ICGEB International Centre for Genetic Engineering and Biotechnology

Developing Knowledge

ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED IN 2011

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2011

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CROATIA	Kristian VLAHOVICEK	Computational genomics of extremophyle microbial ecosystems
HUNGARY	Peter DEÁK	Determining the chemosensitivity of checkpoint mutations
IRAN	Abbas SAHEBGHADAM LOTFI	Over production of alpha1-antitrypsin in yeast and its encapsulation for drug delivery purposes
MOROCCO	Driss IRAQI	Genetic engineering as a strategy for improving drought tolerance in wheat
NIGERIA	Stella Ifeanyi SMITH	Diagnostic methods for the detection of *Helicobacter pylori* and epidemiology of enteric *Helicobacter* infections from patients in Nigeria
POLAND	Jaroslaw DZIADEK	d metabolism in pathogenesis of tubercolosis
SERBIA	Milena STEVANOVIC	Regulation of human *SOX18* gene expression and its role in angiogenesis
SOUTH AFRICA	Bongani M. MAYOSI	Identification of genes that modify the risk of sudden death in inherited arrythmogenic heart disease
SRI LANKA	W.A.J.M. DE COSTA	Expression of candidate genes for salt tolerance in Sri Lankan rice germplasm
SYRIA	Ayman AL-MARIRI	Bioconversion of wastewater sludge to produce *Bacillus thuringiensis* biopesticide
TURKEY	Mahinur S. AKKAYA	Identification and functional assessment of the genes involved in response to yellow rust infection in wheat

ARGENTINA

Title: functional genomics of plant responses to the light environment of crops
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ICGEB Contract No.: CRP/07/007
ICGEB Reference No.: CRP/ARG07-02
Abstract: Crop yield depends on the pattern of plant growth and development, which is in turn affected by the light environment of the vegetation canopy. This project has addressed the complexity of plant responses to the canopy light signals by combining reverse genetics based on the analysis of the transcriptome, forward genetics and physiological studies. We show that crop light signals can reduce grain yield despite intensive breeding and selection for yield. In part, this negative effect could persist due to a balancing action of beneficial effects of crop light signal under stressful condition. In fact, we have shown that crop light signals improve water use efficiency. We have identified novel gene functions and associated processes related to light responses. The findings range from novel functions of the plant photoreceptors to the identification of context-specific functions of genes encoding signalling components, including a proposed link between light signals, hormone signalling and the regulation of seed size. This knowledge provides tools to improve the adjustment of plants to the crop conditions.

Objectives:

The aim of this project is to uncover the identity and function of genes involved in selected plant responses to the signals provided by the light environment of crops.

Results Obtained:

The light environment that plants experience in crops is characterised by a low ratio of red light (R) to far-red light (FR) compared to sunlight out of the canopy. This environment is caused by the optical properties of the green leaves that absorb most of the R that they receive and reflect and transmit more efficiently the FR region of the spectrum. The changes in R/FR ratio are perceived by sophisticated plant photoreceptors called phytochromes. In turn, phytochromes (often in combination with cryptochromes) control several aspects of plant growth and development.

In this project we have observed that low, compared to high R/FR ratios reduce grain yield (grain number and grain weight) in wheat. Unexpectedly, this negative effect of canopy shade light is more severe in modern than in old cultivars of wheat. Therefore, there are negative consequences of the responses to R/FR that have not been eliminated (rather, they have been enhanced) by breeding and selection for yield. One of the reasons why negative consequences on yield could persist despite selection for yield is that positive aspects of the response to R/FR ratio balance negative aspects under certain conditions. In this regard, guided by the results of transcriptome analysis in *Arabidopsis thaliana*, we investigated the effects of R/FR on stomata density and its consequences on transpiration and photosynthesis (which depend on water vapour and carbon dioxide exchange through the stomata). We observed increased water-use efficiency in plants that perceived the low R/FR typical of dense canopies. Therefore, the results of this project indicate that low R/FR have not only negative but also positive effects, which by balancing the former could justify why the negative consequences have not been eliminated by breeding and selection for yield.

The results demonstrate that canopy light signals have large impact on physiologically important aspects of the crop and that these effects are complex. The project has addressed this complexity by using different approaches. We have characterised light responses of the transcriptome under novel experimental settings designed to dissect the complexity into its components. For instance, the comparative analysis of the transcriptome in different organs of tomato plant exposed to different R/FR has helped to identify genes and associated processes differentially affected in different organs. These effects are related to differential impact on end-products like pigments (chlorophyll, anthocyanin) and other compounds (lignin, etc.). Furthermore, the organ specific analysis in tomato and wheat provided a more powerful identification of light-responsive genes than previous studies involving entire seedlings. The combination of current light signals with previous light signal or with temperature fluctuations has also helped to identify genes acting under specific conditions. Some gene pathways previously though to be redundant now appear related to selected conditions. Many of these functions have been evaluated by using mutants and transgenic seedlings in *Arabidopsis thaliana*. The findings range from novel functions of the plant photoreceptors to the identification of context-specific functions of genes encoding signalling components, including a proposed link between light signals, hormone signalling and the regulation of seed size. This knowledge provides tools to improve the adjustment of plants to the crop conditions.

Results Unforeseen in the Original Project:

The results correspond to the original aims but the answers obtained were not always the ones we would have predicted. This confirms the extraordinary richness of plant biology.

Publications:

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Boccalandro, H.E., Rugnone, M.L., Moreno, J.E., Ploschuk, E.L., Serna, L., Yanovsky, M.J., Casal, J.J. Phytochrome B enhances photosynthesis at the expense of water use efficiency in Arabidopsis. 2009. Plant Physiol. **150**, 1083-1092

Sellaro, R., Crepy, M., Trupkin, S.A., Karayekov, E., Buchovsky, A.S., Rossi, C., Casal, J.J. Cryptochrome as a sensor of the blue/green ratio of natural radiation in Arabidopsis. 2010. Plant Physiol. **154**, 401-409

Ugarte, C.C., Trupkin, S.A., Ghiglione, H., Slafer, G., Casal, J.J. Low red/far-red ratios delay spike and stem growth in wheat. 2010. J. Exp. Bot. **61**, 3151-3162

Sánchez, S., Cagnola, J.I., Crepy, M., Yanovsky, M.J., Casal, J.J. Balancing forces in the photoperiodic control of flowering. 2010. Photochem Photobiol Sci. (in press)

BRAZIL

Title: Hemozoin formation as a potential target for chemotherapy of schistosomiasis
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ICGEB Contract No.: CRP/07/008
ICGEB Reference No.: CRP/BRA07-03
Abstract: Schistosomiasis is a major health problem and its treatment is based almost exclusively on the use of praziquantel. Adult Schistosoma digests haemoglobin, releases toxic free heme in the gut and crystallises it into hemozoin (Hz). Our group has previously demonstrated that interference of Hz formation by chloroquine leads to a significant decrease in the overall severity of experimental schistosomiasis. Therefore, the working hypothesis of the present project was that heme crystallisation is a key biochemical pathway and its inhibition should be deleterious to *Schistosoma* parasites. Our main goal in this project was to determine whether Hz formation would be a suitable chemotherapeutic target for rational drug design against schistosomiasis. The results obtained in this project demonstrate that interference with Hz formation in *S. mansoni* represents an important mechanism of schistosomicidal action of antimalarial quinoline methanols and points out the heme crystallisation process as a valid chemotherapeutic target to treat schistosomiasis.
Objectives:
This proposal focused on the process of heme crystallisation into hemozoin (Hz) in the blood-feeding parasite *Schistosoma mansoni* as a potential chemotherapeutic target. The working hypothesis was that this process represented a key protective mechanism against heme toxicity in this organism and that compounds targeting heme crystallisation should be schistosomicidal. Accordingly, the general aim of the project was to investigate whether Hz formation is a suitable chemotherapeutic target for schistosomiasis.
The specific objectives were:
(i) To investigate the biological activity of quinolines *in vitro* and *in vivo* in experimental murine schistosomiasis;
(ii) To investigate the mechanism of Hz formation *in vitro*, induced by organic-aqueous interfaces and in extracellular gut lipid droplets of *S. mansoni*, and the inhibitory activity of quinolines aiming the design of new drugs.
Results Obtained:
Our group has previously demonstrated that heme crystallisation into hemozoin (Hz) within the *S. mansoni* gut is a major heme detoxification route with lipid droplets involved in this process and acting as a potential chemotherapeutical target (Oliveira et al., J. Infect. Dis., 2004; Correa Soares et al., FEBS Letters, 2007). In a work published during the ICGEB grant period (Correa Soares et al., PLoS Negl. Trop. Dis., 2009), we investigated the effects of quinoline methanols such as quinine (QN) in a murine schistosomiasis model by using a combination of biochemical, cell biology and molecular biology approaches. The treatment of *S. mansoni*-infected mice with daily intraperitoneal injections of QN (75 mg/kg/day) from the 11th to 17th day after infection caused significant reductions in worm burden (39%–61%) and egg production (42%–98%). Hz formation was significantly inhibited (40%–65%) in female worms recovered from QN-treated mice and correlated with reduction in the female worm burden. We also observed that QN treatment promoted remarkable ultrastructural changes in male and female worms, particularly in the gut epithelium and reduced the granulomatous reaction to parasite eggs trapped in the liver. Microarray gene expression analysis indicated that QN treatment increased the expression of transcripts related to musculature, protein synthesis and repair mechanisms. The overall significant reduction in several disease burden parameters by the antimalarial quinoline methanols indicates that interference with Hz formation in *S. mansoni* represents an important mechanism of schistosomicidal action of these compounds and points out the heme crystallisation process as a valid chemotherapeutic target to treat schistosomiasis.
We have also advanced on the mechanisms involved on Hz formation *in vitro*, by testing the effect of organicaqueous interfaces, polarity and phospholipid membranes as models of study. In this regard, a work recently published by our group (Stiebler et al., Insect Biochem. Mol. Biol., 2010) demonstrated that Hz formation proceeded optimally at pH 4.8 and 28oC, apparently involving three kinetically distinct mechanisms along this process. Furthermore, in the triatomine insect *Rhodnius proixus*, pharmacological blockage of phospholipid membranes production *in vivo* by azadirachtin reduced haemoglobin digestion and Hz formation. Mössbauer spectrometry analyses of *R. prolixus* midgut showed that Hz represents the only measurable iron species found four days after a blood meal. Autocatalytic heme crystallisation to Hz is revealed to be an inefficient process and this conversion is further reduced as the Hz concentration increases. Also, phospholipid

membranes derived lipids were able to induce rapid Hz formation, regardless of the insect diet composition. These results indicate that phospholipid membrane-driven Hz formation in *R. prolixus* midgut occurs at physiologically relevant physico-chemical conditions and that lipids derived from this structure play an important role in heme crystallisation.

Since Hz formation *in vivo* is strongly associated with amphipatic structures (phospholipid membranes and lipid droplets), this raised the possibility that reduced polarity environments would facilitate heme crystallisation. This seems to require a heme dimer acting as a precursor of Hz crystals that would be formed spontaneously in the absence of the competing water molecules bound to the heme iron. Then, we tested this hypothesis *in vitro* by conducting a number of physico-chemical experiments, aiming to investigate the role of medium polarity on spontaneous heme crystallisation *in vitro*. We assessed the effect of water on the spontaneous Hz formation by using the aprotic solvent dimethylsulfoxide (DMSO) and a series of polyethyleneglycols (PEGs). We observed that both DMSO and PEGs (3.350, 6.000, 8.000, and 22.000) increased the levels of soluble heme in acidic conditions. These compounds were able to stimulate the production of true Hz crystals in the absence of any biological sample. Interestingly, the effects of DMSO and PEGs on Hz formation were positively correlated with their capacity to promote previous heme solubilisation in acidic conditions. Curiously, a short chain polyethyleneglycol (PEG 300) caused a significant reduction in both soluble heme levels and Hz formation. Finally, both heme solubilisation and Hz formation strongly correlated with reduced medium water activity provided by increased DMSO concentrations. Therefore, these data support the notion that reduction of the water activity is an important mechanism on heme crystallisation, which depends on the previous increase of soluble heme levels. A comprehensive review of the topics investigated in our project were reviewed in a recent publication of our group (Stiebler et al., 2011).

Results Unforeseen in the Original Project:

We have not developed the concept of organic-aqueous interfaces provided by the lipids isolated from extracellular gut lipid droplets in *Schistosoma* on heme crystallisation. Also, the effect of commercial and new synthetic quinolines on *S. mansoni* cultured *in vitro* were not assessed during the ICGEB project. Both aspects are currently being investigated by our group.

Publications:

Soares, J.B.R.C., Menezes, D., Vannier, M.A., Ferreira-Pereira, A., Almeida, G.T., Venâncio, T.M., Verjovski-Almeida, S., Zishiri, V.K., Kuter, D., Hunter, R., Egan, T.J., Oliveira, M.F. Interference with hemozoin formation represents an important mechanism of schistosomicidal action of antimalarial quinoline methanols. 2009. PLoS Negl. Trop. Dis., **3**, e477-e493

Stiebler, R., Hoang, A.N., Egan, T.J., Wright, D.W., Oliveira, M.F. Increase on the initial soluble heme levels in acidic conditions is an important mechanism for spontaneous heme crystallisation *in vitro*. 2010. PLoS One **5**, e12694

Stiebler, R., Timm, B.L., Oliveira, P.L., Hearne, G.R., Egan, T.J., Oliveira, M.F. On the physico-chemical and physiological requirements of hemozoin formation promoted by perimicrovillar membranes in *Rhodnius prolixus* midgut. 2010. Insect Biochem. Mol. Biol., **40**, 284-292

Stiebler, R., Soares, J.B., Timm, B.L., Silva, J.R., Mury, F.B., Dansa-Petretski, M., Oliveira, M.F. On the mechanisms involved in biological heme crystallisation. 2011. J. Bioenerg. Biomembr. (in press)

BULGARIA

Title: Hydrophobic binding capacity of tumour specific galectins and anticancer effects of their synthetic ligands on tumour cell lines

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Abstract: Galectins are a family of mammalian lectins, defined by their affinity for β-galactosides and structural similarity. They play important intracellular regulatory roles in RNA splicing, apoptosis, cell proliferation, and cell cycle. There is a growing body of data, indicating that galectins act extracellularly in the regulation of immune and inflammatory responses.

They play important role in cancer, binding to glycoconjugates including carcinoembryonic antigen (CEA), Mac-2 binding protein, etc, that are characteristically overexpressed in some types of cancer. It has been also proposed that Galectins are modulators of tumour progression that increase the metastatic potential of certain cancers.

In this respect they are useful targets for development of new anti-cancer therapies. Since porphyrins (important biological molecules) are currently used as photosentisers in photodynamic therapy (PDT) for cancer treatment, in this project we investigated the galectin-porphyrin complexes, testing galectins as possible porphyrin binding molecules.

The aim of our project was to study the hydrophobic binding capacity of hGal-1 and hGal-3, particularly their interaction with anticancer agents.

Objectives:

(i) Evaluation of the hydrophobic binding capacity of hGal-1 and hGal-3, using modeling and prediction, as well as fluorescent hydrophobic dyes, in addition to their carbohydrate activities;

(ii) Study of the interactions of galectins with metal and non-metal anticancer agents;

(iii) Investigation of the effects of galectins ligands on tumour cell lines. Cytotoxicity assay and apoptosis determination.

Results Obtained:

Hydrophobic binding capacity of hGal1 and hGal3 was evaluated using modelling and prediction, as well with hydrophobic fluorescent dyes TNS, ANS. In this study, we found that additionally to carbohydrate binding domain/site, hGal-1 and hGal-3 have hydrophobic binding sites, similar to plant lectins. Since many porphyrin-based drugs cannot accumulate selectively in tumours, the question that obviously raises is whether hGal-1 and hGal-3 (considered as a modulators of tumour progression, could be employed in porphyrin delivery to tumour cells? Studying of galectin-porphyrin complexes is important, since these proteins could be tested as a possible porphyrin-binding molecules with potential application in PDT.

This research reports the interaction of a human lectin hGal-1 to three metalloporphyrins, and due to its high affinity for ZnTPPS, Mn- and Au-porphyrin compounds, it could be defined as a porphyrin-binding protein. Protein interactions play a key role in understanding the distribution, elimination and transport of small molecules in biological systems. Understanding the molecular basis of drug-protein interactions is critical when designing new therapeutic agents to improve their bioavailability and potency.

Furthermore, in the last decade most investigations on human tumours revealed a direct relationship between hGal-1 expression and tumour stage, for which it becomes a valuable tumour marker, as well as a potential target for therapy. Regarding this, and its binding to cancer antigens, as well as its porphyrin binding capacity, hGal-1 can be considered as a possible candidate for anticancer therapy and could be viewed as a potentially interesting delivery molecule for the treatment of cancer cells.

We also found that hGal-3 may possess the ability to bind hydrophobic anticancer compounds. Possession of hydrophobic binding capacity could have both physiological and therapeutic implications for hGal-3.

We also investigated potential interaction between the hydrophobic and carbohydrate binding sites by examining the interaction of hGal-3 with $ZnTPPS_4$ in the presence and absence of lactose.

The obtained results indicate that there is no interaction between this hydrophobic site and the carbohydrate binding site in the hGal-3 structure, and suggest that conformational changes resulting from lactose binding do not alter $ZnTPPS_4$ affinity. The result is similar to those obtained in analysis of $ZnTPPS_4$ binding to plant lectins that also demonstrates a lack of carbohydrate sensitivity.

The far UV CD experiments of (hGal-3-bohemine complexes and hGal-3-$ZnTPPS_4$ complexes) also revealed that there were changes within the secondary structure of hGal-3 upon binding of the anticancer agents, which explain the fluorescence changes observed.
The results provide data regarding the interactions of human lectins with hydrophobic drug molecules and indicate specifically that hGal-3 possesses a novel hydrophobic site that can interact with two anticancer agents – bohemine and $ZnTPPS_4$.
Bohemine is a synthetic CDK inhibitors, which can regulate the cell cycle and are effective anti-proliferative agents leading to their evaluation as anticancer agents.
$ZnTPPS_4$ is effective in producing ROS, which is a crucial determinant of outcome in PDT, and in induction of cell death in G361, a human melanoma cell line.
Since hGal-3 binds to important cancer antigens and glycoconjugates that are overexpressed on cancer cells, and is uptaken by a number of different types of cells where it can be localised within either or both the cytoplasm and nucleus, the results suggest that hGal-3 potentially could have utility in the targeted delivery of anticancer agents. Surface Plasmon Resonance (SPR) was also applied in order to study the interaction of several porphyrin compounds to hGal-3. Several porphyrin compounds were tested as possible ligands to hGal-3. The experimental data showed that this protein interacts with the three porphyrins: Au porphyrin, Mn porphyrin and Fe porphyrin with high affinity. Interestingly, the Au porphyrin showed the highest binding affinity to the immobilised hGal-3. This motivated the ongoing study aiming to test the cytotoxicity of Au porphyrin, and cisplatin (as a control) using viability assay. The obtained results have shown that cytotoxicity effect of Au porphyrin is higher than cisplatin when applied on prostate cancer cells (PC3). Despite the fact that cisplatin has significant clinical benefit for many solid tumours, the effectiveness has been hampered by its toxic side effects and tumour resistance that often leads to appearance of secondary malignancies. This encouraged the investigators to search for new metal complexes with higher anticancer activities and lower side effects.
The present results suggest that treatment with Au porphyrin may overcome the tumour cell resistance against cisplatin treatment. The obtained results indicate that hGal-3 ligand-Au porphyrin offers considerable potential for development and application in anticancer therapy.

Publications:

Bogoeva, V.P., Russev, G.C. Fluorescence study of steroid hormone binding activity of *Helix pomatia* agglutinin. 2008. Steroids **73**, 1060-1065

D'Auria, S., Petrova, L., John, C., Russev, G., Varriale, A., Bogoeva, V. Tumour-specific protein human galectin-1 interacts with anticancer agents. 2009. Mol. Biosyst. **5**, 1331-1336

Bogoeva, V.P., Varriale, A., John, C.M., D'Auria, S. Human galectin-3 interacts with two anticancer drugs. 2010. Proteomics **10**, 1946-1953 (Erratum in: 2010. Proteomics **110(13)**, 2560)

Bogoeva, V.P., Ivanov, I., Russev, G.C., Atanassova, L. Cytokinin binding capacity of mistletoe lectin-I from *Viscum album* (submitted)

CHINA

Title: Identification of genes involved in regulation of early stamen development through analysis of proteins binding to *CsETR1* promoter in cucumber

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Abstract: We identified *CsSTO* as one of the possible regulators in *CsETR1* expression. We confirmed that *CsSTO* could bind *CsETR1* promoter *in vitro* and *in vivo*; found CsSTO could activate *CsETR1* promoter in a dual-transformation system in Arabidopsis; found *CsSTO* might be a photoperiodic responsive gene. Meanwhile, we systematically analysed a cucumber MADS-box gene, *CsMADS1*. We found this gene has a high sequence similarity with Arabidopsis *AP3*, preferentially expressed in petals and stamens, and can complement the Arabidopsis *ap3* mutant.
These data suggested that *CsMADS1* is a cucumber B class gene. We then found that CsMADS1 can bind *CsETR1* promoter *in vitro* and *in vivo*; can activate *CsETR1* promoter in the Arabidopsis dual-transformation system. We further found that in the Arabidopsis dual-transformation system, induced CsMADS1 could increase expression of Arabidopsis endogenous ethylene receptors. These data suggested that *CsMADS1* is a key regulator responsible for the organ specific downregulation of *CsETR1*. In a parallel experiment, we found although AP3 could bind promoters of all 5 ethylene receptor genes, induced *AP3* expression could not increase expression of these genes, suggesting that AP3 has no regulatory function upon the ethylene receptor genes. This finding provided an example of functional divergence highly conserved B class genes.

Objectives:
Identification of genes involved in regulation of early stamen development through analysis of proteins binding to *CsETR1* promoter in cucumber.

Results Obtained:
Identified *CsSTO* as one of the possible regulators in *CsETR1* expression. The findings provided an opportunity to understand the mechanism of phenomena that short-day condition is facilitating the female flower development.

Results Unforeseen in the Original Project:
Systematically analysed a cucumber MADS-box gene CsMADS1. The findings suggested that *CsMADS1* is a cucumber B class gene, and a key regulator responsible for the organ specific downregulation of *CsETR1*. A parallel experiment on Arabidopsis *AP3* suggested that AP3 has no regulatory function on the expression of ethylene receptor genes, provided an example of functional divergence of highly conserved B class genes.

Publications:

Bai, S.N., Xu, Z.H. From ethylene promotion of female flowers to ethylene inhibition of stamen development: a review on the study of developmental fate of inappropriate organs in unisexual cucumber flowers. 2010. Scientia Sinica Vitae **40(6)**, 469-475

Wang, D.H., Li, F., Duan, Q.H., Han, T., Xu, Z.H., Bai, S.N. Ethylene perception is involved in female cucumber flower development. 2010. Plant J. **61(5)**, 862-872

Sun, J.J., Li, F., Li, X., Liu, X.C., Rao, G.Y., Luo, J.C., Wang, D.H., Xu, Z.H., Bai, S.N. Why is ethylene involved in selective promotion of female flower development in cucumber? 2010. Plant Signal Behav. **5(8)** (in press)

COLOMBIA

Title: Molecular identification of pathogenic effectors in a cassava bacterial pathogen
Principal Investigator: Adriana Bernal, Laboratory of Mycology and Plant Pathology, Department of Biological Sciences, Faculty of Sciences, Universidad de los Andes, Carrera 1 Nos. 18A-10, Bogotá, Colombia. Tel: +57-1 3394949, ext. 2619, E-mail: abernal@uniandes.edu.co
ICGEB Contract No.: CRP/07/011
ICGEB Reference No.: CRP/COL07-01
Abstract: *Xanthomonas axonopodis* pv. *manihotis* (Xam) is the causal agent of bacterial blight disease in cassava. As many animal and plant pathogenic bacteria, Xam possesses a type-III secretion system (TTSS) that allows secretion and translocation of effector proteins into the cytoplasm of the plant cell. These proteins then interact with target proteins of the host plant, facilitating the infection process and interfering with host specific functions (signalling and defence). Much progress has been made in the identification of Type III effector proteins (T3E) in several pathogenic bacteria, but only one effector had been reported for Xam at the beginning of this project. The aim of this project was to identify effector proteins used by Xam to cause disease in cassava and to further characterise these proteins at the molecular level. The number of effectors identified in Xam is comparable to that present in other members of the genus Xanthomonas. The variability of these effectors both at the genus and the subspecies level indicates co-evolutionary processes with cassava plants that need to be further determined. One effector protein was determined as important for maximal virulence in Xam, probably due to its activity as a plant defence suppressor, as determined in this study. The knowledge acquired in the development of this project will help in the development of alternative methods for disease control in cassava.

Objectives:

The aim of this study is to identify and characterise effector proteins from *Xanthomonas axonopodis* pv. *manihotis* (Xam). The specific objectives are:
(i) To isolate effector genes and effector gene fragments from Xam, based on the sequences of effectors reported for other Xanthomonas;
(ii) To identify effector proteins from Xam using an *in vivo* genetic screen that reports the presence of a secretion and translocation signal in the genome of the bacterium;
(iii) To characterise the sequence diversity of a subset of the identified effector genes in plant pathogenic bacteria whose sequences have been reported, and in natural collections of Xam from Colombia;
(iv) To determine the importance of a subset of effector genes in the pathogenesis of Xam.

Results Obtained:

It was necessary to develop a genetic screening system for the detection of effectors in the genome of Xam. The idea was to generate an insertional library of Xam strain CIO151 where a transposon was used to insert an effector gene orphan of the Nterminal Type III secretion signal. This effector should be an avirulence protein; in other words, a protein that is recognised by the plant, thus generating a type of programmed cell death called the hypersensitive response (HR), easily observable a few days post-inoculation of the plant. When clones of the insertional library were inoculated into plants, only those where the insertion had taken place downstream of a Type III secretion signal would generate an HR in the corresponding plant.
For the screening, a heterologous plant species was selected using two criteria: first, the plant should not have a visible cell death against Xam and, second, the plant should recognise a bacterial avirulence gene. After assaying many plant species and several avirulence effectors, we selected two promising systems: the Arabidopsis-AvrRpt2 gene isolated from *Pseudomonas syringae* and the Pepper-AvrBs2 gene isolated from *Xanthomonas euvesicatoria*. A library was generated using the AvrRpt2 gene and approximately 1500 clones were assayed by inoculations in Arabidopsis plants with the corresponding recognition gene Rps2. Two clones were selected as consistently eliciting an HR in Rps2-containing plants. Several PCR-based methods for the identification of flanking regions were used and two proteins were selected as effector candidates: a hypothetical protein and a major membrane lipoprotein. These are currently being validated as novel effectors identified in the genome of Xanthomonas.
In addition to the genetic screen, we used bioinformatics to identify effector proteins present in the genome of Xam. The first bioinformatic approach was a search for homologs of effectors previously reported for other species of Xanthomonas in the genome of Xam obtained by second generation sequencing technologies. This approach resulted in 19 effector genes identified, with some additional genes that were present as potential pseudogenes in the genome. The second bioinformatic approach consisted in the use of three algorithms generated in the last three years for the *de novo* identification of effectors in pathogens, based on specific signatures present mostly in the N-terminus of the protein. This analysis resulted in six candidate novel effector genes that

were detected by at least two of the algorithms. Experimental validation using fusions with AvrRpt2 or AvrBs2 confirmed one of the effectors as a novel gene obtained in this project.
We further characterised several of the effectors identified in this project in their role in virulence of Xam in cassava using a mutagenesis approach. We showed the importance of one of these effectors for maximal virulence in Xam and complementation assays validated these data. Most effectors reported to date are suppressors of plant defence at two levels: they suppress the so called PAMPtriggered Immunity (PTI) or basal defence and some of them have the ability to suppress the Effector-Triggered Immunity (ETI) or specific plant defence, which is elicited by effectors recognised by certain plant cultivars. One effector was able to suppress the deposition of callose in plant cell walls of Arabidopsis, which is a sign of PTI. In addition, it was able to suppress the generation of a Hypersensitive Response (HR), a type of programmed cell death which is a sign of ETI in plants. Therefore, we have demonstrated the ability of this effector to suppress plant defense at both levels.

Results Unforeseen in the Original Project:

The original project was mostly focused on the identification of effectors using the genetic screen in Xam. However, with the advent of Next Generation Sequencing technologies, it was possible to obtain the almost complete genome sequence of the bacterium and the bioinformatic searches were much more productive than the genetic screen. Noteworthy, this approach had been suggested by one of the reviewers of the original proposal.

Publications:

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CROATIA

Title: Computational genomics of extremophile microbial ecosystems
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ICGEB Contract No.: CRP/07/024
ICGEB Reference No.: CRP/CRO07-03
Abstract: The aim of this project is to mobilise the resources and ideas for the application of computational methods in analysis and visualisation of metagenomic data, in the scope of broadening knowledge on mechanisms and diversity of life.
The problems that we would like to tackle through the proposed in silico research, give an answer to the organisation of the genetic information inside the complex systems such as complete environments, and to the genetic adaptation of life to extreme conditions, which will provide us with the insight into molecular adaptations necessary for sustaining life in the most inhospitable places on Earth. In order to answer those questions with the most recent methods, we need the help of information technology, mathematics and statistics on one side, and molecular biology and evolution on the other, connected through a multidisciplinary approach. The knowledge gathered through fundamental research, presented with the new discoveries of the large genome sample analysis, will provide us with an insight into evolutionary and ecological relationships inside an environment. The applications of the proposed project results arise from the enormous potential for industrially and medically applicable genes, enzymes or whole metabolic pathways.
Objectives:
(i) Data acquisition. Most of the metagenomic sequences are stored in the publicly accessible databases such as GenBank (NCBI, NIH) or EMBL (EBI, EMBL). In most cases they present a single readout of the automatic DNA analyser, which produces fragments of approx. 500 base pairs in length. Since the number of such fragments originating from an environment can easily reach more than one million, it is necessary to organise a local system (relational database) for the storage and retrieval of large amounts of data. This kind of system enables quick access to nucleotide sequences and provides a framework for all further computational and statistical analyses.
(ii) Sequence assembly. Sequences stored in a database and originating from the automatic DNA analysers need to be assembled into the longest possible contigs in order to piece together as many complete genes (coding regions) as possible from the original data. Therefore, it is necessary to eliminate sequence fragments originating from experimental contamination during amplification and DNA sequencing (contaminants include vectors, poly-A fragments, sites for cloning, etc.). Next, it is necessary to align remaining sequences in order to find the clusters which overlap and assemble longer sequence fragments. The assembled DNA sequences will then be translated into protein sequences by finding the correct reading frame. Finally, translations will have to be functionally characterised.
(iii) Sequence alignment. For sequence alignments and similarity-based comparisons we will use locally installed program packages BLAST (NCBI, NIH) and FastA (Univ. of Virginia) for the heuristic sequence similarity search. FastA package provides a programming tool for the rigorous pairwise comparison according to the Smith-Waterman algorithm.
(iv) Functional characterisation. We will functionally characterise the assembled protein sequences in several ways: primarily by similarity search against the databases containing previously functionally characterised sequences (COG, Clusters of Orthologous Genes, and databases from the KEGG collection, Kyoto encyclopedia of genes and Genomes). Moreover, previous experience of the principal investigator in the field of functional characterisation through the protein domain architecture prediction will aid in application of classification algorithms based on the machine learning methods (i.e. Support Vector Machines).
(vi) Analysis of the codon usage patterns in the coding regions. One of the hypotheses we want to explore is whether the organisation of the genetic information at the level of extreme environment metagenome resembles that at the level of the each particular genome. Therefore, by analysing predicted expression levels through codon usage patterns we intend to investigate whether genes that are presumed important for a particular environment exhibit some form of expression optimisation within respective organisms. Furthermore, codon usage provides a way to reveal the horizontal transfer of genes in bacteria; horizontally transferred genes are often associated with pathogenic features or antibiotic resistance, which clearly illustrates connection between the field of computational genomics and urgent problems of public health.
(vii) Analysis of the metabolic pathways abundance. The frequency of particular gene in the environment can be associated with its importance for the survival of an organism, which is especially the case in extreme environments. We will examine the relevance of the above statement by comparing environments in terms of relative abundance of diverse metabolic

pathways.

Results Obtained:

(i) Data processing and collection. We have collected the data available in public databases from 7 different metagenome sequencing projects, in various stages of sequence assembly: (i) Metagenome from Soudan Mine; (ii) Sediment microbial community from the Salton Sea, CA. Hyper-saline; (iii) Acid Mine Drainage microbial communities from Richmond mine; (iv) Aquatic microbial communities Yellowstone National Park Hot Springs (3 samples); (v) Marine microbial communities Guerrero Negro, Baja California Sur, Mexico hypersaline mats.

Datasets were pre-processed to eliminate vector contaminants and reads over 200bp were selected, to facilitate assembly. Sequence reads were converted into the format suitable for Celera assembler and a database framework was set-up to store assembled fragments.

(ii) EST and WGS reads assembly into larger genomic fragments. Shotgun sequences were assembled using Celera assembler into contigs and scaffolds. Owing to the sample size, metagenomes are often sequenced with low pass count (1-2x) and therefore often cannot be assembled into long uninterrupted sequence chunks.

(iii) ORF finding and functional classification of protein sequences. Assembled sequences were functionally classified using a set of specially developed programmatic tools for parallel processing of BLAST search results. Metagenome transcripts were assigned a functional category (based on COG functional classification) with a 3-nearest neighbour algorithm.

(iv) Development of various software tools to analyse metagenome data. Within the scope of the analysis of metabolic and signalling pathways is the investigation of protein interactions that can help elucidate many processes at the systems level, especially in large data sets such as metagenomes. Unfortunately, the only type of available data from metagenomes are mainly sequences, rather than complete 3D structures of proteins that would be more useful for studying protein interactions. We have in parallel started to develop methods for predicting protein interactions directly from sequences that would largely circumvent the lack of structural data and greatly improve the ability to study protein interactions in metagenomes. The first step in development of the prediction algorithm was the development of a software tool, PSAIA (Protein Structure and Interaction Analyser) in order to predict and investigate protein-protein interaction sites. Systematic investigation of protein interaction sites will serve as a basis for a machine-learning algorithm and a predictor for protein interaction sites in metagenomic sequences.

(v) Functional characterisation of novel genes from various metagenomes. The main hypothesis is that microbial communities behave as meta-genomes, with concerted evolution and selection for similar codon usage patterns across the entire ecological niche. We have demonstrated that codon usage patterns are non-random in each metagenomic data-set and that there is a pronounced and statistically significant bias in use of synonymous codons across each metagenome, moreover that different metagenomes show different CU bias. We have further explored the effect of microbial genomes optimising codon usage for translational efficiency (by selecting for synonymous codons that match the tRNA abundance and contribute to mRNA stability) in order to predict the expressivity of functionally characterised genes and to rank the gene functions according to their predicted expressivity within the entire metagenome. This revealed the existence of metagenome-wide optimisation for lifestyle-specific genes (e.g. Sargasso sea metagenome shows optimisation for amino acid transport across the membrane while the whale carcass metagenome selects for energy conversion functions, Figure below). We have established the proof of concept in well-characterised metagenomes, such as the Sargasso sea metagenome, and are progressing towards establishing functional category sets in metagenomes from extreme environments.

(vi) Adaptation of existing and further development of various software tools and methods for high-throughput data analysis. Integral part of this objective is also to develop other novel methods for knowledge extraction from biological information originating in large-scale –omics experiments. In an interdisciplinary approach, the bioinformatics group has established itself as a leader in development of machine learning algorithms for knowledge discovery and data mining. During the first year we have demonstrated a successful application of a data mining algorithm termed *Random forests* in predicting the protein interaction sites directly from primary sequence, and we have further applied a similar principle in predicting the relationship between histone modification and gene expression levels in eukaryotes. The results have recently been published in PNAS, and are a product of a collaboration set-up between our group and Vingron group at the Max Planck Institute for Molecular Genetics in Berlin, Germany (with the first author, our Ph.D. trainee Rosa Karlic).

Results obtained during the three-year funding period are collected in a manuscript that is currently under review in Molecular Systems Biology.

Publications:

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HUNGARY

Title: Determining the chemosensitivity of checkpoint mutations
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Abstract: Cells accumulate many alterations in their genome during malignant transformation leading eventually to tumours with distinctive genotypes. Some of these mutations disrupt cell cycle control and DNA repair mechanisms and make the cancerous cells more sensitive to genotoxic agents. This phenomenon is exploited in chemotherapy treatments. Unfortunately, the agents and treatment protocols used at present are not specific enough to leave normal cells unharmed, so thus chemotherapy works essentially along a delicate compromise between efficiency and toxicity.
One way to increase effectiveness and lower toxicity is to identify and use anticancer agents with high specificity toward distinctive genetic lesions of the targeted tumour. For this approach, we tested the selectivity of currently used chemotherapy agents for individual cell cycle and DNA repair mutations in *Drosophila melanogaster*. Most of the homozygous mutants appear to be more sensitive to these drugs compared to heterozygotes. This finding underlines the notion that checkpoint and DNA repair defects in cancer cells determine their sensitivity to cytotoxic drugs. Furthermore, stand out toxicity values were obtained in certain drug-mutant combinations indicating that some agents have selectivity for certain genetic defects. Because the checkpoint and DNA repair genes are conserved from fruit flies to humans, the selective agents identified in our tests could be used for correlating effectiveness of chemotherapy in human tumours with defined genetic defects these tumours possess.
Objectives:
The aim of this project was to determine the sensitivity of previously characterised *Drosophila* mutants, each disrupting a particular cell cycle checkpoint or DNA repair gene, to FDA-approved anticancer agents. Our rationale was that testing different checkpoint mutations for chemosensitivity in model organisms could lead to the identification of chemotherapeutic drugs with high specificity for particular checkpoint defects. Any compound with such specificity can then be used for correlating therapeutic effectiveness in human tumours with checkpoint defects in those same tumours. This kind of data obtained in experimental organisms, together with better diagnostics of human tumours, might lead to effective, tailor-made chemotherapy, in which we will know what works in advance and apply drugs accordingly.
Results Obtained:
A relative toxicity tests were used to determine the toxicity of 16 FDA-approved cytostatic drugs in 16 Drosophila cell cycle checkpoint or DNA repair mutants. The test is based on the principle that if checkpoint or DNA repair mutants develop in constant exposure to DNA damaging agents, the mutants die at concentrations that the wild type (heterozygous) siblings tolerate.
For each test experiments, heterozygotes were crossed in *Drosophila* test vials containing 5 ml of standard fly medium. The progeny at first larval stage was treated with different drug concentrations. The crawling and foraging larvae efficiently absorb the drugs, since they stay in contact with them through their body wall and digestive tracks. For statistical analysis, surviving adults were scored daily at each drug concentration and identified as homo- or hemizygous and heterozygous animals. The ratio of homo- or hemizygotes was calculated relative to the total number of progeny. These primary data were used to calculate relative toxicity (homozygous mutant progeny/total progeny) and median relative lethal concentration (R-LC50). An R-LC50 represents a drug concentration where the ratio of homozygotes or hemizygotes amounts to 50 % of the expected ratio in untreated tests.
Most of the homozygous mutants appear to be more sensitive to certain anticancer drugs than the heterozygotes. This finding underlines the notion that checkpoint and DNA repair defects are common in cancer cells and that these defects determine, or at least strongly influence their sensitivity to cytotoxic drugs. Furthermore, stand out toxicity values were obtained in certain drug-mutant combinations, in which the toxicity of compounds were several orders of magnitude higher compared to others, and the homozygotes were killed at concentrations where the heterozygotes were not affected. These results indicate that some agents have selectivity for certain genetic defects. These agents have the potential to improve the therapeutic success by targeting unique cellular defects found only in tumour cells. *RecQ* and *mei-41* mutants appear to be very sensitive to most drugs tested, but on the contrary to this, loss of *Rad18-like* function confers resistance to all drugs tested. Because the checkpoint and DNA repair genes are conserved from fruit flies to

humans, the selective agents identified in our tests could be used for correlating effectiveness of chemotherapy in human tumours with defined genetic defects these tumours possess.

Results Unforeseen in the Original Project:

Though most of the homozygous mutants appear to be more sensitive to at least some anticancer drugs than the heterozygotes, there is, however, one exception: the *Rad18-like* mutation confers resistance to all drugs tested. The *Rad18-like* gene has sequence similarity to *Rad18,* which encodes an ubiquitin ligase with a central role in translesion synthesis and post-replication repair. *Rad18-like* mutants express their characteristic X-ray irradiation sensitivity.

Publications:

Pál, M., Tósoki, R., Varga, K., Nagy, O., Deák, P. Mutations of checkpoint and DNA repair genes confer diverse sensitivity to anticancer agents in *Drosophila melanogaster*. 2011. (in preparation)

IRAN

Title: Over production of Alpha1-antitrypsin in yeast and its encapsulation for drug delivery purpose

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Abstract: Human alpha-1 antitrypsin (AAT) is an important and one of the major members of the serine protease inhibitor (serpins) super family. As it inhibits a broad range of proteases, especially neutrophil elastase, it plays an important role in maintaining protease-antiprotease homeostasis in lung. AAT deficiency causes emphysema in adults and liver disease in children. The US Food and Drug Administration (FDA) has approved the use of plasma derived AAT for augmentation therapy in deficient patients. The current commercial AAT, which is obtained from human blood, has two main problems: contamination with viruses and source limitation. Because of these, researchers look for other methods to produce large quantities of pure and safe AAT and using recombinant DNA technology in different hosts can be an alternative. One of these considered hosts is yeast *pichia pastoris* (*P. pastoris*) that is a suitable bioreactor for protein production. The main aim of this study is to overproduce AAT in this host by different strategies and to investigate ability of poly (D, L-lactide-co-glycolide) nanoparticles to carry this protein as a potential therapeutic agent for the protection of lung tissue against free proteolytic activity.

Objectives:

The main goal of this study is the production of recombinant human AAT protein in the yeast *P. pastoris* and evaluating it, first in order to use it in research applications and secondly, in continuation of this study for making a suitable drug for patient with its deficiency. Because this protein exists in high prevalence in blood (2gr/lit) and also it should be used for their whole life in deficient patients, it should be economical for patients and producers. One solution is overexpression of this protein in optimised condition.

Results Obtained:

After three years we have obtained nearly all of desired goals according to our planning. In overall, the work was done in different sections:

Cloning, expression and purification of AAT in methylotrophic yeast *P. pastoris*, and evaluation of recombinant AAT. For these purposes, AAT gene was isolated from Human hepatocellular carcinoma (HepG2) cell line and cloned in suitable vectors for *P. pastoris*. The vectors were transformed to yeast (*P. pastoris* X33). Positive yeast clones were detected in YPDS plates containing 100 µg/ml Zeocin and confirmed with PCR and AOX and GAP specific primers. The recombinant X33 were cultured on YPD medium. Other culture media used were the following: BMGY and BMMY supplemented with 2% phenyl methyl sulphonyl fluoride (PMSF) for protease inhibiting. The supernatant was used for analysis of protein expression using SDS-PAGE and silver staining and confirmed with western blot analysis. The enzyme-linked immunosorbent assay (ELISA) was used for quantitative determination of secreted AAT levels in the medium. The recombinant AAT was purified with affinity chromatography and its inhibitory activity was assayed against elastase. Isoelectrofocusing (IEF) showed that its Ip is among 5.1 to 5.2.

Elevating the expression of AAT in methylotrophic yeast P. pastoris. For this step four strategies were used, including optimisation of codon usage for yeast, using two AOX1 and GAP promoters, two native and α-MF signal sequence, and finally construction of multicopy clones. The results indicated that increasing the gene dosage to two copies has positive effects on the AAT expression. On other hand, optimisation of AAT codons in favor of *P. pastoris* and use of native AAT signal sequence instead of yeast signal sequences are another beneficial strategies for increasing AAT expression in this host. Comparison of two different promoter indicated that on a small scale constitutive promoter can express AAT more efficient but on a larger scale, inducible promoter could be more beneficial because of its adjustable property.

Production of AAT using high-cell density fermentation of recombinant *P. pastoris.* For this purpose, some studies were done on the cultivation media to find an animal component free, chemically defined media, which are ideal in the manufacture of human pharmaceutical proteins such as AAT. The media used in fermentation, which is done in three steps: Glycerol batch phase (GBF), Glycerol fed-batch phase (GFP) and Methanol fed-batch phase (MFP).

AAT encapsulation by PLGA nanoparticles. In this section the human AAT loaded PLGA nanoparticles was prepared using oil in oil (O/O) emulsion solvent evaporation techniques. The results were investigated through scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). The ELISA test was used for assaying protein release and protein integrity. The results showed that PLGA NPs and AAT-loaded PLGA NPs indicated that both types of

particle have spherical shapes and relatively smooth surfaces. In comparison to the control NPs, AAT-loaded NPs were of a smaller size (with an average size of 550 nm) and lower yield with about 50% efficiency. The resulting AAT release profiles indicated three phases: the first was an initial burst phase (day 1 to 3) with 60% of the AAT released. The second phase was an intermediate one (day 4 to 9) with a low and continuous release including approximately a 36% of release and, finally, a third phase (till day 36) was detected with a moderate burst. According to the FTIR spectroscopy there are no chemical interactions between polymer and protein and physical links put them together.

Publications:

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MOROCCO

Title: Genetic engineering as a strategy for improving drought tolerance in wheat
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Abstract: Wheat is the most important cereal crop and a staple food in Morocco. The average annual per capita consumption of cereals in Morocco is estimated at over 200 kg per person. Drought is the most important environmental stress affecting the wheat crop, causing a severe decrease in performance. Moreover, the transfer of resistance to abiotic stresses such as drought, using traditional approaches is limited because of the complexity of the characteristics of tolerance However, genetic transformation can help in improving this trait, while overcoming the difficulties of classical improvement. Therefore, this project was formulated with the objectives of genetic transformation of bread wheat and durum wheat with genes known to be involved in drought tolerance (barley *HVA1* gene) and molecular characterisation of transgenic plants, and testing of the wheat transgenic plants for tolerance to drought under controlled environments. We also train students in plant transformation techniques in order to develop the next generation plant breeders and to enhance public awareness of the potential of genetic engineering. Accordingly, as a first step, a set of Moroccan wheat varieties were selected and suitable media for developing embryogenic callus from mature embryo explants of those varieties had been identified. The collected embryogenic calli were bombarded with 1µm gold particles coated with plasmid DNA (plasmid pBY520) containing *HVA1* gene at a pressure of 1100 psi. After shooting, the induction of embryogenic tissue in the absence of selective agent "basta" was successful for all varieties studied. However, during the selection (on basta), the percentage of survival reduced drastically as there were subcultured on the selective media. Finally putative transgenic plants were obtained both for durum wheat and bread wheat. The confirmation of gene integration by molecular biology techniques was performed using PCR technique and transgenic plants were identified both in durum wheat (at T0 to T5 generation plants, var. 'Irden') and bread wheat (var. 'Marchouch'). Since, barley *HVA1* gene known to impart tolerance both to drought and salinity, a screening system for both the traits have been adapted and tested. The suitable concentration of soil moisture levels and salt concentrations which induce stress for non-transformed plants are identified Preliminary analysis showed that some of the transgenic plants showed improved level of tolerance compared to non-transgenic control plants. Further physiological analysis is in progress. A total of five students (4 Master and 1 B.Sc.) completed training/research in this project while two Ph.D. students are continuing their studies toward their PhD degree.
Objectives:
(i) Transformation of bread wheat and durum wheat with genes known to be involved in drought tolerance and molecular characterisation of transgenic plants;
(ii) Testing of the wheat transgenic plants for tolerance to drought under controlled environments;
(iii) Training of students/researchers in genetic transformation of wheat.
Results Obtained:
(i) Suitable media for obtaining embryogenic cali from immature and mature embryo explants of Moroccan bread wheat and durum was identified;
(ii) Transgenic bead wheat and durum wheat plants with barley HVA1 gene were obtained;
(iii) Methods for screening for tolerance to drought and salt tolerance was standardised and used for screening non-trangenic plants to identify suitable NaCl concentration and soil moisture level;
(iv) Additionally, this project helped INRA-Morocco to attain capacity to adapt biolistic transformation technology in wheat.
Results Unforeseen in the Original Project:
Optimisation of media for obtaining embryogenic callus and regeneration using matured embryos in case of bread wheat and durum wheat.
Publications:
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Iraqi, D., Udupa, S.M. Genetic transformation of durum wheat with HVA1 gene of barley using biolistic approach (in preparation)

NIGERIA

Title: Diagnostic methods for the diagnosis of *Helicobacter pylori* and epidemiology of enteric *Helicobacter* infections from patients in Nigeria

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Abstract: *Helicobacter pylori* is the causative agent of gastritis, peptic ulcer and a risk factor in the development of gastric cancer. It has been reported to be common in the Tropics. The study was aimed at looking at methods that would enable faster and accurate diagnosis of *H. pylori* in Nigeria as well as screening for enteric *Helicobacter* spp from faeces and patients with gastroduodenal symptoms.

Objectives:

(i) To develop rapid and reliable methods of diagnosis of *H. pylori* in Nigeria by culture, stool antigen kit and PCR for the direct detection of the organism from biopsies as a confirmatory method;

(ii) To screen our isolates for antibiotic susceptibility testing using the disk diffusion and E-test methods and to evaluate the effectiveness of a genotypic strategy for the direct detection of CLA and tetracycline resistances in gastric biopsy samples, with a confirmation using the FISH technique from patient stomach biopsy, this will involve designing of probes for the Tet R1, R2 and R3 resistances;

(iii) To evaluate the prevalence of *Helicobacter* sp intestinal infection in asymptomatic subjects and establish the correlation of the infection with intestinal disorders.

Results Obtained:

A total of 296 patients were screened for *H. pylori* using CLO test, *Helicobacter pylori* stool antigen test (HpSA), histology, culture, serology, direct Gram stain, FISH and PCR methods.

Out of 296 patients screened, *H. pylori* was present in 33.8% of CLO test, 48% HpSA, 16% culture, 44% serology, 23% direct Gram stain, 37% histology, 66.1% FISH, the various PCR genes studied ranged from 4-52%. Amongst patients with diarrhoea, *Helicobacter* spp was present in 15% using glmM gene, 62% using 16S rRNA. Amongst patients with gastroduodenal symptoms, 48.5% were positive by UBT, by 16S rRNA gene 39% were positive for *Helicobacter* spp, while only 5.3% were positive for *H. pylori* using the glmM gene.

The FISH technique is the most promising technique for the diagnosis of *H. pylori* in Nigeria, although only a few number of samples were screened compared to the other methods, but the results from the FISH technique were comparable to histology and CLO tests. This is the first report in Nigeria using the FISH method for *H. pylori* diagnosis.

The presence of *Helicobacter* spp/*H. pylori* from stool of patients with diarrhoea and also those presenting with gastroduodenal symptoms is the first report in Nigeria, although the sample size might be too small to draw a conclusion.

Results Unforeseen in the Original Project:

A high number of patients were positive for *H. pylori* using the FISH technique and this showcases the uniqueness of the FISH technique. In addition, although the *H. pylori* isolates could not be maintained viable after confirmation due to incessant power outages experienced in the part of the world, the FISH technique enables one to screen for antibiotic resistance directly from the biopsy as evidenced by the resistance patterns to clarithromycin and tetracycline. It would also enable one in the proper management of *H. pylori*. It is quite surprising however that the *ure*A gene was not found useful for the diagnosis of *H. pylori* since most *H. pylori* strains are rapid urease producers.

Publications:

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POLAND

Title: Steroid metabolism in pathogenesis of tuberculosis
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Abstract: As reported, cholesterol plays a crucial role during the uptake of mycobacteria by macrophages. However, the significance of cholesterol modification enzymes encoded by *Mycobacterium tuberculosis* for bacterial pathogenicity remains unknown. Here we explored whether the well known cholesterol modification enzymes, cholesterol oxidase and ketosteroid dehydrogenase are important for virulence of the tubercle bacillus. Homologous recombination was used to replace both genes from the *M. tuberculosis* genome with the non-functional copies. The resultant mutants were attenuated in peritoneal macrophages. The mice infection experiments confirm the significance of cholesterol oxidase in the pathogenesis of *Mycobacterium tuberculosis*. Moreover, we show that *M. tuberculosis* grown in media containing carbon source other than cholesterol is able to accumulate cholesterol in the free lipid zone of its cell wall. This cholesterol accumulation decreases the permeability of the cell wall for the primary antituberculosis drug, rifampin, and partially masks the mycobacterial surface antigens. Furthermore, *M. tuberculosis* was able to grow on mineral media supplemented with cholesterol as the sole carbon source. Targeted disruption of the kstD gene inhibited growth due to inactivation of the cholesterol degradation pathway. Our findings that *M. tuberculosis* is able to accumulate cholesterol in the presence of alternative nutrients and use it when cholesterol is the sole carbon source *in vitro* may facilitate future studies into the pathophysiology of this important deadly pathogen.
Objectives:
The main of this project was to answer the following questions:
(i) Is *Mycobacterium tuberculosis* able to use cholesterol as a carbon and energy source?
(ii) Does *M. tuberculosis* actively accumulate cholesterol in the cell wall?
(iii) What is the role of cholesterol metabolism in *M. tuberculosis* pathogenesis?
(iv) What is the role of cholesterol oxidase and ketosteroid dehydrogenase in the pathogenesis of tubercle bacilli?
Results Obtained:
The sequencing of mycobacterial genomes revealed the presence of putative choD orthologs in *M. tuberculosis*, *M. bovis*, *M. leprae* and *M. smegmatis*. Moreover, both *M. smegmatis* and *M. tuberculosis* genomes contain putative genes coding for other enzymes of cholesterol catabolism. Microbial cholesterol oxidase (EC 1.1.3.6) catalyses the oxidation and isomerisation of cholesterol to cholestenone (4-cholesten-3-one), which is an initial step in the cholesterol degradation process. The resulting cholestenone might be further catabolised by a number of enzymes to final inorganic compounds. We have previously shown that neither wild type *M. smegmatis* strains nor *M. smegmatis* delta-ksdD mutant strains (which accumulates intermediates of the cholesterol biodegradation process - androstendion, AD) growing in the presence of cholesterol are able to accumulate cholestenone in amounts that are detectable by GC (Brzostek et al., 2005). To verify the enzymatic activity of the putative cholesterol oxidase enzyme of *M. tuberculosis*, choDTb was cloned under control of the heat shock promoter (Phsp65, choDTb) and introduced into *M. smegmatis* delta-ksdD, which is able to transform cholesterol to AD. The host strain and resulting mutant that overproduces ChoDTb (*M. smegmatis* delta-ksdD-PhspchoDTb), were cultured in the presence of cholesterol. The degradation of cholesterol and accumulation of the steroid intermediates were monitored by GC within 72 h. The investigated strains were able to use cholesterol and accumulate AD, however, the temporary accumulation of cholestenone was observed exclusively in the strain that overexpressed ChoDTb.
To assess the role of cholesterol oxidase in the pathogenesis process of *M. tuberculosis*, a strain carrying an internal deletion in the choD gene was prepared using the two-step recombination protocol of Parish & Stoker (2000). Moreover, a control strain carrying the delta-choD gene complemented with intact choD controlled with the heat shock promoter (PhspchoDTb) was constructed. The wild type *M. tuberculosis* strain and two mutant strains (delta-choD and delta-choD-PhspchoD) grew with the same doubling time in Middlebrook 7H9/OADC broth (data not shown) and were used to infect *in vitro* mouse peritoneal macrophages at a ratio of 0.1:1. The number of viable bacteria used for infection and recovered from macrophages 6 and 8 days post-infection was analysed by c.f.u. The dramatic decrease in the number of viable bacilli was observed in the case of the delta-choD mutant, but not in the wild type or complemented strains.
Mycobacteria introduced intravenously into mice disseminate rapidly in the organism and can be identified in spleens and lungs. The number of viable bacteria isolated from these organs shows a

progression of infection. Three *M. tuberculosis* strains were used to infect intravenously C57BL/6 mice: (i) wild type; (ii) delta-choD and (iii) delta-choD-PhspchoD. At least ten mice of each group were euthanised 10 weeks post-infection and the number of viable bacteria in isolated spleens and lungs was determined by c.f.u. The wild type bacilli and complemented mutant carrying an intact choD were maintained *in vivo* over this 10-week period, with about 10/5 bacilli isolated from lungs and 10/4 from spleens. The delta-choD strain was cleared out from the lungs of the majority of mice and only about 103 delta-choD-bacilli were recovered from the spleens of the infected animals. The experimental infection of C57BL/6 mice with the mycobacterial strains listed above was also performed for 7 and 13 weeks (3 mice in the group) with similar results being obtained.
It is well known that fast-growing mycobacteria degrade natural sterols and use them as a source of carbon and energy. However, the ability of tubercle bacilli to utilise cholesterol was not observed until recently. To determine conclusively whether *M. tuberculosis* could accumulate cholesterol, we followed the fate of tritium-labeled cholesterol supplemented into bacterial cultures. Moreover, microscopic analysis revealed filipin binding of cells grown in the presence of cholesterol, but not control cells. Analysis of individual bacilli indicated that the dye-bound cholesterol was deposited in the cell envelope, not intracellularly. We hypothesised that cholesterol could accumulate in the most external layer of the cell wall, the free lipid zone, which is more loosely formed than the other parts of the mycobacterial cell wall. To verify this hypothesis, we isolated and carefully washed cells grown in the presence of cholesterol, and subjected them to extraction of the free lipid zone. The obtained extracts and defatted cells were analysed by thin layer chromatography, which revealed that cholesterol was, indeed, deposited in the free lipid zone, together with phospholipids, glycolipids and sphingolipids. The accumulation of cholesterol results in decreased permeability of the mycobacterial cell wall what was measured by uptake of antituberculosis drug - rifampin. Moreover, we observed that *M. tuberculosis* delta-kstD mutant growing on mineral medium supplemented with cholesterol as the sole carbon source accumulated 9OHAD in a time-dependent manner, whereas this intermediate was not observed in wild type and delta-kstD- PhspkstD strains grown in the same medium. Moreover, the time-dependent accumulation of 9OHAD was affected by supplementation of glycerol as an alternative carbon source. Both the decreased growth of the delta-kstD mutant and its accumulation of 9OHAD on mineral medium supplemented with cholesterol shown that *M. tuberculosis* can utilise cholesterol as a carbon and energy source. Moreover, we also found direct evidence that cholesterol degradation in *M. tuberculosis* is performed exclusively by AD/ADD intermediates, with KstD playing an essential role in this process.

Results Unforeseen in the Original Project:

We did not expect that tubercle bacilli are able for both cholesterol degradation and accumulation dependent on availability of carbon sources.

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SERBIA

Title: Regulation of human *SOX18* gene expression and its role in angiogenesis
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ICGEB Contract No.: CRP/07/019
ICGEB Reference No.: CRP/YUG07-01
Abstract: SOX18 transcription factor is an important regulator of vascular development playing a role in endothelial cell specification or differentiation, angiogenesis and atherogenesis. The aim of this proposal was to study regulation of the human *SOX18* expression in endothelial cells and to gain additional insights into transcriptional regulatory networks relevant to angiogenesis that involve this transcription factor.
We have demonstrated that transcription factors Sp3 and ZBP-89 act as negative regulators, whereas NF-Y up-regulates *SOX18* promoter activity. Additionally, we have performed TF Protein Array using membrane that contains transcription factors (TFs) relevant to angiogenesis and demonstrated that 22 out of 42 TFs have the ability to bind to probe encompassing *SOX18* promoter region. By combining *in vitro* binding assays, mutational analysis and functional assays we have demonstrated that Egr1 acts as a potent positive regulator of *SOX18* gene expression. We have focused our further research on bZIP transcription factors belonging to CREB/ATF and AP-1 family. By functional assays, we have shown that CREB over-expression caused significant down-regulation.
Further, we have demonstrated that the angiogenic factor VEGF and the inflammatory cytokine TNF increase, while the nonsteroidal anti-inflammatory drugs ibuprofen and NS398 decrease the SOX18 protein level. These results for the first time demonstrate that *SOX18* expression is modulated by factors and drugs known to positively or negatively regulate angiogenesis. This opens the possibility of pharmacological manipulation of *SOX18* gene expression in endothelial cells to stimulate or inhibit angiogenesis.
In order to identify novel SOX18 target genes, we have performed gene expression profiling of HUVEC (human umbilical vein endothelial cells) overexpressing either *wt* or dominant negative (DN) form of SOX18 protein by applying GeneChip® Gene 1.0 ST Array System (Affymetrix). Gene ontology analysis revealed that most of the affected genes are involved in immune response and inflammation. The most prominent change in the expression was observed for CXCL10, an interferon-inducible chemokine. *In silico* analysis of the 5′ flanking region of CXCL10 gene revealed the presence of 6 putative SOX binding sites, indicating that CXCL10 might be a direct SOX18 target. Further functional analyses are needed in order to confirm direct role of SOX18 in the regulation of CXCL10 gene expression.
Objectives:
The experiments described in this proposal are designed to study transcriptional regulation of the human *SOX18* gene expression and to gain additional insight into regulatory networks relevant to angiogenesis that involve this transcription factor. Accordingly, it was proposed:
(i) To perform functional analysis of the control elements involved in transcriptional regulation of *SOX18* expression in endothelial cells;
(ii) To identify transcription factors involved in regulation of *SOX18* gene expression;
(iii) To study the effects of pharmacological inhibitors of angiogenesis and cytokines on *SOX18* expression in endothelial cells;
(iv) To identify novel SOX18 downstream target genes in endothelial cells.
Results Obtained:
The regulation of the human *SOX18* gene expression was studied in HeLa cells and two cell lines of endothelial origin, EA.hy926 and HUVEC. By *in vitro* binding assays we demonstrated that Sp3, ZBP-89 and NF-Y are able to specifically bind within probe deriving from *SOX18* proximal promoter, spanning the region -200 to -162 relative to ATG. Further, in co-transfection assays we demonstrated that transcription factors Sp3 and ZBP-89 act as negative regulators, whereas NF-Y up-regulates *SOX18* promoter activity.
Additionally, we have performed TF Protein Array using membrane that contains transcription factors (TFs) relevant to angiogenesis and demonstrated that 22 out of 42 TFs have ability to bind to probe encompassing *SOX18* promoter region. By this TF Protein Array we have identified 22 TFs, including EGR1, CREB1, GATA1, PAX6, PPAR, HNF4 and STAT1, which could be involved in transcriptional regulation of *SOX18* gene expression.
Given the pivotal role of EGR1 in the transcriptional response of endothelial cells to angiogenic growth factors involved in angiogenic switch, this TF was selected for further comprehensive functional analysis. By combining *in vitro* binding assays, mutational analysis and functional assays

we have demonstrated that Egr1 acts as potent positive regulator of *SOX18* gene expression. We have focused our further research on bZIP transcription factors belonging to CREB/ATF and AP-1 family. By functional assays, we have shown that CREB over-expression caused significant down-regulation, while over-expression of c-Jun, JunB and ATF3 led to significant up-regulation of *SOX18* promoter activity.

Further, we have demonstrated that the angiogenic factor VEGF and the inflammatory cytokine TNF increase, while the NSAID ibuprofen and NS398 decrease the SOX18 protein level. These results for the first time demonstrate that SOX18 expression is modulated by factors and drugs known to positively or negatively regulate angiogenesis. This opens the possibility of pharmacological manipulation of *SOX18* gene expression in endothelial cells to stimulate or inhibit angiogenesis.

In order to identify novel SOX18 target genes, we have performed gene expression profiling of HUVEC overexpressing either *wt* or dominant negative (DN) form of SOX18 protein by applying GeneChip® Gene 1.0 ST Array System (Affymetrix). Over-expression of *wt* SOX18 protein resulted in up-regulation of 2 and down-regulation of 3 genes, while over-expression of DN form caused down-regulation of 20 genes and up-regulation of 5 genes. Gene ontology analysis revealed that most of the affected genes are involved in immune response and inflammation, including CXCL10, CXCL11, CCL20, RSAD2, IDO1, CCL5, TLR3, OASL, Mx2, IFIT1 and IFIH1. The most prominent change in expression was observed for CXCL10, an interferon-inducible chemokine. The CXCL10 was both, up-regulated by over-expression of *wt* SOX18, and down-regulated by its DN form. *In silico* analysis of the 5′ flanking region of CXCL10 gene revealed the presence of 6 putative SOX binding sites, indicating that CXCL10 might be a direct SOX18 target. Further functional analyses are needed in order to confirm direct role of SOX18 in regulation of CXCL10 gene expression.

Results Unforeseen in the Original Project:

Gene ontology analysis revealed that most of the potential *SOX18* targets genes identified in this study are involved in immune response and inflammation. The most prominent change in expression was observed for CXCL10 that has anti-proliferative effect on endothelial cells *in vitro* and angiostatic and anti-tumour effect *in vivo*. Further work is needed to reveal biological relevance of the data obtained.

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SOUTH AFRICA

Title: Identification of genes that modify the risk of sudden death in inherited arrhythmogenic heart disease

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ICGEB Contract No.: CRP/07/020
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Abstract: Cardiac arrhythmias are a feature of many disease states that are a major cause of morbidity and mortality worldwide. We postulated that individuals at high risk may have a genetic predisposition to developing ventricular fibrillation under specific conditions. Such susceptibility or modifier genes are difficult to identify in the context of complex diseases, but may be better studied in the context of monogenic diseases characterised by a high risk for lethal arrhythmias, such as the Long QT Syndrome (LQTS), Hypertrophic Cardiomyopathy (HCM) and Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC/D).

The major aim of the study was to utilise founder families to identify modifier genes capable of increasing or decreasing the risk for SCD in patients affected with inherited arrhythmogenic heart disease. We had access to unique founder families in Cape Town, which are ideal for the study of modifier genes in light of their homogeneous genetic background. Specifically, 22 ancestrally related LQTS families, and three groups of ancestrally related HCM families (with three, seven and eleven families per group) had been identified, and clinical investigations already performed in the LQTS and HCM families. By contrast, about 50 ARVC/D probands who had been identified at the beginning of this project remained to be genetically characterised for the primary disease-causing mutations in known ARVC/D-causing genes.

Objectives:

We conducted a two-phase investigation through this grant. First, we performed genetic characterisation of the 50 ARVC/D probands to determine the prevalence of the known genetic mutations that cause ARVC/D in the newly established ARVC/D Registry of South Africa. Second, a candidate gene approach was used to identify genes that modify the susceptibility to LQTS and HCM.

Results Obtained:

We have fully characterised the first 50 patients enrolled in the ARVC Registry of South Africa in terms of clinical characteristics, survival experience, and spectrum of plakophylin-2 gene mutations. Whilst the clinical characteristics are the same as elsewhere in the world, we have found that the survival is extremely poor in South Africans affected with ARVC. This is possibly related to the low uptake of implantable cardioverter defibrillators (ICDs). The most significant discovery was that of a novel founder effect in the plakophilin-2 gene. Almost 50% of ARVC patients with plakophilin-2 gene mutations (which account for a quarter of South African patients with ARVC) carry the same recurrent mutation (C1162T). These results provide a basis for the study of modifier genes in this condition, provided the four families with the founder effect can be extended. It is estimated that there are 200 individuals at risk of carrying the founder mutation who live in South Africa (Watkins 2009).

We also studied the genotype-phenotype correlations of the founder families with HCM. We found that troponin T mutation is associated with an abnormal blood pressure response to exercise, a factor that may account for the higher mortality in troponin T carriers despite minimal hypertrophy (Heradien 2009).

The search for modifier genes for the LQTS resulted in the discovery of NOS1AP as a modifier gene for LQTS (Crotti 2009). With respect to HCM, the effect of the functional +1675 G/A polymorphism (rs1403543) and additional single nucleotide polymorphisms in the 3' untranslated region of the Ang II type 2 receptors [AT2R] gene (AGTR2) on a heritable composite hypertrophy score in HCM founder families was investigated. There was a significant association between rs1403543 and hypertrophy, with each A allele decreasing the average wall thickness by ~0.5 mm. This study confirms a hypertrophy-modulating effect for AT2R in HCM and implies that +1675 G/A could potentially be used in a panel of markers that profile a genetic predisposition to LVH in HCM (Carstens 2010).

Publications:

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SRI LANKA

Title: Expression of candidate genes for salt tolerance in Sri Lankan rice germplasm
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ICGEB Contract No.: CRP/06/012
ICGEB Reference No.: CRP/SRI06-01
Abstract: In this project, two cDNA libraries were constructed from salt-stressed shoot tissue of the salt-tolerant Sri Lankan rice variety At354 at phase I and phase II of salt stress development. Differential hybridisation, followed by DNA sequencing identified 14 up-regulated and 17 down-regulated cDNA clones in response to salt stress during phase I. Differential hybridisation of 960 cDNA clones from Phase II cDNA library identified 34 up-regulated and 14 down-regulated clones. However, DNA sequencing was successful for only 13 cDNA clones, which included 11 up-regulated and 2 down-regulated clones. The identified differentially-expressed candidate genes of rice variety At354 belonged to a variety of functionality classes such as transcription, hormone-related functions, signalling, translational machinery, transport proteins, osmoprotectants, ROS scavengers, photosynthesis, general metabolism, protein transport/turnover and defence-related functions. Relative gene expression of three selected up-regulated candidate genes for salt tolerance during phase I showed significant increases in salt-stressed At354 relative to the unstressed control. Screening of 102 Sri Lankan rice varieties identified six highly-tolerant varieties at phase I and ten tolerant varieties at phase II. Possible physiological mechanisms of tolerance in these tolerant varieties were identified.

Objectives:

The overall objective of this project was to identify candidate genes for salt tolerance from Sri Lankan rice germplasm, quantify their expression (at phase I and phase II of salt stress development) under salt stress and relate their expression levels to whole-plant salt tolerance. Our original objectives were as follows:

Component 1 (Activity 1): Identification of differentially expressed genes in response to salt stress in a known salt-tolerant rice variety from Sri Lankan rice germplasm by differential hybridisation of full length cDNA clones;

Component 2 (Activity 2): Screening of Sri Lankan rice varieties for whole-plant salt tolerance;

Component 3 (Activity 3): Quantifying the expression, under salt stress, of selected candidate genes for salt tolerance in a limited number of rice varieties differing in the degree of whole-plant salt tolerance using quantitative real time PCR.

Results Obtained:

Component 1 (Activity 1): The objective of this activity was achieved in full. Two cDNA libraries were constructed from salt-stressed shoot tissue of the known salt-tolerant Sri Lankan rice variety At354 at phase I and phase II of salt stress development. Differential hybridisation identified 48 and 66 different cDNA clones carrying up- and down-regulated genes respectively at phase I. A second round of different hybridisation confirmed 22 and 30 different cDNA clones as carrying up- and down-regulated genes respectively at phase I. DNA sequencing gave successful results for 14 up-regulated and 17 down-regulated cDNA clones. Differential hybridisation of 960 cDNA clones from the cDNA library of salt-stressed shoot tissue at phase II identified 34 up-regulated and 14 down-regulated clones. However, DNA sequencing was successful for only 13 cDNA clones, which comprised of 11 up-regulated and 2 down-regulated clones.

Candidate genes whose expressions were either up- or down-regulated due to salt stress in shoot tissues of variety At354 belonged to a variety of functionality classes such as transcription, hormone-related functions, signalling, translational machinery, transport proteins, osmoprotectants, ROS scavengers, photosynthesis, general metabolism, protein transport/turnover and defence-related functions.

Component 2 (Activity 2): The objective of this major activity was achieved in full.

(i) Based on reduction of leaf and total biomass growth at phase I of salt-stress development (i.e., 24 hours after increasing salt-stress to 100 mM Na^+), the following six rice varieties were identified as "highly-tolerant" to salinity during phase I: At303, Bg350, Bg450, H9, Hetada Wee, Pola Al. In addition, 23 rice varieties were identified as "tolerant" to salinity during phase I.

(ii) Based on a combination of five different measures salt-tolerance, which included during phase II of salt development (including leaf area and total biomass reduction at two stages during phase II and the appearance of salt-toxicity symptoms), the following 10 rice varieties were identified as "tolerant" to salinity during phase II: At303, Bg380, Bg350, Pachchaperumal, Bg450, Nona Bokra, H9, Pokkali, MI273 and Podi Wee A8, At401. In addition, the varieties Bg94-1, Murungakayan were identified as "moderately tolerant" to salinity during phase II.

(iii) The varieties which were tolerant or moderately tolerant to salinity during phase II employed two different mechanisms to tolerate salt-ion toxicity (i.e., Na^+ Exclusion from the shoot and intra-cellular compartmentation), which is the principal stress during phase II. Na^+ exclusion was identified as the principal mechanism responsible for phase II salt-tolerance of the following varieties: At303, Bg380, Bg350, Pachchaperumal, Bg450, Nona Bokra, Murungakayan and Bg94-1. Intra-cellular compartmentation was identified as the principal mechanism responsible for phase II salt-tolerance of the following varieties: At401, Podi Wee A8, MI273 and Pokkali.

Component 3 (Activity 3): The objective of this major activity was partially achieved because of lack of time. Relative gene expression of three selected up-regulated candidate genes *(OSPHI-147, OSPHI-194*, and *OSPHI-928)* for salt tolerance during phase I showed significant increases in salt-stressed At354 relative to the unstressed control. We expect to continue this activity by extending it to a range of rice varieties differing in their degree of salt-tolerance as outlined in the original proposal.

Publications:

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SYRIA

Title: Bioconversion of wastewater sludge to produce *Bacillus thuringiensis* bioinsecticide
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ICGEB Contract No.: CRP/07/021
ICGEB Reference No.: CRP/SYR07-02
Abstract: Dry sludge samples from Damascus (urbanistic region), Aleppo (industrial region), and Homs (rural region) Wastewater Treatment Plants were collected and subjected to different chemical-physical analysis. Cultivation of *Bacillus thuringiensis* subsp. *aegypti* C-18 (Bt) was performed in shake flask cultures and 3 L capacity fermentor using 3 media: semisynthetic LB, LB + salts, and wastewater sludge media. In shake flask fermentation, significant differences between sludge and LB media were found concerning the effect of culture volume on colony count during 24 h of incubation at 30 and 37 °C. The highest production of colonies was obtained when *B. thuringiensis* had been incubated for 72 h in both sludge and LB media either at 30 °C or 37 °C. The optimisation conditions for Bt production in bench scale fermentor indicated that the highest production was recorded at 200 rpm, pH 8, 72 h of fermentation, and 30 °C. The entomotoxicity of Bt cultured on sludge media, especially sludge from Homs region, against the potato tuber moth and the European grapevine moth was higher, even at low spore concentrations, compared to LB and LB + salts media. The UV resistance to UV-A and UV-B radiations of LB and sludge media without the addition of UV blockers or absorbers was studied. The results demonstrated that the wastewater sludge media possessed higher UV resistance in comparison to LB and LB + salts media.
Objectives:
(i) Characterisation of wastewater sludge from Wastewater Treatment Plant;
(ii) Initiation of shake flask fermentation experiments to produce Bt insecticide using sludge as a culture medium;
(iii) Optimisation of the shake flask experiments by a bench scale fermentation using 3 L capacity fermentor;
(iv) Field evaluation of the produced Bt bio-insecticide (resistance to solar UV radiation).
Results Obtained:
Wastewater sludge could be used to produce Bt insecticide either in shake flask or bench fermentor. Seventy-two hours of fermentation and pH 8 at 30 ºC were the optimum conditions of fermentation. The entomotoxicity (measure of bio-pesticidal action) of Bt cultured on sludge media against the potato tuber moth and the European grapevine moth was higher, even at low spore concentrations. The wastewater sludge media possessed higher UV resistance, in term of residual insecticidal activity, compared to LB and LB + salts media.
Results Unforeseen in the Original Project:
Wastewater sludge from rural region outperformed other sludges from industrial and urbanistic region in term of entomotoxicity and UV resistance.
Publications:
None

TURKEY

Title: Identification and functional assessment of the genes involved in response to yellow rust infection in wheat

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ICGEB Contract No.: CRP/07/022
ICGEB Reference No.: CRP/TUR07-03

Abstract: The research of the understanding of fundamental biological processes of both the susceptibility (S) and resistance (R) of plant pathogen or pathogen to plant, in other words: fundamental biological processes of plant-pathogen interactions are needed to be carried out at the molecular level. The molecular level studies are still very limited for the proposed pathogen and plant interactions relevant to the subject matter of this proposal; yellow rust (stripe rusts) disease causing pathogen *Puccinia striiformis* f.sp. *tritici* (PST) and its host, bread wheat (*Triticum aestivum*), since they are both difficult organisms to study for the following reasons:
On the pathogen side:
(i) PST is a obligate biotrophic pathogen, it cannot be cultured on plates as detached from its host, rather its urediospores must be grown and maintained on the infected, undetached compatible wheat lines;
(ii) Other than very recently accumulating EST sequences, there is no genome sequence available for any race of PST yet;
(iii) An effective mean of transformation of this pathogen does not exist;
(iv) Without any doubts, the most interesting pathogen within the host cell, such as haustoria of which is very difficult to isolate sufficient amount of haustoria uncontaminated with chloroplasts.
On the host organism, bread wheat side:
(i) Bread wheat has a huge hexaploid genome, it is difficult and labour-intensive to perform map-based cloning for all the resistance alleles of the R-genes;
(ii) Its genome sequencing is proceding however is still far from completion.
We have conducted our studies towards the identification and functional assessment of the host genes putatively involved in response to yellow rust infection in wheat. We have proposed to study some of the wheat genes previously found in our laboratory upon compatible and incompatible yellow rust responsive, either by differential display method (Bozkurt et al. 2007) and/or by screening of Wheat-Affymetrix gene chip (Bozkurt et al. 2009), or the genes identified as putative interactors of the fragments of a candidate yellow rust resistance gene (Yr10) in the yeast library via dual-yeast two-hybrid method. Towards this aim, we have proposed to assess the expression level analyses of some of the selected genes/plant homologs by qRT-PCR and apply Virus Induced Gene Silencing (VIGS) approach to confirm the roles and biological functions. Additionally, we had anticipated to obtain the gene sequences of the pathogen, only expressed in the host after a particular number of days of inoculations or infections, either by the proposed "enrichment method" or by first isolating the pure haustoria, and then sequencing the haustorial transcriptome. Also, we propose to conduct the determination of putatively involved miRNAs by screening microarray composed of mature miRNAs. Moreover, the aim of identification of new genes or proteins of the plant responding to the pathogen inoculation was pursued by proteomics approach on the barley and powdery mildew causing *Blumeria graminis* f. sp. *tritici* pair. Another additional and important goal of our project was the training of graduate students, in order to help them in obtaining their degree while working towards the understanding of the function of genes in plant pathogen interactions during disease formation and/or resistance response.
As a result, we have accomplished most of our objectives:
(i) determination of expression levels of genes;
(ii) functional analysis of some genes using VIGS gene function analysis method mostly on barley-powdery mildew plant pathogen pair;
(iii) isolation of pure yellow rust haustoria from infected plants;
(iv) analysis and the evaluation of miRNA array chip screening, and determination of plant genes responding to both compatible an incompatible pathogen infections by proteomics approach.
We also wish to add that the ICGEB grant helped us to obtain funds from other national grant agencies: TUBITAK and METU.

Objectives & Results Obtained:

(i) Some of the wheat genes previously found in our laboratory upon compatible and incompatible yellow rust response either by differential display method (Bozkurt, et al. 2007) and/or by screening of Wheat-Affymetrix gene chip (Bozkurt, et al. 2010), or the genes identified as putative

interactors of the fragments of a candidate yellow rust resistant gene (Yr10) in the yeast library *via* dual-yeast two-hybrid method by qRT-PCR.
(ii) In order to confirm and assess the biological roles of the genes, we have had proposed to apply Virus Induced Gene Silencing (VIGS) approach.
Part of this study led to a publication of two manuscripts (Dagdas, et al. 2010 and Demircan, et al. 2010). Here we are not presenting the details of the results available in the manuscripts published; however, the yet to be published part of the results are presented below under the draft manuscript "RAD6 encodes E2 Ubiquitin conjugating enzyme, a negative regulator of plant cell death and defense".
(iii) We had anticipated to obtain the gene sequences of the pathogen, only expressed in the host after particular number of days of inoculations or infections, either by proposed "enrichment method detailed in the proposal application" or by first isolating the pure haustoria, and then sequencing the haustorial transcriptome. We were able to obtain haustoria with a collaboration of Dr. C. Rampitsch from Canada.
(iv) Also, determination of putatively involved miRNAs by screening microarray composed of mature miRNAs was proposed to be conducted. The details of the work will be presented under the draft title of "Determination of plant microRNAs putatively responding to *Blumeria graminis* f.sp. *hordei* in barley and *Puccinia striiformis* f. sp. *tritici*" and the work is going to be published after performing confirmation experiments.
(v) Moreover, the aim of identification of new genes or proteins of the plant responding to the pathogen inoculation was pursued by proteomics approach on the barley and powdery mildew causing *Blumeria graminis* f. sp. *hordei* pair. The details of this study will be presented under the draft title of "Proteome of compatible and incompatible *Blumeria graminis* f. sp. *hordei* inoculated barley" in a manuscript still to be published as detailed here below.
(vi) As importantly as the above goals of our project, another aim was to train graduate students and help them earn their degrees while working toward understanding of the function of genes in plant pathogen interactions during disease formation and/or resistance response.

Results Unforeseen in the Original Project:

The unforeseen outcome was the great difficulty to obtain rust uredinospores of *Puccinia striiformis* f. sp. *tritici* in large amounts in the conditions we are in Turkey; such as unexpected electrical outage, loosing plants and spores in growth due to which. Therefore, we are unable to report the transcriptome sequences at this stage.

Publications:

Dagdas, Y.F., Dagdas, G., Unver, T., Akkaya, M.S. A new ZTL-type F-box functions as a positive regulator in disease resistance: VIGS analysis in barley against powdery mildew. 2009. Physiol. Mol. Plant Pathol. **74**, 41-44

Demircan, T., Akkaya, M.S. Virus induced gene silencing in *Brachypodium distachyon*, a model organism for cereals. 2010. Plant Cell, Tissue and Organ Culture **100(1)**, 91-96

Bozkurt, T.O., McGrann, G.R., MacCormack, R., Boyd, L.A., Akkaya, M.S. Cellular and transcriptional responses of wheat during compatible and incompatible race-specific interactions with *Puccinia striiformis* f. sp. *tritici*. 2010. Mol. Plant Pathol. **11(5)**, 625-640

Yildirim,-Ersoy, F., Ridout, C.J., Akkaya, M.S. Detection of physically interacting proteins with the CC and NB-ARC domains of a putative yellow rust resistance protein, Yr10, in wheat. 2011. J. Plant Diseases and Protection **118(3/4)** (in press)