range of 60-90°C, considerable stability at 80°C, while calcium ions did not appear essential for the enzyme stability, which is not so common for amylases from *B. amyloliquefaciens*. The other example is amylase from strain designated as *Bacillus* sp. 16B. Optimum pH for this enzyme was 4.0-6.5 unlike the most of *Bacillus* strains. Reaction products distributions analysed by TLC shown G2, G3 and in lesser extent G4 were the only end product of hydrolysis of all starches tested.

Cloning of different amylases genes and heterologous expression in *E. coli* were performed. Using defined media for *E. coli* and controlled fermentation, amylase activity (IU/mL) obtained was 7 fold

higher than in shake flask. Furthermore using feed batch operation mode the obtained amylase activity (IU/mL) was 50 fold higher than in the shake flask, which corresponds to approximately 0.5 g/L of extracellular amylase. This high productivity enables us to perform concentrated raw starch hydrolysis study. Additional step for scale-up of productivity was using C43(DE3) instead of BL21(DE3) expression cells where 2 fold higher amylase activity (IU/mL) which corresponds to approximately 1 g/L of amylase produced extracellularly.

Several approaches were developed for downstream processing of proteins in order to get economical procedure for amylase isolation. One of them exploited new mixed mode resins with unique selectivity that allows the use of hydrophobic and cation exchange interaction to achieve effective purification. Pure proteins were obtained with high recovery from *Bacillus* sp. and *E. coli* directly from fermentation broth under high salt concentration, while pigments from broth were eluted in flow through fraction. This is not the case with common ion exchangers where pigments are reducing usability of resin significantly and thus increase overall costs of purifying target protein.

Response surface methodology was used to explore the action of amylase on the cold hydrolysis of high concentrated raw corn suspension to determine the optimum hydrolysis conditions leading to maximum efficiency and starch utilisation ratio. Final hydrolysis degree of 100% was obtained even for the hydrolysis of 30% starch suspension after 22 h and can be modulated by changing enzyme doses vs. incubation time upon need.

#### **Results Unforeseen in the Original Project:**

Up to five genes coding for amylase-related putative enzymes were identified after the genomes sequencing of selected amylase producing strains. Some of them contain carbohydrate binding modules suggested to play important role in raw starch hydrolysis. Identification of starch binding domains or surface binding sites might explain the reasons for ability of some amylases to act on raw starch.

#### **Publications:**

**Božić, N., Šokarda Slavić, M., Gavrilović, A., Vujčić, Z.** Production of raw-starch-digesting  $\alpha$ -amylase isoform from *Bacillus* sp. under solid-state fermentation and biochemical characterisation. 2014. *Bioprocess Biosyst. Eng.* **37**, 1353-1360

**Glgorijević, N., Stevanović, N., Lončar N., Baošić, R., Vujčić, Z., Božić, N.** Thin layer chromatographic comparison of raw and soluble starch hydrolysis patterns of some  $\alpha$ -amylases from *Bacillus* sp. isolated in Serbia. 2014. *J. Serb. Chem. Soc.* **79**, 411-420

**Šokarda-Slavić, M., Božić, N., Vujčić, Z.** Growth temperature of different local isolates of *Bacillus* sp. on the solid state affects different production of raw starch digesting amylases. 2014. *Arch. Biol. Sci.* **66**, 483-490

**Lončar, N., Fraaije, M.W.** Not so monofunctional - a case of thermostable *Thermobifida fusca* catalase with peroxidase activity. 2015. *Appl. Microb. Biotechnol.* **99(5)**, 2225-2232

**Lončar, N., Šokarda Slavić, M., Vujčić, Z., Božić, N.** Mixed-mode resins: taking shortcut in downstream processing of raw-starch digesting  $\alpha$ -amylases. 2015. *Sci. Rep.* **5**, 15772 (doi: 10.1038/srep15772)

**Šokarda-Slavić, M., Pešić, M., Vujčić, Z., Božić, N.** Overcoming hydrolysis of raw corn starch under industrial conditions with *Bacillus licheniformis* 9945a  $\alpha$ -amylase. 2015. *Appl. Microb. Biotechnol.* (in press)

**Šokarda Slavić, M., Lončar, N., Milošević, J., Vujčić, Z., Božić, N.** Purification and characterisation of a novel  $\alpha$ -amylase from a newly isolated *Bacillus* strain 16B (in preparation)

## SLOVENIA

**Title:** The role of progesterone metabolism in endometriosis

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**Abstract:** Endometriosis is a complex disease that is defined as the presence of endometrial glands and stroma outside the uterine cavity. It is more frequent than cancer and diabetes, and affects up to 10% of all premenopausal women and 35-50% of women with infertility. Development of endometriosis relates to increased estrogen action and progesterone deprivation, where estrogens stimulate proliferation and progesterone differentiation. Disturbed progesterone action in endometriosis is usually explained by decreased levels of the progesterone receptors (PR-AB), while metabolism of progesterone in endometriosis has not yet been studied. In peripheral tissues the active progesterone is metabolised by the actions of 5alpha-reductases types 1 and 2 (SRD5A1, SRD5A2), 5beta-reductase (AKR1D1), 20alpha-hydroxysteroid dehydrogenases (20alpha-HSDs), 3alpha-HSDs and 3beta-HSDs. Differential expression of genes encoding these enzymes may, therefore, lead to low concentrations of protective progesterone. Moreover, studies in breast cancer revealed that progesterone metabolism may result in increased levels of 5alpha-pregnanes, which exert proliferative effects via binding to specific membrane bound receptors. This project evaluated the hypothesis that in endometriosis metabolism of progesterone leads to diminished levels of this protective steroid and formation of pro-proliferative 5alpha-pregnanes.

### Objectives:

- (i) Expression analysis of genes encoding progesterone metabolising enzymes and PR-AB in endometriotic epithelial cell line Z12 and in control endometrial epithelial cells HES and HIEEC to provide essential information on differences in expression levels between diseased and normal cell lines.
- (ii) Progesterone metabolism study in endometriotic and control endometrial cell lines employing <sup>3</sup>H labelled or unlabelled progesterone, separation and identification of metabolites by HPLC and LC-MS/MS analyses, to clarify progesterone metabolism and potential formation of pro-proliferative 5-alpha-pregnanes.
- (iii) Evaluation of potential proliferative effects of these progesterone metabolites on epithelial endometriotic and control endometrial cell lines by real-time cell analyser.

### Results Obtained:

(i) Gene expression analysis of genes encoding progesterone receptors and progesterone metabolising enzymes. Progesterone exerts its protective effects via PRB thus diminished progesterone action may be associated with enhanced pre-receptor metabolism in endometriotic cell line. Our quantitative real-time PCR analysis revealed that gene *PGR* encoding both receptors, PRA and PRB, is expressed in Z12, HES and HIEEC cells. In Z12 cell line PRB was detected also at the protein level by Western blot analysis, which supported our hypothesis that enhanced progesterone metabolism may reduce occupancy of PRB, which may thus be associated with pathophysiology of endometriosis. We next evaluated expression of all genes encoding progesterone metabolising enzymes. Expression of *SRD5A2* and *AKR1D1* was not detected in endometriotic cell line Z12 and in control cell lines HES and HIEEC; the expression of progesterone metabolising genes *AKR1C1*, *AKR1C2* and *AKR1C3* that encode 3alpha, 3beta and 20alpha-HSDs, was significantly higher in control epithelial endometrial cells HES and HIEEC than in endometriotic epithelial cells Z12. Genes *HSD17B2* in HES cell line and *SRD5A1* in HIEEC cell lines were not differentially expressed. The regulation of *PGR* expression differed in Z12 cells compared to HES and HIEEC control cells. There were higher *PGR* mRNA levels in Z12 compared to HES cells but lower mRNA levels in Z12 compared to HIEEC cell line, similarly as we also observed in tissue samples (Hevir et al. 2011). In contrast, the expression of genes encoding progesterone metabolising enzymes *AKR1C1*-*AKR1C3* in model cell lines did not correlate with the expression profile previously seen in tissue (Hevir et al. 2011). This discrepancy may result from low agreement between mRNA levels, protein levels and enzymatic activities, therefore further metabolism studies were performed as they better reflect the actual situation in these model cell lines.

(ii) Progesterone metabolism studies. We established an LC-MS/MS method to separate 17 P metabolites in pg quantities. Fifteen metabolites were detected in the investigated cell lines. Progesterone metabolism was much faster in HES cell line as compared to Z12 cell line. The major metabolites of 50 nM progesterone formed in Z12 cells were 5alpha-pregnane-3,20-dione, 5alpha-pregnane-3beta-ol-20-one and 5alpha-pregnane-3alpha-ol-20-one. The ratios between 5alpha-pregnanes and 4-pregnenes were 13.1, 9.8 and 10.4 after 4, 8 and 24-hour metabolism. The major metabolites of 500 nM progesterone formed in the control cell line HES were 4-pregnene-

20alpha-ol-3-one, 5alpha-pregnane-3alpha,20alpha-diol, 5alpha-pregnane-20alpha-ol-3-one, and 5alpha-pregnane-3beta,20alpha-diol. This data suggests that progesterone is metabolised to 4-pregnene-20alpha-ol-3-one by AKR1C1-AKR1C3 enzymes. Progesterone is probably also metabolised by SRD5A1 to 5alpha-pregnane-3,20-dione and further by the AKR1C1-AKR1C3 enzymes to 5alpha-pregnane-3alpha-ol-20-one and the major metabolite 5alpha-pregnane-3alpha-20alpha-diol. The ratios between 5alpha-pregnanes and 4-pregnenes were 2.5, 8.4 and 114.4 after 4, 8 and 24-hour metabolism. These ratios remained more or less constant over time in Z12 cell line and increased in HES cell line. As the Kniss's group that provided HES cells reported in 2014 (Kniss et al. 2014) that these cells are contaminated with HeLa cell line, we had to include additional control cell line HIEEC, an epithelial cell line that originates from normal endometrium of pre-menopausal women and has so far been well characterised (Chapdelain et al. 2006) and to perform additional evaluation of gene expression and quantitation of progesterone metabolism. These experiments were performed in comparison with endometriotic cell line Z12. In HIEEC cell line we observed different metabolic profiles of 500 nM progesterone after 4, 8 and 24 hours. In all time points progesterone was metabolised mainly to 4-pregnene-20alpha-ol-3-one and to lower quantities of 5alpha-pregnane-20alpha-ol-3-one, 5alpha-pregnane-3beta-ol-20-one, 5alpha-pregnane-3,20-dione, 4-pregnene-3alpha-ol-20-one, 5alpha-pregnane-3beta,20alpha-diol, and 5alpha-pregnane-3alpha-ol-20-one. This data suggests that progesterone is first metabolised by the AKR1C1-AKR1C3 enzymes to form 4-pregnene-20alpha-ol-3-one; simultaneously progesterone is probably also reduced by SRD5A1 to form 5alpha-pregnane-3,20-dione, which is further metabolised to mono-hydroxyl and dihydroxy-metabolites. Our data show that less 5alpha-pregnanes are formed as compared to 4-pregnenes in this control cell line with the ratios between 5alpha-pregnanes and 4-pregnenes of 0.7, 0.7 and 1.5 after 4, 8 and 24 hours, respectively. In contrast, the major metabolite of 50 nM progesterone in Z12 cells was 5alpha-pregnane-3,20-dione, followed by 5alpha-pregnane-3beta-ol-20-one, 5alpha-pregnane-3alpha-ol-20-one and 4-pregnene-3alpha,20beta-diol. This data supports the proposed metabolic pathway where progesterone is first reduced by SRD5A1 to form 5alpha-pregnane-3,20-dione and then by AKR1C1-AKR1C3 enzymes to produce 5alpha-pregnane-3alpha/beta-diol metabolites. In this manner in Z12 cell line less 4-pregnene metabolites are formed as compared to 5alpha-pregnanes, with the ratios between 5alpha-pregnanes and 4-pregnenes of 6.3, 7.0 and 4.1 after 4, 8 and 24 hours, respectively. The importance of these different metabolic profiles in control HIEEC cells and endometriotic Z12 cells was further studied by evaluating the effects of individual metabolites on proliferation of these cell lines.

(iii) The effects of progesterone metabolites on proliferation of the model cell lines. The effects of progesterone metabolites on the proliferation of the model cell lines HIEEC and Z12 were evaluated in real-time using xCELLigence RTCA DP analyser. First we optimised conditions for further experiments, where we selected 5000 cells/well as the optimal cell density of both cell lines and the media with phenol red and 10% FBS as the optimal growth media. We further evaluated the effects of 5alpha-pregnane and 4-pregnene metabolites on proliferation of these cell lines. The cell lines were incubated with progesterone and its metabolites in 1 mM and 10 mM concentrations, with DMSO as a negative control and estradiol in 10 nM, 1 mM and 10 mM concentrations as a positive control. In two independent experiments compounds progesterone, 5alpha-pregnane-3,20-dione, 5alpha-pregnane-3beta-ol-20-one, 4-pregnene-20alpha-ol-3-one, and 5alpha-pregnane-3beta,20alpha-diol showed no significant effects on proliferation of HIEEC cell line. Also estradiol did not affect proliferation of this cell line, which is in agreement with the published data (Chapdelain et al. 2006). Further evaluation of progesterone and its metabolites 5alpha-pregnane-3,20-dione, 5alpha-pregnane-3beta-ol-20-one, 4-pregnene-20alpha-ol-3-one, 4-pregnene-3alpha-ol-20-one and 5alpha-pregnane-3alpha-20-one in endometriotic cell line Z12 in three independent experiments showed no significant effects on proliferation, although estradiol as a positive control stimulated cell proliferation. This data is in contrast to our unpublished results on quantification of cell proliferation with WST reagent showing that several P metabolites in 10 nM to 1 mM concentrations stimulate proliferation of Z12 cells at 24 and 48 hours.

#### **Results Unforeseen in the Original Project:**

This project revealed that the diseased and control cell lines differ in progesterone metabolism, where more 5alpha-pregnanes are formed in endometriotic cell line Z12. To conclusively clarify the potential proliferative effects of progesterone metabolites further studies comparing different methodologies, 1) impedance based real-time cell proliferation assay with 2) metabolic-based cell-proliferation assay need to be done in breast cancer cell lines MCF-7 and T-47D, where the proliferative effects of progesterone metabolites have been observed originally (Wiebe et al. 2005), and in parallel in endometriotic cell line Z12. Due to specific morphology of cells the impedance based method may namely not always correlate with other methodological approaches (Vistejnova et al. 2009). This CRP project thus clarified progesterone metabolism in model cell line of endometriosis and with the novel LC-MS/MS method provided an essential tool for determination of progesterone metabolites in different cell lines and physiological fluids.

#### **Publications:**

**Sinreih, M., Zukunft, S., Sosič, I., Cesar, J., Gobec, S., Adamski, J., Lanišnik Rižner, T.** Combined liquid chromatography-tandem mass spectrometry analysis of progesterone metabolites. 2015. PLOS One, **10(2)**, e0117984

**Sinreih, M., Anko, M., Adamski, J., Lanišnik Rižner, T.** Progesterone metabolism in model cell line of peritoneal endometriosis Z12. 2015. (in preparation)

## SOUTH AFRICA

**Title:** Genotyping and autonomic analysis of founder families with cardiomyopathy

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**Abstract:** In South Africa, cardiovascular disease (i.e., heart disease and stroke) is the second most important cause of mortality after HIV/AIDS and tuberculosis, and was responsible for 21% of all deaths in the year 2000. The causes of heart disease among South Africans remain largely non-ischemic, with hypertension, rheumatic heart disease, and cardiomyopathy being the major causes of cardiovascular disease in the country. We have shown that, between 1999 and 2006, there has been an increase of 23% in deaths attributed to cardiomyopathy, pericardial disease and valvular heart disease.

The cardiomyopathies pose the greatest research challenge of all the cardiovascular diseases in South Africa, because of their greater prevalence in poor communities; the difficulty in diagnosis that often requires highly specialised cardiological investigations that are lacking in resource poor environments; poor access to life-saving interventions such as heart transplantation and implantable cardioverter defibrillators; and the high mortality associated with these often irreversible disorders of heart muscle.

We have shown that all forms of cardiomyopathy, including the dilated, hypertrophic, restrictive, and right ventricular arrhythmogenic forms, are found in African populations. The majority of the studies have concentrated on the pathogenesis and management of the dilated, hypertrophic and the restrictive forms of the disease with some progress being made in improving the diagnosis and treatment of these conditions. There is however little information that is available to guide the diagnosis and treatment of patients with the right ventricular arrhythmogenic forms of the disease. The initial studies show arrhythmogenic right ventricular cardiomyopathy (ARVC) is prevalent in all communities of South Africa and is associated with a high mortality at a young age.

We have established the ARVC Registry of South Africa under the auspices of the Cardiac Arrhythmia Society of South Africa (CASSA) as a national research programme to study the clinical characteristics and molecular genetics of ARVC in African populations. The ARVC Registry is based in the Department of Medicine at Groote Schuur Hospital under the directorship of the applicant, with Professor Andrzej Okreglicki (Cardiac Electrophysiologist and President of CASSA) serving as the co-director. There are 209 patients who have been referred to the ARVC Registry over the past 8 years, 88 of whom are considered to have a definite diagnosis of ARVC.

The initial analysis of the first 50 South African ARVC patients has yielded a number of important findings, which include the discovery of a new founder effect that is associated with a recurrent mutation on the same haplotype background in the plakophilin 2 (PKP2) gene. The population consists of four South African ARVC families all segregating the same PKP2 mutation (C1162T) caused by a founder effect. The index cases, who carry the founder mutation, potentially have over 100 relatives who are available for study, half of whom may be expected to be the disease-gene carriers. Large numbers of individuals who carry a founder mutation are a proven model for study the genotype-phenotype correlations of inherited diseases, specifically as it relates to severity of disease and outcome.

Founder effects are common in the Afrikaner population of South Africa. Professor Peter Schwartz from Pavia, Italy, who is a collaborator in this application, has studied South African long QT syndrome (LQTS) families segregating for the same KCNQ1 mutation (A342V) leading to the discovery of a significant amount of previously unforeseen information, which ranges from an unusually high clinical severity not explained by electrophysiological characterisation of the mutation, to the importance of tonic and reflex control of heart rate for risk stratification, to the identification of the first modifier genes for the clinical severity of LQTS.

We hypothesise that additional founder effects may be present in other genes that cause ARVC, such as desmoplakin, desmoglein, desmocollin and plakoglobin. There is experimental and clinical evidence that alterations in autonomic control of the heart, highlighted by depressed baroreflex sensitivity (BRS) – a marker of reduced cardiac vagal activity – are associated with increased risk of arrhythmias and sudden death in myocardial infarction and the long QT syndrome. We postulate that individual differences in autonomic responsiveness are associated with a higher or lower propensity for life-threatening arrhythmias, as assessed by reflex control of heart rate in the South African ARVC founder population. Finally, given that changes in sympathetic tone are a trigger for events in ARVC, we propose that genetic influences on autonomic function may modify the severity and outcome of ARVC patients.

**Objectives:**

The aims of this study were:

- (i) to identify new founder effects in arrhythmogenic right ventricular cardiomyopathy (ARVC);
- (ii) to perform a genealogical analysis of the founder families to identify the original founder and all living relatives;
- (iii) to assess the relationship between autonomic function and risk for cardiac events in founder families, and
- (iv) to identify modifier genes for severity of disease and outcome in founder families.

**Results Obtained:**

We completed mutation analysis of 5 desmosomal protein genes (plakophilin-2 [PKP-2], desmoglein-2 [DSG-2], desmoplakin [DSP], desmocollin-2 [DSC2], and plakoglobin [JUP]) in 130 cases of ARVC. We discovered a new family with the founder mutation C1162T in PKP2, thus bringing to 5 the number of kindreds with a PKP2 founder effect. No founder effects were found in other genes. 23/130 (17.7%) ARVC cases with a disease-causing mutation in desmosomal protein genes. We also conducted other genetic analyses: (i) mutations screening of the phospholamban gene proved to be negative in ARVC; (ii) we discovered a disease-causing mutation in the dystrophin gene in cardiomyopathy with Marfanoid phenotype, and (iii) exome sequencing in ARVC families with no known disease-causing mutation identified deleterious mutations in two novel genes that are undergoing tests to establish causality.

**Publications:**

**Shaboodien, G., Watkins, D.A., Pillay, K., Beighton, P., Heckmann, J.M., Mayosi, B.M.** Limb-girdle weakness in a Marfanoid Man: Distinguishing calpainopathy from Becker's Muscular Dystrophy. 2015. *Pract. Neurol.* **15**, 152-154

**Fish, M., Shaboodien, G., Burke, M.A., Seidman, C., Crotti, L., Schwartz, P.J., Mayosi, B.M.** Mutation analysis of the phospholamban gene in South Africans with dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and peripartum cardiomyopathy. *Sci. Rep.* (submitted)

## TUNISIA

**Title:** Microarray-based system for the detection of North African mutations

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**Abstract:** Building indigenous research capacity enables scientists to be more responsive to public needs in their countries. The objective of this project was the development of a microarray system that enable low cost, rapid and specific identification of known variants associated with heterogeneous recessive diseases in population of the North African region. This population, of over 150 million residents, shares several features, as history, population mixture and social behaviours that promote the spread of founder mutations. During this project, we establish a mutation database for North African Mediterranean countries. By developing an oligonucleotide microarray, we are able to diagnose known mutations in a single reaction based on nucleic acid hybridisation coupled to an enzyme-mediated reaction of primer extension. As first application, we focus on identifying the 58 variants responsible for recessive hearing loss, the most common sensory impairment. An extension of the chip coverage of other mutations causing Charcot-Marie-Tooth is the second step. In the absence of known mutations, targeted next generation sequencing is performed to validate microarray data and identify novel mutations and genes. Later, updates of our microarrays with the novel mutations will be useful to increase their detection rates.

**Objectives:**

Epidemiological studies place North African region among the countries with higher rate of consanguinity and incidence of recessive disorders. The most current strategy for the genetic testing in these countries relies on PCR-RFLP and gene-specific Sanger sequencing. However, the extreme genetic heterogeneity makes molecular diagnosis time consuming and expensive using these methods. Our project aims to implement a multiplex rapid genetic test that is affordable to patients of the south shore of the Mediterranean. We proposed the development of a DNA microarray-based system for the detection of sequence variants associated with hearing loss and Charcot-Marie-Tooth. The availability of an affordable extensive mutation screening test will result in improved diagnosis, more accurate genetic counseling, and eventually an improved management of hereditary disease. The identification of the causal variants reduces the psychological and social impacts and provides families with prognostic information, recurrence risks, and improved habilitation options. Finally, our microarray-based tests can be used as a first screening step toward the identification of novel genes.

**Results Obtained:**

Mutations detected in Northern African Mediterranean families were retrieved from international literature. Data are included in a database (<http://www.tdvd.org/north-africa/mutation.php>). These data are very useful for the development of target high-throughput screening methods based on microarray and next generation sequencing.

In order to develop a microarray diagnosis system, we first optimise PCR amplification for genomic DNA mutated regions. Using different primers, polymerases, MgCl<sub>2</sub> concentrations, we optimised the amplification of regions associated with hearing impairment (36 DNA regions, 58 mutations) and Charcot-Marie-Tooth (21 DNA regions, 24 mutations), in twelve and seven multiplex PCRs, respectively. The reaction conditions for all the amplicons are uniform, enabling a semi-automated workflow by using 96-well plates and liquid handling robots.

Several criteria were applied for the selection and design of optimal oligonucleotide probes (length, secondary structure, presence of repeats, mismatches). In our preliminary assays, the oligonucleotides were spotted at three different concentrations (100, 50 and 25 µM). Our analysis showed no significant differences using the three different probe concentrations and actually oligonucleotides are spotted at concentration of 50 µM. At the 5' end of each oligonucleotide probe, we added 12 (12 C-C bonds, i.e. approximately 20Å in length) or 6 carbons and an amino linker to ensure the attachment of oligonucleotide probes to the glass surface and to overcome the steric hindrances of target DNA binding to the oligo. Our analysis showed no significant differences using the C6 or C12 linkers. Actually oligonucleotide probes with 6 carbons spacer are ordered and spotted on modified slides. At the beginning of our project, we used slides coated with 3-aminopropyltrimethoxysilane plus 1,4-phenylenedi-isothiocyanate. However, we noted higher background auto-fluorescence and the presence of comets. These spots cause problems when it comes to quantification of the array since they are not circular. Later oligonucleotide probes were printed on slides coated with epoxysilane or aldehydesilane. Using these chemistries, the spot morphology is perfect. In addition, spot and background fluorescence measurement showed that signal-to-noise ratio is high which enables the identification of alleles with a high confidence level. As planned the first application of our microarray system was the identification of variants responsible for recessive hearing loss. For 57 mutations, only one extremely well designed probe

(sense or antisense) in terms of thermodynamic behaviour was spotted. However, during assay development, a few of the designed oligonucleotides failed to perform the APEX reaction. For oligonucleotides forming self-annealing secondary structures that fail to hybridise or facilitate self-priming and extension, we design new versions of such primers by incorporating a mismatch or a modified nucleotide at the 5' or internal part of the primer. These changes reduce primer self-complementarity without compromising hybridisation and primer extension. Our method was evaluated in around 500 assays using DNA from available 101 homozygote patients and mutation carriers. Duplicate PCR and minisequencing reactions were performed. Accurate identification for all genotypes was achieved based on both absolute signal intensity and the log<sub>2</sub> scale of fluorescence ratio. Based on this analysis, we have evidence that: (i) Oligonucleotide probes are highly selective; (ii) fluorescent signals are high with minimal background intensity and, (iii) reproducibility from spot to spot is high (Figure 1).

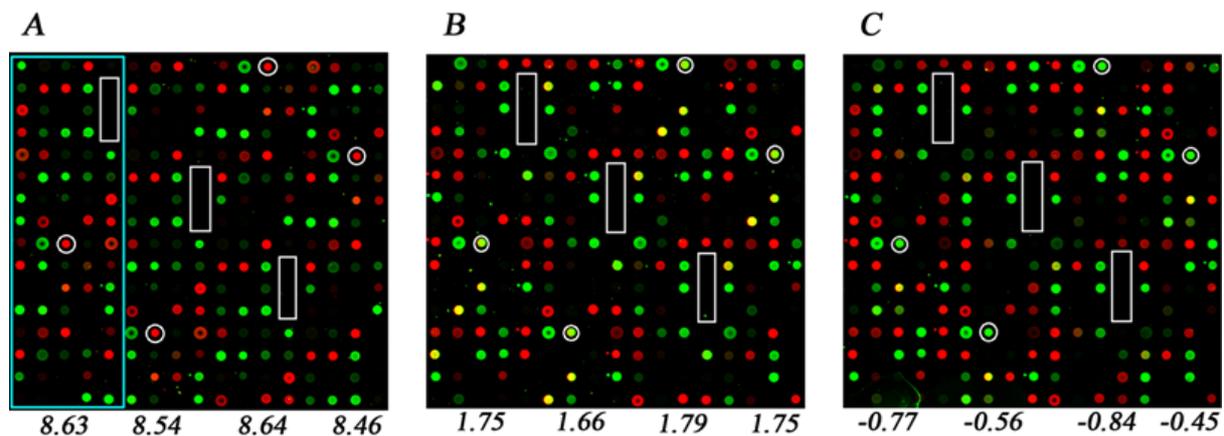


Figure 1: Three NADf scanned Arrays (16X17). Each array show four identical blocks of spotted oligonucleotides deposited on the chip in a sequential manner from left to right. Every two blocks are separated by three DNA free spots that are surrounded by a rectangle. Here, APEX were performed with Cy5-ddATP and ddUTP meanwhile Cy3-ddGTP and ddCTP. Array A: Normal genotype for ESRRBc.913 Array B: Heterozygous for ESRRBc.T913C mutation, The sense strand is extended by both the WT base T and base C, complementary for the mutation. Array C: Homozygous for ESRRBc.T913C. The numerical numbers below each array indicate the log<sub>2</sub> ratio of median (Cy5/Cy3) for each spot corresponding to the ESRRBc.T913C oligonucleotide probe.

To increase the capacity of the multiplex genotyping procedure, we spotted sixteen arrays by slide and used for minisequencing a system forming separate reaction chambers. This miniaturised format allows simultaneous genotyping of 8 samples at minimised reagent costs. The whole procedure can be completed in a single day. All the equipment and reagents required for performing the assay are generally available. In addition, the use of Cy3 and Cy5 dyes makes this chip compatible with most of the scanners used routinely for CGHarray. Actually, we start the conception of ready to use kit. Each kit will contain 12 PCR ReadyMix for 8 DNA distributed on one Ready-to-use 96-well plate. The kit contains also primer extension mixture and sixteen arrays slide.

Blind analyses were realised to evaluate the accuracy of our microarrays in 48 HI and 36 CMT unrelated patients. For the CMT patients, only one heterozygous mutation was detected suggesting a high genetic heterogeneity of this pathology in North Africa. In the absence of known mutations, the TruSight One sequencing panel was used to validate microarray data and to identify novel mutations. Novel variants are being confirmed by segregation analysis. For hearing impairment, the blind analysis showed mutant alleles in 13 patients by the NADf chip. Sanger sequencing confirmed the presence of these mutations. For one Usher patient without any known mutation, Sanger sequencing revealed a novel mutation c.91C>T in *USH1C* gene. We subsequently designed a novel probe for this and have added it to the NADf chip after being validated with the Usher patient harboring this novel mutation. This increased detection rate of our microarray from 27% to 29%.

#### Results Unforeseen in the Original Project:

Three DNA samples of the probands without any known North African mutations were analysed with whole exome sequencing. Three variants were detected and they were not present in the EVS and dbSNP databases and Tunisian healthy-hearing controls. In one patient, a novel *COL11A2* mutation p.Ala37Ser was identified. The amino acid residues Ala37 was highly conserved in different species. In a second patient, we identified a p.Gln424Pro mutation in a novel gene *DCDC2*. In a collaborative study, we reveal *DCDC2* to be a player in hair cell kinocilia and supporting cell primary cilia length regulation likely via its role in microtubule formation and stabilisation. In the third patient, a missense mutation in a novel gene of SLC22 family was identified.

Continuous updates of our microarray with these novel and future identified mutations could be easily performed to increase the efficiency and the rate of detection of our microarrays.

**Publications:**

**Chakchouk, I., Grati, M., Bademci, G., Bensaid, M., Ma, Q., Chakroun, A., Foster, J. 2<sup>nd</sup>, Yan, D., Duman, D., Diaz-Horta, O., Ghorbel, A., Mittal, R., Farooq, A., Tekin, M., Masmoudi, S., Liu, X.Z.** Novel mutations confirm that COL11A2 is responsible for autosomal recessive non-syndromic hearing loss DFNB53. 2015. *Mol. Genet. Genomics*. **290**, 1327-1334

**Chakchouk, I., Ben Said, M., Jbeli, F., Benmarzoug, R., Loukil, S., Smeti, I., Chakroun, A., Gibriel, A.A., Ghorbel, A., Hadjkacem, H., Masmoudi, S.** NADf chip, a two-color microarray for simultaneous screening of multigene mutations associated with hearing impairment in North African Mediterranean countries. 2015. *J. Mol. Diagn.* **17**, 155-161

**Grati, M., Chakchouk, I., Ma, Q., Bensaid, M., Desmidt, A., Turki, N., Yan, D., Baanannou, A., Mittal, R., Driss, N., Blanton, S., Farooq, A., Lu, Z., Liu, X.Z., Masmoudi, S.** A missense mutation in DCDC2 causes human recessive deafness DFNB66, likely by interfering with sensory hair cell and supporting cell cilia length regulation. 2015. *Hum. Mol. Genet.* **24**, 2482-2491

## TURKEY

**Title:** Microarray analysis and functional characterisation of drought stress related genes in wheat

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**Abstract:** Drought can severely impair plant growth and is responsible for significant wheat yield reductions in cultivated areas. The identification and elucidation of functional characteristics of the genes that play an important role in the complex drought-response mechanism in wheat will be helpful for plant breeders to improve more productive wheat in spite of the water-limited conditions. Based on the physiological data achieved from our screening, the most promising drought stress tolerant and non-tolerant wheat varieties were selected. An RNA-Seq experiment was performed using tolerant and non-tolerant wheat genotypes to identify differentially expressed transcripts under different drought stresses. 311 GB of 100bp paired end Illumina reads were assembled because in the database there was no reference genome for wheat. Hence *de novo* assembly was performed before comparative transcriptome analysis. Root proteome profiling was performed by an advanced proteomic approach to obtain information on the molecular mechanisms of bread wheat in response to drought and also show complementarities between two levels of cellular organisation. A total of 191 proteins were identified as differentially expressed to drought stress. Responses of antioxidative defence system of drought tolerant and non-tolerant cultivars were also investigated for different drought stresses. All of these results will allow us to get a better idea about the possible role of these genes and proteins in complex drought-response mechanism. Our data provides the most complete sequence resource accessible for the study of crops and development of functional molecular markers. *De novo* assembly of transcriptome data will also be useful for the identification of candidate genes that may benefit applied plant breeding programs.

### Objectives:

- (i) Drought stress treatment and measurement of plant water status in terms of the physiological consequence of drought stress. The initial screening of 12 bread wheat genotypes consisted of three independent replications on physiological differences;
- (ii) To discover differentially expressed genes under drought conditions in drought tolerant and non-tolerant wheat varieties. RNAseq approach was used to identify unique or novel gene expression patterns in bread wheat (*Triticum aestivum* L), drought sensitive and drought tolerant genotypes that originated and cultivated in Central Anatolia. Three different hydroponic cultures were applied to 3 selected wheat genotypes for transcriptomal profiling by next generation sequencing technology (Illumina HiSeq 2000, USA);
- (iii) Proteomics: drought stress induced proteome changes were analysed in root tissue via advanced proteomic approach by a high-definition mass spectrometer with nano electrospray ionisation source coupled to a high-performance liquid chromatography system (nanoUPLC–ESI–qTOF–MS). Plant samples were treated with 7 different hydroponic cultures to perform root proteome analysis;
- (iv) Antioxidant activity in response to drought stress between drought-tolerant and non-tolerant wheat genotypes was identified. Antioxidant enzyme experiments; changes in the activities of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX)] and peroxidation levels of lipids in cell membranes (TBARS content) of wheat varieties to drought were investigated with the application of different drought stress conditions;
- (v) To confirm the RNAseq data by qRT-PCR experiments and compare the RNAseq data with proteome.

### Results Obtained:

Physiological data achieved from screening were used for the selection of the wheat genotypes and the most promising drought stress tolerant and non-tolerant wheat varieties (two drought tolerant and one non-tolerant) were selected. A total of 72 samples (3 varieties\*3 replicates\* 2 different tissue\* 2 different drought stress\*2 control tissue) were used for RNAseq experiments. *De novo* short read transcriptome assembly was performed for the comparative bioinformatics analysis. By analysing 311 gigabases of 100 bp paired end Illumina reads from an hexaploid wheat poly(A) RNA library, we identified common and new differentially expressed transcripts. Selected differentially expressed genes were confirmed by Real Time PCR. Probable pectinesterase/pectinesterase inhibitor 42 (Cell wall biogenesis/degradation), Zinc finger CCCH domain-containing protein 36, Metacaspase-5 (induce programmed cell death), Arogenate dehydratase-5, Extensin-like protein, Germin-like protein 9-1, Phosphoglycerat

e/bisphosphoglycerate mutase, Ferritin (ferric iron binding; oxidoreductase activity), Serine/threonine protein phosphatase 2A, Protein GIGANTEA, F-box protein FBW2, Mitogen-activated protein kinase 18, Polyadenylate-binding protein RBP45B, MPK4, TIP1 genes were found to be differentially expressed in response to 4h and 8h drought stressed root and leaf tissues of tolerant and non-tolerant genotypes. We performed root proteome analysis by an advanced proteomic approach, high definition mass spectrometer with nano electrospray ionisation source coupled to a high-performance liquid chromatography system (nanoUPLC-ESI-qTOF-MS) to identify drought-related proteins. A total of 191 proteins were differentially expressed in tolerant and non-tolerant wheat genotypes.

Responses of antioxidative defense system to drought stress were comparatively studied in three wheat cultivars. The SOD constitutes the first line of the enzymatic defence system via conversion of the toxic O<sub>2</sub> - to the more stable H<sub>2</sub>O<sub>2</sub>. CAT and POX are the major scavengers of H<sub>2</sub>O<sub>2</sub>, which is produced through dismutation of O<sub>2</sub> - in peroxisomes, chloroplasts and cytosols. Under drought stress, increasing in CAT and POX activities of tolerant genotype showed that, Müfitbey has a more effective induced H<sub>2</sub>O<sub>2</sub> scavenging capacity as compared to its control and increasing in activities of CAT and POX might help to overcome cellular damage by reducing the toxic level of H<sub>2</sub>O<sub>2</sub>. Sensitivity of non-tolerant Atay to drought stress may be associated with a decreased activities and/or inadequate increase of CAT and POX resulting in a higher H<sub>2</sub>O<sub>2</sub> accumulation. On the other hand, drought tolerance of Müfitbey may be related with induced CAT and POX activities. In this project, similarities between protein and RNA levels help increase our confidence in novel biomarkers, differences may also reveal other post-transcriptional regulatory junctures. All these analyses will allow us to get a better idea about the possible role of these genes in the drought-response mechanism. The drought-related genes that are functionally characterised could be introduced into agronomically important wheat cultivars.

#### **Results Unforeseen in the Original Project:**

In contrast to microarray methods, sequence-based approaches directly determine the cDNA sequences. For this reason, they provide a far more precise measurement of transcript levels and their isoforms than the other methods. *De novo* assembly of Illumina short reads was a useful approach for transcriptome sequencing in our project. *De novo* assembled data generated by this Project will be an effective resource for accelerating and improving drought-related gene discovery of this important crop.

#### **Publications:**

**Cevher Keskin, B., Yildizhan, Y., Külen, O., Yüksel, B., Onarici, S.** The investigation of drought-associated genes in bread wheat through comparative transcriptome profiling. 2011. *Curr. Opin. Biotechnol.*, **22(1)**, S138

**Cevher Keskin, B.** ARF1 and SAR1 GTPases in endomembrane trafficking in plants. 2013. *Int. J. Mol. Sci.* **14(9)**, 18181-18199

**Cevher Keskin, B., Yildizhan, Y., Kulen, O., Onarici, S.** Quantitative expression analysis of TaMPK4 and TaTIP1 genes in drought tolerant and non-tolerant *T. aestivum* cultivars. *Plant Biotechnol. Rep.* (submitted)

**Cevher Keskin, B., Kulen, O., Yildizhan, Y., Onarici, S., Baykal, A.T., Sezerman, U.** An advanced proteomics approach in wheat (*Triticum aestivum* L.) genotypes in response to drought stress (in preparation)

**Cevher Keskin, B., Yildizhan, Y., Kulen, O., Onarici, S., Yuksel, B., Özer, B., Sağıroğlu, M.S., Sezerman, U.** The discovery of drought responsive genes by *de novo* assembly of the wheat (*Triticum aestivum* L.) RNAseq data (in preparation).