Characterization of genetic abnormalities and study of the mechanisms of origin of chromosomal rearrangements in human embryonic stem cells

Nguyen Thi Ha*, Prof. Dr Karen Sermon* and Dr Claudia Spits*

**Vrije Universiteit Brussel, Department of Embryology and genetics, Laarbeeklaan 101, 1090 Brussels (Belgium)*

Abstract

Human embryonic stem cells (hESCs) are pluripotent cells, unlimited capable of undifferentiated proliferation *in vitro*. The application of hESCs as a renewable source of a wide range of cell types is a promising approach to treat numerous diseases and injuries and to provide new models for research. Continuous maintenance and expansion of hESCs in culture can lead to adaptation and the acquisition of chromosomal abnormalities. Chromosomal alterations of hESCs can affect the validity of experimental results and hamper possible therapeutic applications, suggesting the need for rigorous genetic analysis of hESCs during prolonged culture.

All hESC lines examined in this work were derived from embryos that were donated by patients undergoing *in vitro* fertilization (IVF) and preimplantation genetic diagnosis (PGD). These hESC lines were cultured on inactivated mouse embryonic fibroblasts (MEFs), in serum replacement (SR) medium, at 37 $^{\circ}$C and 10% CO_2.

Eighteen polymorphic microsatellites located one at each band of chromosome 18 were used to detect uniparental disomy (UPD) and loss of heterozygosity (LOH) and to confirm some of the aberrations that were found by array-CGH in a previously published study. PCR was performed using different DNA inputs for conventional PCR and small-pool PCR. Our results confirmed our previous study and showed partial and whole chromosome 18 deletions in four hESC lines: VUB01, VUB04_CF, VUB13_FXS and VUB26_QUATRO. Nevertheless, we did not find UPD or LOH. Furthermore, we detected microsatellite instability (MSI) in two hESC lines VUB01 and VUB04_CF and were able to quantify the allele frequencies and number of abnormal cells at different passages in culture.

To understand the mechanisms of origin of chromosomal abnormalities, we studied the effect of cell density and media compositions on the levels of dNTP precursors and on the induction of fragile sites. To do this, two different hESC lines were cultured at different cell densities, in dilution series at a factor of 5. After 6 days, cells were collected and spun onto slides, which were stained to visualize DNA double strand breaks. This study may contribute to improving hESC culture media and systems.

Analysis of APOBEC3G single nucleotide polymorphisms in a cohort of HIV-1 concordant and discordant couples in Dakar, Senegal

Teresia Muthoni Njoroge[1]*, Prof. Dr L. Kestens[1], Dr Wim Jennes[1] and Ir L. Heyndrickx[1]

**Current address to be submitted*
[1]*Institute of Tropical Medicine, Immunology Unit, Department of Microbiology, Nationalestraat 155, 2000 Antwerpen (Belgium)*

Abstract

This study is part of the collaborative project on correlates of HIV-protective immunity (COPRIM) carried out between the Institute of Tropical medicine in Antwerp and the Cheikh Anta Diop University in Dakar, Senegal. The general objective is to explore biological factors that render HIV-exposed seronegative individuals resistant to HIV-1 infection. APOBEC3G, a cytidine deaminase, is an important host factor that inhibits HIV-1 replication. Our main objective in this thesis was to analyze APOBEC3G genetic variants and assess their influence on HIV-1 susceptibility, transmission and disease progression.

One hundred and twenty nine HIV-positive, 35 exposed-seronegative and 76 HIV-negative subjects in HIV-concordant, HIV-discordant and HIV-negative couples from Dakar, Senegal, were enrolled in the study. APOBEC3G exons 3 and 4 were amplified by PCR, sequenced and screened for single nucleotide polymorphisms (SNPs). Effects of the identified SNPs on resistance and susceptibility to HIV-1 infection were analyzed by comparing allelic frequencies between seropositives and seronegatives. The role of these SNPs in HIV-1 transmission ability was assessed by comparing allelic frequencies between transmitters in HIV-concordant couples and non-transmitters in HIV-discordant couples. Finally, we analyzed the effect of the SNPs on the CD4+ T cell count of HIV-1 positive subjects who were not on anti-retroviral therapy.

Four SNPs were identified in exons 3 and 4. Three SNPs; C4368T (rs3736685), C4354G (rs6001417) and A4553G (rs8177832; H186R) are almost in complete linkage disequilibrium with an allelic frequency of 0.38. They are weakly associated with reduced HIV-1 transmission ability in non-transmitters (P=0.094) and correlate negatively with the CD4 count in HIV-infected subjects (P=0.006). SNP T4100C (rs5757465; F119F) has an allele frequency of 0.04 and is negatively linked to the other three SNPs. This SNP is weakly associated with increased susceptibility to infection (P=0.100) and correlates positively with the CD4 count of HIV-infected subjects (P= 0.069).

Our findings suggest that APOBEC3G genetic variants influence HIV susceptibility, transmission ability and disease progression. These results warrant further investigations to identify the impact of these mutations on APOBEC3G function.

Dynamics of *P. vivax* infections in a cohort of Peruvian patients treated with chloroquine and primaquine

Richard Christopher Delgado Ratto[1]*, Prof. Dr U. D'Alessandro[1], Prof. J.P. Van Geertruyden[1]

[*]*Laboratory of Malaria/Unit of Molecular epidemiology, Intstitute of Tropical Medicine, Alexander von Humboldt Universidad Peruana Cayetano Heredia (Peru)*

[1]*Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerpen (Belgium)*

Abstract

The control and eradication (if possible) of *Plasmodium vivax*, the most prevalent of the malaria species in South America and Asia, represent one of the biggest challenges for the affected countries. The high number of recurrent cases after the radical treatment (chloroquine plus primaquine) and the high prevalence of asymptomatic cases reported in the Amazon Basin, complicate even more the efforts for controlling the disease. In the present study, we explore using molecular and serological tools the dynamics of the parasite population in a cohort of patients treated with CQ+PQ treatment during one year in a community from the Peruvian Amazon. The diagnosis of the patients done by microscopy was confirmed by species-specific PCR and the *P. vivax* positive samples were genotyped using 15 microsatellites. Analysis of the genetic structure of *P. vivax* population was done. The presence of newly established infections was estimated detecting the presence of antibodies against the Circumsporozoite protein (CSP).

A promising methodology for the estimation of the drug efficacy in cohorts using CQ+PQ is described. Most of the recurrences in the cohort were due to relapses representing a high risk of drug resistance against primaquine in the Amazon Basin. By the way, we found a high number of asymptomatic/subpatent infections that, despite the low intensity of transmission, may reflect a clinical immunity within the people.

The limited genetic diversity and clonal structure of *P. vivax* population found in these areas may represent a high risk for the rise and spread of drug resistance, nevertheless also may stimulate positively the development of clinical immunity by the people in the Amazon Basin.

Recombinant bacteriophage endolysins as new antibacterial agents

Beker Feto Gemmeda[1]*, Prof. Dr R. Lavigne[1] and Maarten Walmagh[1]

** Current Address to be submitted*

[1]K.U.Leuven, Lab. Voor Gentechnologie, Kasteelpark Arenberg 21, 3001 Leuven (Belgium)

Abstract

To be submitted…

Characterization of *Staphylococcus aureus* SecA1 in view of its potential use as antibacterial target

Kalejaiye Titilola Dayo[1]*, Prof. Dr J. Anné[1] and Dr Kenneth Segers[1]

**Current address to be submitted*

[1]*KULeuven Rega-Instituut, Afdeling Bacteriologie, Minderbroederstraat 10, Blok x, bus 1030, 3000 Leuven (Belgium)*

Abstract

The story of antibiotic resistance and virulence in *S. aureus* is, as has been stated by others, one of "depressing evolutionary progression" (Deresinski, 2005). In an era when increased bacterial resistance to existing antibiotics threatens to impede successful chemotherapy, identification of inhibitors of novel antibacterial targets is critical (Economou, 2001). Such targets should be highly conserved among prokaryotes, not conserved among eukaryotes, essential, and well-defined. The secretion pathway (Sec pathway) meets all of these criteria (Alksne *et al.*, 2000) since bacteria secrete a variety of newly synthesized proteins across the cytoplasmic membrane via the Sec system (Pretz *et al.*, 2004). SecA protein, an ATPase, is the central component of the translocation machinery for newly synthesized proteins in *Escherichia coli*. The hydrolysis of ATP generates the driving force for the translocation of preprotein. (Ahn and Yun, 2009; Natale *et al.*, 2005, van der Wolk *et al.*, 1993).

In this study, we have characterized the enzymatic properties of the preprotein translocation motor SecA1 from *S. aureus*. (saSecA1). The saSecA1 gene was isolated by PCR and cloned into the pET23d expression vector for overexpression of the recombinant protein in *E. coli*. A mutant saSecA1 protein (W727A saSecA1) was generated by site-directed mutagenesis. After overexpression of the recombinant proteins and purification by Ni-NTA affinity chromatography, the enzymatic properties of both proteins were characterized by evaluating the reaction conditions at which the intrinsic ATPase activity was optimal. The developed colorimetric assay (in 96-well format) was validated for screening of potential saSecA1 inhibitors using known small-molecule ecSecA inhibitors.

In addition, we have shown that the intrinsic ATPase activity of saSecA1 is stimulated by inverted membrane vesicles containing overexpressed recombinant ecSecYEG. However, this membrane ATPase activity of saSecA1 was not stimulated by the *E. coli* preprotein ProPhoA(Cys$^-$). Finally, we have shown by site-directed mutagenesis that amino acid W727 has no detectable effect on the intrinsic ATPase activity of saSecA1. This suggests that W727 plays no major role in the regulation of the ATPase activity of saSecA1, in contrast to the corresponding amino acid (W775) in ecSecA, which has been shown to be a regulator of the ecSecA ATPase activity.

Passive protection of piglets against diarrhea induced by F4-positive enterotoxigenic *E.coli* strains

Okello Emmanuel[1]* and Prof. Dr H. De Greve[1]

**Department of Veterinary Surgery and Reproduction* *School of Veterinary Medicine, Makerere University, PO Box 7062, Kampala (Uganda)*
[1]*Vrije Universiteit Brussel Institute of Molecular Biology and Biotechnology, Building E, room E.6., Pleinlaan 2, B-1050 Brussels (Belgium)*

Abstract

To be submitted…

Localization and expression of aminopeptidase N in pig gastrointestinal tract and respiratory system

A.S.M. Lutful Ahasan[1]*, Prof. Dr B. Goddeeris[1,2], Prof. Dr. E. Cox[1], Vesna Melkebeek[1] and Philippe Belot[1]

** Chittagong Veterinary and Animal Sciences University, Khulshi-4202, Chittagong (Bangladesh)*
[1]U. Gent, Laboratory of Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Salisburylaan 133, 9820 Merelbeke, U. Gent (Belgium)
[2]Afdeling Gentechnologie, Kasteelpark Arenberg 30 - bus 2456 , 3001 Heverlee, KU Leuven (Belgium)

Abstract

To be submitted…

Phage tail-associated cell wall hydrolase : therapy for methicillin-resistant *Staphylococcus aureus* (MRSA)

Ashenafi Shiferaw Bulle[1]*, Prof. Dr H. De Greve[1] and Prof. Dr J.-P. Hernalsteens[1]

** Current address to be submitted…*
[1]*Vrije Universiteit Brussel Institute of Molecular Biology and Biotechnology, Building E, room E.6., Pleinlaan 2, B-1050 Brussels(Belgium)*

Abstract

Phage tail-associated cell wall hydrolases from *S. aureus* phages Phi11 and PhiETA (Siphovirus) were studied to evaluate their action against methicillin-resistant *S. aureus* (MRSA) which becomes resistant to more and more antibiotics. The tail-associated cell wall hydrolases are a lytic factor involved in local cell wall degradation and subsequently allowing the injection of phage DNA into the host cytoplasm. Putative gene 49 and gene 61, of phage Phi11 and PhiETA respectively, is located in a morphogenic module. It has two lytic domains homologous to a CHAP domain (corresponding to an amidase function) at its N-terminus and a lysozyme domain (belonging to the subfamily 2 (LYZ2)) at its C-terminus.

In this study, the full length cell wall hydrolase and its domains were cloned and expressed in *E. coli* C43 (DE3) (pRIL). However, only the lysozyme domains from both phages were expressed and purified as soluble and active proteins. The lysozyme domains from Phi11 (amino acids 407–632) with or without a histidine tag and the lysozyme domain from PhiETA (amino acids 405–624) caused efficient lysis of *Micrococcus luteus,* but fail to cause lysis of *S. aureus*. These data strongly suggest that if conditions for expression of the amidase domain and the full length cell wall hydrolase will be optimized, in order to get soluble and active proteins, gene products from ORF 49 and 61 of Phi11 and PhiETA respectively will be therapeutic candidates against methicillin-resistant *S .aureus* (MRSA). This is the first report so far on phage tail-associated cell wall hydrolase from phage Phi11 and PhiETA.

Keywords: - Bacteriophage, methicillin-resistant *S. aureus* (MRSA), tail-associated cell wall hydrolase, lysozyme

Investigation of mitochondrial carrier proteins of *Trypanosoma congolense* strains characterized for their sensitivity/resistance to isometamidium

Evans Njeru Simon[1]*, Dr Vincent Delespeaux[1] and Prof. L. Maes[1]

** Current address to be submitted…*
[1]*Instituut Tropische Geneeskunde, Nationalestraat 155, 2000 Antwerpen (Belgium)*

Abstract

Tsetse – transmitted African trypanosomiasis is one of the major constrains on expansion of livestock industries in Africa, with *Trypanosoma congolense* being the most pathogenic trypanosome species infecting livestock. The treatment and prophylaxis of African animal trypanosomiasis in cattle, sheep and goats is dependent on the use of three main trypanocidal drugs, namely; diminazene aceturate, homidium and isometamidium. Isometamidium has been used in the field for several decades both prophylactically and therapeutically in the treatment of livestock suffering from *T. congolense* infections. The drug usually accumulates and compartmentalizes in the kinetoplast DNA (kDNA) of the parasite. There is a growing concern that the effectiveness of chemotherapy will be significantly reduced due to the emergence of drug resistance occasioned by the inappropriate drug use practices, with drug resistance presently reported in 17 African countries.

Trypanosomes causing animal diseases have not been extensively studied at molecular level as those causing human infections, hence not much is known on the genome of *T. congolense*, the trypanosome in which drug resistance is well documented. There is need to therefore employ molecular techniques so as to unravel the genetic alterations that could possibly contribute to ISM resistance. Although ISM is known to accumulate preferentially in kDNA of *T. congolense*, no research has been conducted to explore any would be genetic modifications of possible transporter (s) located in the inner mitochondrial membrane that would reduce the accumulation of the drug within the kDNA of this parasite.

In this study, the role of mitochondrial carrier proteins in mediating ISM resistance in *T. congolense* was investigated. By use of Polymerase Chain Reaction – Restriction Fragment Length Polymorphism – Single Strand Conformation Polymorphism (PCR – RFLP – SSCP) technique, mitochondrial carrier protein - encoding genes for T. *congolense* parasites that were ISM sensitive and ISM resistant were screened for possible point mutations that if present; could possibly have an effect on drug uptake by the parasites. Our findings in this study suggests that the SSCP analysis of the genes coding for putative mitochondrial carrier proteins in ISM resistant *T. congolense* strains showed no polymorphism or sequence modifications when compared to those of ISM sensitive strains. Although no point mutations were revealed in the mitochondrial carrier proteins investigated here, it may be possible that other alternate modes of ISM resistance could be employed by the parasites to enable them reduce drug accumulation in their kinetoplast. It is therefore imperative to consider investigating for example, the differential expression of genes coding for importers and exporters and find their correlation with the two phenotypes.

Generation of nanobodies against surface exposed epitopes of *Trypanosoma congolense* with the aim of developing a reliable diagnostic tool for Nagana.

Steven Odongo[1] and Prof. Dr S. Magez[1]

[1] *VIB Department of Structural Biology, Vrije Universiteit Brussel, Building E.8.01, Pleinlaan 2, 1050 Brussel (Belgium)*

Abstract

African trypanosomes responsible for Nagana in livestock have lead to tremendous annual economic losses in the sub-Saharan Africa. Efforts to wipe the disease from the African continent have been futile due to the many technical and logistical problems hindering the control of the disease agents.

Our study is focused on alternative diagnostic methods for Nagana using Nanobodies. A field diagnostic test we hope to develop in the near future from these Nanobodies would be an antigen capture test (e.g. pick pen) based on detection of trypanosomes VSG antigen in the infected sample. Specifically, we focused on the generation of anti-VSG Nanobodies for application in field diagnosis of *Trypanosoma congolense*, the main causative agent of Nagana in cattle. Nanobodies are used in order to guarantee the sensitivity of the test. Their minute size (≈15kDa) and extended 'finger'-like CDR3 enable them to recognise unique epitopes in crevices, limiting possible competition for the same epitopes by conventional IgG antibodies that will also be present in the infected sample.

In this study, we generated three anti-VSG Nanobodies (**NbTcVSG1**, **NbTcVSG2** and **NbTcVSG3**) from an alpaca cDNA immune library. In brief, general selection for constructs containing anti-VSG Nanobodies was done through biopanning of display library on sVSG followed by screening of the enriched library and production of the Nanobodies. After successful production and purification, binding ability of each of the Nanobodies to living trypanosomes was assessed by FACS, immune fluorescence microscopy and antigen capture dot blot assay. The results obtained from these binding assays showed positive binding to parasites by all the three Nanobodies. However, signals intensities obtained on dot blot showed that there are qualitative differences in the affinities of the Nanobodies for parasites antigens present in the infected blood. It could be seen that **NbTcVSG1** seems most efficient followed by **NbTcVSG3** and **NbTcVSG2** being the least.

The study conducted here successfully generated three diagnostic tools that can already be used to aid diagnosis of Nagana caused by *T. congolense* using Immunofluorence microscopy. Further studies will be performed to determine the cross-reactivity of these Nanobodies i.e. ability to bind other species of African trypanosomes or *T congolense* strains. Information on K_{on} and K_{off} of the Nanobodies will also be obtained in order to assess their affinities for VSG antigen. Last but not least, protocols to adapt the application of the technique in the field will be optimized.

Characterization of the antagonistic activity of *Pseudomonas putida.* BW11M1 against phytopathogenic *Xanthomonas*

Maha Madakalapuwage Sandya Malkanthi[1]* and Prof. Dr R. De Mot

* *Current address to be submitted…*
[1]*K.U.Leuven, Centre of Microbial and Plant Genetics, Faculty of Bioscience Engineering, Kasteelpark Arenberg 20, B-3001 Heverlee (Belgium)*

Abstract

Xanthomonas represents a major genus of phytopathogenic bacteria affecting diverse crops. The availability of few effective chemical bactericides, development of resistance against such agents and environmental concerns associated with their use, fosters the search for alternative compounds targeting *Xanthomonas*. Such molecules may be found among secondary metabolites that mediate antagonism among plant-associated bacteria. In this study, the antagonism of the non-pathogenic rhizosphere isolate *Pseudomonas putida* BW11M1, displaying a broad anti-*Xanthomonas* activity, was explored.

Both phenotypic and genetic analysis revealed that strain BW11M1 produces at least two different non-protein compounds that differentially inhibit the growth of *Xanthomonas.* A first compound targets a subset of species/pathovars represented by *Xanthomonas alfalfae* subsp. *alfalfae (Xaa)*, while growth of other xanthomonads such as *Xanthomonas translucens* pv. *cerealis (Xtc)* is affected by a different metabolite. In a previous study, it was shown that genes for non-ribosomal peptide synthesis are required for the *Xaa*-inhibitory activity, indicating the involvement of production of a putative cyclic lipopeptide (CLP). In the current work, screening of a transposon mutant library of strain BW11M1 for altered inhibition of *X. translucens* pv. *cerealis* yielded several mutants with reduced activity.

Characterization of the inactivated genes conferring a phenotype of slightly diminished anti-*Xtc* activity and abolished anti-*Xaa* activity identified some additional mutants apparently affected in CLP production. These mutants were also affected in swarming and lack haemolytic activity, which strongly suggests that these phenotypes rely on CLP production.

A second group of mutants displays strongly reduced *Xtc* antagonism but retains anti-*Xaa* activity. Some of the mutated genes show homology to genes involved in production of toxoflavin by *Burkholderia glumae*, suggesting that a related secondary metabolite with antimicrobial activity may be produced by *P. putida* BW11M1.

In search of quorum sensing regulated processes in the intestinal pathogen *Salmonella* Typhimurium

Pham Thi Khanh Van[1], Prof. Dr J. Vanderleyden[1] and Ir. Kint Gwendoline[1]

[1]*K.U.Leuven, Centre of Microbial and Plan Genetics, Kasteelpark Arenberg 20, 3001 Leuven (Belgium)*

Abstract

Salmonella Typhimurium is a common agent for non-typhoidal salmonellosis causing food-borne infection in humans. The infection can be acquired from human interaction with contaminated food through eating or handling, for example, poultry, eggs, and animals. Studies are being conducted to find the solution to losses associated with this organism. Our studies hope to contribute to the solution of the problem of salmonellosis due to *Salmonella* Typhimurium. We investigated a protein of *Salmonella* Typhimurium known as LuxS which catalyzes the production of the AI-2 signaling molecule. This reaction is simultaneously involved in the metabolic activated methyl cycle. Both LuxS and AI-2 are involved in type II quorum sensing postulated as a cell-cell communication system for interspecies signaling.

This thesis research conducted on LuxS focusses on its modification, translocation, and regulatory factors. For investigation of LuxS modification, thiol-trapping technique was performed to understand whether this modification is an *in vivo* process related to oxidation of Cys-83 of LuxS. In addition, immunoprecipitation technique was also performed to isolate different forms of LuxS for subsequent MS/MS analysis. In translocation studies, we investigated the possible involvement of the Tat pathway in LuxS translocation using a LuxSPhoA reporter fusion strain. Proteins investigated for possible involvement in regulation of LuxS translocation and/or expressions were RpoS (alternative sigma factor) and H-NS (histone-like nucleoid structuring protein). For investigation of LuxS translocation, we employed a PhoA activity assay and an ampicillin resistance assay using LuxSPhoA and LuxSBla fusions, respectively. In addition, investigation of RpoS and H-NS involvement in LuxS expression was analyzed by western blot.

In this study, we neither elucidated the type of modification on LuxS nor isolated different forms of LuxS proteins. Further translocation studies were not able to show involvement of Tat pathway. But we have shown here that H-NS might be involved in the translocation of LuxS across the cytoplasmic membrane. Similar translocation studies on RpoS presently do not suggest that this protein an effect on LuxS translocation.
With this finding, the mechanism by which H-NS regulates LuxS translocation should be studied further. Ultimately, new insights in the role of LuxS in *S.* Typhimurium might be used for drug-development.

Functional analysis of the *RON3* and *ORB1* genes that determine leaf size and shape in *Arabidopsis thaliana*

Mathew Tinyang Katloi[1]*, Prof. M. Van Lijsebettens[1] and Prof. Dr G. Angenon[2]

[1]*Ghent University VIB Dep. Plant systems biology, Technologiepark 927, 9052 Gent (Belgium)*
[2]*Institute for Molecular Biology and Biotechnology, Building E, room E7.04b, Pleinlaan 2, B-1050 Brussels (Belgium)*

Abstract

Since *Arabidopsis* has become an increasingly handy and useful model plant for molecular genetics, *Arabidopsis thaliana* (L.) Heyn {Landsberg *erecta* (L*er*) and Columbia (Col) ecotypes} was entirely used as the experimental model plant in this project. The experimental system was the leaf, which characteristically arises as a primordium from the SAM. The leaf initiation and development is a function of among other factors, the auxin. Auxins are known to take part in axis formation during embryogenesis, leaf initiation, venation patterning, phyllotaxis and dorsoventrality in leaves. They are also involved in root meristem maintenance, hypocotyl and root elongation. Defects in auxin transport and depletion of local auxin maxima lead to low rate of leaf initiation or complete failure of lateral organ formation (Reinhardt *et al*., 2003). Leaf mutants with growth defects were selected from an EMS-mutagenized collection (Berna *et al*., 1999) for gene cloning (Peters *et al*., 2004) and functional analysis of *RON3* and *ORB1* genes. *RON3* and *ORB1* belong to the Ron and the Orb class of *Arabidopsis* leaf mutants respectively, in the EMS-mutagenized leaf collection (Bernà *et al*., 1999). The RON3 (classified as a HRGP in the TAIR database) was cloned in the "Chromatin and Growth Control" research unit. It has been found to play a role in auxin signalling and transport. Studies on *ORB1* have revealed its function in pigmentation, leaf development and flowering.

In this project, the function of *RON3* in auxin signalling was characterized by using pATHB8-GUS and pDR5-GUS (auxin specific) markers, and analyzing the root architecture. RON3-GFP fusion protein was localised by confocal microscopy and overepression of the *RON3* was analysed by QPCR. Similarly, *ELF6* was identified as the first candidate of *ORB1* and sequenced to locate the point mutation. Moreover, the role of the *ORB1* in the control of plant morphology was studied through leaf size and growth, flowering, root and hypocotyl growth analyses.

The results of this project have exclusively shown that *RON3* functions in the auxin signalling and transport related biological processes, and that RON3 is located either in the nuclear envelope or in the nucleus, thus the RON3 is not a HRGP, and further analysis can lead us to pinpointing the precise location and the specific function of the RON3 gene. In addition, we have demonstrated that *ORB1* is not *ELF6* and we have gone further to suggest that *PMR6* should be our next putative candidate gene of *ORB1*. The morphological analysis of *ORB1* has demonstrated that *orb1-2* and *orb1-3* are the strongest and the weakest *orb1* alleles respectively. Our data has also shown that *ORB1* is essential for initiation, growth and development of the rosette leaves, controls flowering time and takes part in primary root growth.

Alternative selectable marker gene systems for banana transformation: testing of candidate selective agents

Julius Sebastian Missanga[1]*, Prof. Dr R. Swennen[1] and Dr Gabriella Kovacs[1]

** Address to be submitted*
[1]K.U.Leuven, Faculty of Applied Bioscience and Engineering, Laboratory of Tropical Crop improvement, Division of crop Biotechnics, Kasteelpark Arenberg 13 bus 2455, 3001 Leuven (Belgium)

Abstract

Selectable marker genes are important for the production of genetically modified (GM) plants. Since during genetic transformation the percentage of cells which incorporated the gene of interest is low the use of a selectable marker gene is a key factor to identify the successfully modified cells. The most frequently used selectable marker genes are still antibiotic and herbicide resistance genes with almost exclusively bacterial origin. The usage of them in GM crops has created some concern that the resistance genes might be escaping and be transferred to other plants or even into pathogens. Therefore, extensive research is ongoing to find alternative selectable marker genes to replace the bacterial originated antibiotic and herbicide resistant marker genes. Since most of the cultivated banana varieties are seedless, sterile and clonally propagated the escape of the transgene exert a minimal risk. Moreover, the evaluation of the transgene product by thorough biosafety, allergenicity and toxicity risk assessment is a prerequisite as far as the crop is taken as food. One of the projects at the Laboratory of Tropical Crop Improvement is focusing on the applicability of the *anthranilate synthase* (*asa*)/5-methytryptophan (5MT) and *acetohydroxyacid synthase* (*ahas/als*)/chlorsulfuron (CS) alternative selection systems in banana transformation. The objective of this thesis work was to test the toxicity of 5MT and CS on non-transformed banana cell cultures to determine their optimal selective concentrations. The effect of different 5MT and CS concentrations on the growth and viability of non-transformed embryogenic cell suspensions (ECSs) of two banana cultivars 'Grande Naine' and 'Williams' was tested. ECS (200 µL at 33% SCV) of each cultivar were placed on solid ZZ medium supplemented with a selective agent at different concentrations. Five replicates per concentration were subjected to growth measurement and visual evaluation while two additional replicates were used for FDA viability assay. All parameters were monitored at regular time points for one month period. ECS cultures placed on ZZ medium without any additional selective agent served as control. The measured fresh weight at day 0 was set at 1.0 for each treatment and at subsequent time points the fresh weight was expressed relative to this value, called relative growth. The inhibitory effect on the growth and viability of banana cultures of 5MT was compared to geneticin, an antibiotic selective agent frequently used in banana transformation. During the four consecutive experiments applying gradually decreasing amount of 5MT as a selective agent a concentration range between 5-15µM/L was found to be applicable for both banana cultivars. 5MT applied at this range proved to be as effective selective agent as the antibiotic geneticin. It was also proved that the effect of 5MT on growth and viability of the banana ECS was not influenced by adding timentin (an antibiotic applied during selection process to inhibit the growth of *Agrobacterium*). As for CS such a concentration range could not be determined as during the two consecutive experiments the applied concentrations were too high. Nevertheless, the applicability of the CS as an effective selective agent for banana cell cultures was proven.

Construction of banana transformation vectors with two alternative selectable marker genes and transient transgene silencing in banana

MD. Mizanur Rahman[1]*, Prof. Dr R. Swennen[1] and Dr. G. Kovacs[1]

**Current address to be submitted*
[1]*K.U.Leuven, Faculty of Applied Bioscience and Engineering (FABSE), Division of Crop Biotechnics, Laboratory of Tropical Crop Improvement, Kasteelpark Arenberg 13, bus 2455, B - 3001 Leuven (Belgium)*

Abstract

Banana plants are becoming worriedly susceptible to newly emerging pathogens and environmental stresses. Since edible bananas are vegetatively propagated they are incapable of genetic modification of their genome, therefore, genetic modification could be the only way out of this vulnerable situation. Success of genetic improvement largely depends on the efficient identification and isolation of transformed cells and selectable marker genes (SMG) are applied to serve this purpose. Because of negative public notion against exclusively used antibiotic resistance selectable marker genes with bacterial origin, scientists are looking for plant genes which could be used as SMG.

Anthranilate synthase (AS) is a key enzyme in plants for biosynthesis of essential amino acid tryptophan and it is regulated by the end product through feedback inhibition. Rice *oasa1d* gene is a mutant *anthranilate synthase* gene which is insensitive to feedback inhibition by tryptophan (Trp) or its analogues e.g. 5MT.

Acetolactate synthase (ALS) is a major enzyme for biosynthesis of branched chain amino acid isoleucine, leucine and valine. ALS is sensitive to inhibition by six groups of herbicides. Point mutation could make the enzyme insensitive to these herbicides. Mutated Arabidopsis ALS gene- *csr1-1* was insensitive to chlorsulfuron herbicide.

Both of these mutated plant genes proved to be a valuable alternative of antibiotic selectable marker genes in different plants, therefore this research was focusing on the construction of *Agrobacterium* transformation vectors containing the *oasa1d* or *als (csr1-1*) genes each cloned under the control of either nos or MT3 promoter together with *neo* gene driven by nos promoter using a modified version of the pSAT versatile vector system for cloning.

During this thesis work SMGs were cloned under the control of one the above mentioned promoters into the cloning cassettes of different pSAT sub-cloning vectors. Then these newly obtained SMG cassettes were excised from the sub-cloning vectors with rare cutting restriction enzymes. The other gene cassette consisting of 'Pnos-*neo*-nosT' was excised and then inserted into the final transformation vectors, pPZP-RCS1 and pPZP-RCS2, respectively, also using rare cutting enzymes. Due to unforeseen cloning difficulties the final transformation vectors containing two gene cassettes (*neo* cassette together with one of the SMG cassettes) could not be completed.

Transient trans gene silencing

RNA silencing is a basic and major regulatory mechanism in three kingdoms of life. In plants, this mechanism is more diversified and plays a major role in the plant defense system against invading genetic material especially resulting from viral attack or to withstand varying biotic and abiotic stresses. Manipulation of this natural system offers an effective reverse genetics tool to study the function of a particular or all genes in a gene family or the genes for which forward genetic mutants have yet not been identified. *In silico* sequence data analysis combined with reverse genetics allows every gene to be studied. In polypolid plants like banana, it is difficult to obtain mutants for a specific gene and thus gene function study, which could be solved by RNA silencing.

No literatures were found regarding RNA silencing experiments in banana during the thesis work. A number of factors could be accountable for this lack of research initiatives. Banana transformation is more laborious and time consuming process than other crops. None of the well characterized candidate viruses for VIGS is suitable for banana and mechanical transfer of viruses to banana is not possible. At this point to analyze RNA silencing in banana, stably integrated reporter transgenes (e.g. *gusA*) provide an attractive alternative for native genes since they offer an easily detectable phenotype combined with precise quantification of the level of down regulation both at the RNA and protein/enzyme level. At the start of this thesis work stably *gusA* transformed banana ECS was under development at the Lab of Tropical Crop Improvement, but not available yet as it requires time to develop. Only remaining feasible approach was to assess RNA silencing in banana through transient expression of a reporter gene and transient co-expression of a reporter gene silencing construct.

The objectives of this work were to co-transform and re-transform banana ECS with the *GUS (gusA)* gene and a GUS silencing construct and to assess GU*S* silencing by histochemical and fluorometric assays.
In GUS silencing experiment, the *gusA* transformation vector pFAJ3000, the *gusA* silencing vector pIMHKUL5 and its basic cloning vector pSTARGATE were used. Embryogenic cell suspension culture (ECS) of banana variety 'Williams' were co-transformed and re-transformed with pFAJ3000 and pIMHKUL5 or pSTARGATE. GUS activity was analyzed though histochemical and fluorometric assay. Transient GUS expression and fluorometric assays revealed that re-transformation was not effective for transient trans gene silencing in banana but with co-transformation about 1.7 fold reduction in GUS activity was found both assays.

Genome-wide analysis of mutations involved in improved xylose fermentation for bioethanol production in industrial strain of yeast *Saccharomyces cerevisiae* TMB3400

Tewodros Firdissa Duressa[1], Prof. Dr Johan Thevelein[1] and Dr. Françoise Dumortier[1]

[1]*KULeuven, Kasteelpark Arenberg 31, box 2438, 3001 Heverlee (Belgium)*

Abstract

A recombinant industrial strain of Saccharomyces cerevisiae TMB3400, which has improved capacity of ethanolic xylose fermentation, was developed previously by metabolic engineering and chemical mutagenesis. However, the mutations that underlie the improved ethanol production by this strain were not identified. Therefore, we conducted this study to identify these mutations, because the identified genetic changes can be transferred to new industrial strain and may facilitate the construction of industrial strains of S. cerevisiae with optimal efficiency in ethanolic xylose fermentation.

Two mapping strategies, namely Artificial Marker Track Exclusion Mapping (AMTEM) and bulk segregant analysis by whole genome sequencing, were utilized to identify the mutations. Using AMTEM, we analyzed chromosome IV for linkage with growth on xylose, and we did not find any region that was linked to improved growth. However, we observed few regions in the chromosome which seem to show biased inheritance and need further linkage analysis with more segregants. For mapping by the bulk segregant analysis, we were able to screen 25 superior ethanol-producing segregants from crosses between segregants of strains TMB3400 and parent TMB3399. However, we did not conduct linkage analysis because the sequencing results were not received at the moment this paper was written. Genotyping the 25 segregants may at least verify the underlying mutations which were identified in previous studies. It is also probable that the whole genome analysis will detect new mutations.

We observed that some segregants that show superior growth on xylose do not yield superior ethanol production. Therefore, segregant screening with regard to xylose should take into account both growth and fermentation parameters. We also observed that the two mapping strategies have their own pros and cons in investigating the mutations. AMTEM is a quick and cheap mapping method but addresses the whole genome provided that each genomic region is represented in the artificial marked strain. The bulk segregant analysis allows detection of regions in the whole genome whether or not the regions are present in the reference genome (Wenger, et al., 2010). Besides, it uses strains with similar genetic background for linkage analysis, which may narrow down the genetic variation and as a result may facilitate better the detection of the mutations. Therefore, concerted use of the mapping strategies may be more powerful to identify the loci and mutations associated with improved ethanolic xylose fermentation.

Characterization of candidate Black Sigatoka responsive banana genes: a step towards improved intragenic plants

Cynthia Elizabeth Vargas Garcia[1]*, Prof. Dr R. Swennen[1] and Dr. S. Remy[1]

**K.U.Leuven, Centre of Microbial and Plan Genetics, Kasteelpark Arenberg 20, 3001 Leuven (Belgium)*
[1]K.U.Leuven, Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Kasteelpark, Arenberg 13, 3001 Leuven (Belgium)

Abstract

To be submitted…