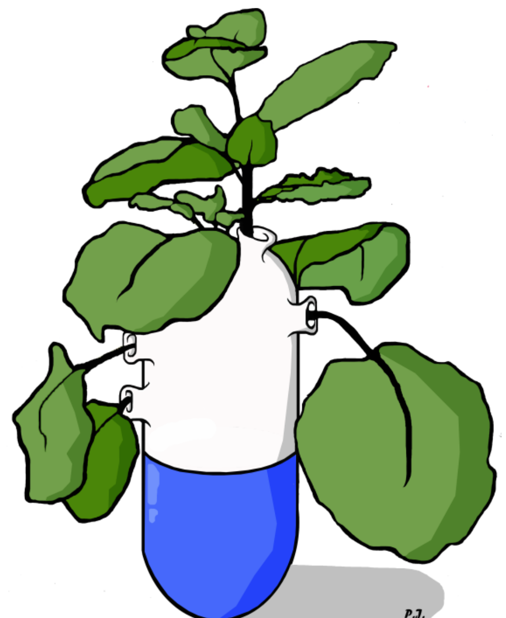
# molecular farming

Using Plants to manufacture High Value Products

COST FA0804

# Valencia 5-8th May 2013

## Final Conference



IBMCP

Instituto de Biología Molecular y Celular de Plantas

# Welcome to the final meeting of the Molecular farming COST FA0804

## Molecular farming COST FA0804 coordinators:

Prof. Kirsi-Marja Oksman-Caldentey (Chairperson), VTT Technical Research Center of Finland.
Prof. Julian K-C Ma (Vice-Chairperson), Hotung Molecular Immunology Unit, St George's University of London.

## Local Organizing Committee:

Diego Orzáez
Asun Fernández del Carmen
Paloma Juárez
Estefanía Huet Trujillo
Marta Vázquez Vilar
Alejandro Sarrión Perdigones
Jose Manuel Julve
Antonio Granell

# Scientific Programme

5-8th May 2013, Valencia

| **May 5th, Sunday** | |
|---|---|
| 17:00 - 18:00 | Registration and Poster Set-up |
| **Opening** | |
| 18:00 - 18:15 | Welcome and Meeting Opening: Kirsi-Marja Oksman, Diego Orzaez. |
| 18:15 - 19:15 | Opening lecture: First FDA approved plant-made Enzyme Replacement Treatment.<br>Yoseph Shaaltiel (PROTALIX Biotherapeutics, Israel) |
| 19:15 - 20:00 | Welcome Reception |

| **May 6th, Monday** | |
|---|---|
| 8:30 - 9:00 | Registration and Poster Set-up |
| **Session 1. WG2: Production Systems and Process Development.<br>Chair: Stefan Schillberg** | |
| 9:00 - 9:15 | Introduction by WG2 chair Stefan Schillberg |
| 9:15 - 10:00 | Manipulating Corn Germplasm to Overexpress Recombinant Proteins.<br>Elizabeth Hood (Arkansas State University, USA) |
| 10:00 - 10:30 | Production of plant-made pharmaceuticals and biomaterials using transient expression systems.<br>Anatoli Girich (Nomad Bioscience GmbH, Germany) |
| 10:30 - 11:00 | Multimerization of plant-produced proteins-tools for enhancing the immune response as well as the mechanical properties of protein-derived structures.<br>Udo Conrad (IPK, Germany) |
| 11:00 - 11:30 | Coffee break |
| 11:30 - 11:50 | Production of a pharmaceutical antibody in tobacco hairy roots – FP7 CoMoFarm project.<br>Suvi Häkkinen (VTT Technical Research Centre, Finland) |
| 11:50 - 12:10 | Plant VNPs for sensitive antibody detection.<br>Fernando Ponz (CIT-INIA, Spain) |
| 12:10 - 12:30 | Influence of E7 epitope from Human papillomavirus type 16 oncoprotein inserted in loops of PVX coat protein on virus particles formation and their expression level in plants.<br>Helena Plchova (Institute of Experimental Botany AS CR, Czech Republic) |
| 12:30 - 13:00 | Poster viewing |
| 13:00 - 14:45 | Lunch |

| **Session 2. WG1: Strategic Development of Molecular Farming.<br>Chair: Paul Christou** | |
|---|---|
| 14:45 - 15:00 | Introduction by WG1 chair Paul Christou |
| 15:00 - 15:45 | Molecular Farming and the Bioeconomy<br>Alfredo Aguilar (European Federation of Biotechnology) |
| 15:45 - 16:15 | Plant-derived Biopharmaceuticals: What's next?<br>Rainer Fischer (Fraunhofer IME / Institute for Molecular Biotechnology, RWTH Aachen University, Germany) |
| 16:15 - 16:45 | Field trials with plants making pharmaceuticals: a case study in Germany<br>Inge Broer (University of Rostock, Germany) |
| 16:45 - 17:10 | Coffee break and Poster viewing |
| 17:10 - 17:30 | Risk assessment and regulation of Molecular Farming<br>Joachim Schiemann (Institute for Biosafety in Plant Biotechnology, Germany) |
| 17:30 - 17:50 | Success and failure in commercial Molecular Farming development<br>Julian Ma (The Molecular Immunology Unit, St. George's University of London, UK) |
| 17:50 - 18:10 | Access to essential medicines in low and middle income countries<br>Harry Thangaraj (Infections and Immunity Research Centre, St. George's University of London, UK) |
| 18:10 - 19:00 | Panel Discussion: Strategic development of molecular farming. Moderator: Julian Ma |
| 21:00 | Dinner |

| **May 7th, Tuesday** | |
|---|---|
| 8:30 - 9:00 | Registration / Poster viewing |
| **Session 3. WG3: Target molecules assessment.<br>Chair: Dirk Bosch** | |
| 9:00 - 9:15 | Introduction by WG3 chair Dirk Bosch |
| 9:15 - 10:00 | Lysosomal storage diseases: therapeutic applications for plant products.<br>Hans Aerts (Academic Medical Center (AMC), The Netherlands) |
| 10:00 - 10:30 | Application of undifferenciated plant cells for the reliable large scale production of natural substances.<br>Gilbert Gorr (Phyton Biotech, Germany) |
| 10:30 - 11:00 | BryoTechnology™: Moss-made products on their way to clinic and market.<br>Andreas Schaaf (Greenovation Biotech GmbH, Germany) |
| 11:00 - 11:30 | Moss-based production of asialo-erythropoietin devoid of Lewis A epitopes.<br>Timo Lorenz (University of Freiburg, Germany) |
| 11:30 - 12:00 | Coffee break and Posters viewing |
| 12:00 - 12:20 | Structure-based design and experimental engineering of a plant virus nanoparticle for the presentation of immunogenic epitopes and as a drug carrier.<br>Marcello Donini (ENEA, Italy) |
| 12:20 - 12:40 | Russell-like bodies sequestering recombinant proteins.<br>Eva Stöger (University of Natural Resources and Applied Life Sciences, Austria) |
| 12:40 - 13:00 | Molecular farming database.<br>Dirk Bosch (Plant Research International, The Netherlands) |

| May 7th, Tuesday | |
|---|---|
| 13:00 - 13:45 | Panel Discussion: Molecular Farming Platforms and Products. Moderators: Dirk Bosch and Stefan Schillberg |
| 13:45 - 15:00 | Lunch and Poster viewing |
| **Session 4. Highlights of STSMs.<br>Chair: Diego Orzaez** | |
| 15:00 - 15:20 | Characterization of a putative substrate for the tobacco matrix-metalloproteinase NtMMP1.<br>Rita Santos (ITQB–Instituto de Tecnologia Quimica e Biologica, Portugal) |
| 15:20 - 15:40 | Fighting randomness: flow cytometric sorting of tobacco BY2 protoplasts.<br>Lauri Reuter (VTT Technical Research Centre, Finland) |
| 15:40 - 16:00 | Purification and characterization of the plant-produced human Surfactant Protein D (hSP-D).<br>Daniela Salgado (IME Fraunhofer, Germany) |
| 16:00 - 16:20 | Generation of Nanobodies for Plant-Made Measures against Malaria.<br>Henning Pennekamp (University of Darmstadt, Germany) |
| 16:20 - 16:40 | Coffee break and Posters viewing |
| 17:45 - 20:00 | City Tour |
| 21:00 | Conference Dinner |

| May 8th, Wednesday | |
|---|---|
| 8:30 - 9:00 | Registration / Poster viewing |
| **Session 5. Future Perspectives.<br>Chair: Kirsi-Marja Oksman** | |
| 9:00 - 9:45 | Towards Sustainable Human existence through Plant Synthetic Biology.<br>June Medford (Colorado State University, USA) |
| 9:45 - 10:15 | The use of a non-replicating transient expression system for the rapid production of virus-like particles in plants.<br>George Lomonossoff (John Innes Centre, UK) |
| 10:15 - 10:45 | GoldenBraid2.0: A comprehensive DNA assembly framework for multigene engineering in Molecular Farming and Plant Synthetic Biology.<br>Marta Vázquez Vilar (IBMCP, Spain) |
| 10:45 - 11:15 | Coffee break and Poster viewing |
| 11:15 - 11:45 | Maurice Moloney (Rothamsted Research, UK) |
| 11:45 - 12:00 | Remarks by the Cost Action Rapporteaur Henrique Guedes Pinto |
| 12:00 - 12:20 | Concluding remarks by the Cost Action Chair and Meeting Closure |
| 12:20 - 13:30 | Meeting of the Evaluation Panel |
| 13:30 - 15:00 | Management Committee meeting |

# ABSTRACTS – Oral Sessions

**Novel ERT for Gaucher Disease: Clinical program with Plant Cell Expressed Recombinant Glucocerebrosidase - taliglucerase alfa**

Shaaltiel, Y.*

Zimran, A.; Petakov, M.; Terreros Muñoz, E.; Solorio-Meza, S.; Amato, D.; Duran, S.; Giona, F.; Heitner, R.; Rosenbaum, H.; Giraldo, P.; Mehta, A.; Pastores, G.; Fernhoff, P.; Szer, J.; Cox, T.; Mengel, E.; Chertkoff, R.; Golembo, M.[16]; Almon-Brill, E.

Protalix Biotherapeutics, Israel.

*Corresponding author, e-mail: shaaltiel@protalix.com

Taliglucerase alfa is a carrot cell-expressed recombinant human β-glucocerebrosidase developed and produced by Protalix Biotherapeutics for the treatment of Gaucher disease (GD). A pivotal, phase III double-blind, randomized, parallel dose group clinical trial was recently completed. This trial treated 31 naïve adult symptomatic patients. Safety endpoints were drug-related adverse events and antibody formation. Primary efficacy endpoint was reduction in spleen volume. Secondary endpoints were change from baseline in hemoglobin levels, liver volume, and platelet count. Patients were enrolled and equally randomized to either 30 or 60 U/kg of taliglucerase alfa administered every other week for 9 months. Taliglucerase alfa was found to be safe and efficacious in a clinically relevant and statistically robust manner. An open label, Phase III switch over trial is ongoing. The objective of this study is to assess the safety and efficacy of taliglucerase alfa in 30 patients, (>2 years), with GD, previously treated with imiglucerase for at least 2 years at a stable maintenance regimen for at least six months. Safety endpoints are adverse events, clinical laboratory tests, electrocardiogram, echocardiography, pulmonary function tests, and antibody formation. Efficacy endpoints are changes in spleen, liver volume, platelet and hemoglobin levels. Interim analysis of data of first 15 patients, which completed the study protocol, demonstrated overall improvement or stability in main disease parameters. There was no evidence of increased safety concerns in patients switched to taliglucerase alfa. Additional clinical trials, including early access programs, and a pediatric study are currently on-going. The data generated with taliglucerase alfa during the development program were also positioned relative to historical data and therapeutic goals generated for imiglucerase, for which extensive experience is available. The results for the main clinical parameters from the pivotal study were compared with data summarized from selected references. All parameters were found to be within a comparable range established from the historical analysis, suggesting similar efficacy between taliglucerase alfa and imiglucerase. Comparison to the therapeutic goals for GD established by Pastores et al demonstrated that treatment with taliglucerase alfa is effective and meets the therapeutic goals as defined. taliglucerase alfa was approved by FDA ENVISA (Brazil) and Israeli authority during the last few month.

## Manipulating Corn Germplasm to Overexpress Recombinant Proteins

Hood, E.*[1] and Howard, J.[2]

[1]Arkansas State University, [2]Applied Biotechnology Institute

* Corresponding author, e-mail; ehood@astate.edu

Achieving high levels of protein accumulation in target plant tissues facilitates low cost production. Many molecular mechanisms are available to promote high level recombinant gene expression and protein accumulation. Our group also uses genetics of corn to promote high level protein accumulation in seed once the recombinant protein has been expressed. The transformation material for corn is the Hi-II tissue culture variety and transgenes must be moved from this starting material into elite inbred varieties in order to grow productive hybrids in the field. In our hybrid production material, we have improved our exo-cellulase (CBH I) lines 40-fold and our endo-cellulase (E1) lines at least 20-fold over initial levels. Our interest is in understanding the mechanism of this increase in protein accumulation and our approach is to use transcriptome sequencing. Near iso-genic lines have been produced that express our gene at high levels and at low levels in the same generation. We are comparing the transcriptome of these disparate lines to identify traits/loci that co-segregate with high expression in order to direct selection based on DNA markers rather than empirical observations of each ear.

## Production of plant-made pharmaceuticals and biomaterials using transient expression systems

Giritch, A.*

Nomad Bioscience GmbH, Weinbergweg 22, 06120 Halle (Saale), Germany

*Corresponding author, e-mail: giritch@nomadbioscience.com

Plant-based transient expression systems provide rapid and versatile platform which allows the expression of genes of interest without transgenic modification of plant genome. **magnICON**® developed by Icon Genetics company is based on the agrobacterial delivery of viral expression vectors using vacuum infiltration. The system is being used in different laboratories for the production of recombinant proteins in the research and industrial scale. High performance of **magnICON**® was demonstrated for various pharmaceutical proteins including vaccine antigens, recombinant enzymes, single chain and full-length monoclonal antibodies *etc*. However, **magnICON**® utilizes the vacuum infiltration of plants grown in trays and thus it brings additional costs and technical limitations making it best suitable for the production of (high cost) pharmaceutical proteins. Nomad Bioscience company developed novel expression system (**NOMADIC**®**)** that utilizes the agrobacterial spraying delivery of expression vectors to plants grown in soil. **NOMADIC**® process is less expensive and easier scalable than **magnICON**®, it is best suited for the production of low-cost proteins such as technical enzymes, animal health products, protein purification matrices *etc*. Further important application of our technology is a modification of agronomic traits using the transient expression of genes of interest. Spraying plants with agrobacteria delivering genes that encode plant flowering factors, defence proteins, plant hormone biosynthesis enzymes or RNAi molecules, multiple desirable agronomic traits have been engineered. Compared to stably transformed plants, our transient approach provides significant improvement in speed and flexibility.

## Multimerization of plant-produced proteins-tools for enhancing the immune response as well as the mechanical properties of protein-derived structures

Conrad, U.*

Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung Gatersleben (IPK)

*Corresponding author, e-mail: conradu@IPK-Gatersleben.de

The synthesis of native-sized proteins is a pre-requisite for exploring the potential of high molecular weight proteins in therapy and material technology. Stabilized trimers of soluble ELPylated HA have been produced in plants. ELPylation of these trimers does not influence the trimerization. The trimeric form of Avian flu ELPylated haemagglutinin was found to enhance the HA-specific immune response compared to the monomeric form. Plant-derived AIV HA trimers elicited potentially neutralizing antibodies interacting with both homologous virus-like particles from plants and heterologous inactivated AIV. Multimers of the spider silk flagelliform protein FLAG were produced in the endoplasmic reticulum of tobacco plant leaf cells with an intein-based posttranslational protein fusion technology. The repeated ligation of FLAG monomers resulted in the formation of large multimers. Synthetic, high molecular weight spider silk proteins larger than 250 kDa based on the assembly of protein monomers *via* posttranslational intein-mediated *trans*-splicing *in planta* were achieved. The resulting multimeric structures form microfibers, thereby demonstrating their great potential as a biomaterial. Spider silk-ELP fusion proteins with N-terminal lysine or glutamine tags have been produced *in planta*, purified by membrane-based Inverse Transition Cycling, connected by a bacterial transglutaminase resulting in multimeric spider silk-ELP derivatives of a molecular weight more than 250 kDa fitting to the range of native-sized spider silk proteins. Layers of spider silk-ELP monomers, pure ELP, fibroin and spider silk-ELP- multimers have been produced and shown to have surfaces displaying a homogenous structure with low roughnesses at nanoscale, thus allowing characterization by AFM-based nanointendation. The highest elastic penetration modul has been measured for the multimers. We conclude, that the increase of the molecular weight can enhance the stiffness. All 3 examples demonstrate the capacity of plant-based expression systems to produce and to purify protein multimers of high molecular weight for human medical, veterinary and nanotechnological applications.

# Production of a pharmaceutical antibody in tobacco hairy roots – FP7 CoMoFarm project

*Häkkinen, S.T. [1]; Raven, N. [2]; Henquet, M. [3]; Laukkanen, M. L. [1]; Anderlei, T. [4]; Bosch, D. [3]; Oksman-Caldentey, K.-M. [1]; Schillberg, S. [2]; Ritala, A. [1]

[1]VTT Technical Research Centre of Finland, P.O. Box 1000, 02044 VTT, Finland,

[2]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Forckenstrasse 6, 52074 Aachen, Germany

[3]Plant Research International, Wageningen UR, P.O.Box 16, NL-6700 AA Wageningen, The Netherlands

[4] Kühner, Dinkelbergstrasse 1, 4127 Birsfelden, Switzerland

*Corresponding author, e-mail: suvi.hakkinen@vtt.fi

In the concept of plant molecular farming, diagnostic and therapeutic antibodies are important targets. In the frame of the EU-funded CoMoFarm project (www.comofarm.org) contained high-yielding production systems based on plants and plant cells for the large-scale production of pharmaceutical proteins was developed. One of the target proteins was the antibody M12, which binds to vitronectin and thus it is a potential drug vehicle to solid tumors.

Tobacco hairy roots were studied as one potential plant-based production platform. Hairy roots were initiated by infecting *N. tabacum* cv. Petite Havana SR1 plants producing apoplast targeted M12 antibody with *Agrobacterium rhizogenes* LBA9402. When it comes to downstream processing, secreted product offers many advantages for easier extraction and purification. However, often recombinant proteins perform instable or go through degradation processes in culture medium and thus optimization of secretion conditions is needed. In this project, a statistical experimental design was used in order to optimally trigger secretion of M12 from tobacco hairy roots. Several chemical agents including phytohormones, macronutrients and signalling compounds were tested and the most remarkable effect was shown by NAA and $KNO_3$, together with a stabilizing agent polyvinylpyrrolidone, leading to a 30-fold increased secretion compared to non-treated hairy roots. Microscopy analyses of hairy root cross sections revealed that the optimized medium induced lateral root formation in hairy roots with morphological changes in inner cortex and root pericycle.

As a result, altogether 57 % of the total M12 produced was secreted; yielding 5.9 mg secreted M12 in one litre of secretion induction medium. Both secreted and intracellular forms were extracted and subjected to Protein A purification. The purified hairy root produced M12 was proven to be functional by vitronectin ELISA. The glycan analyses of secreted and intracellular forms of recombinant M12 showed that three major typical plant complex-type glycans were present.

**Plant VNPs for sensitive antibody detection**

Ponz,F.*

Departamento de Protección Vegetal, CIT-INIA, Apartado 8111, 28080 Madrid.

*Corresponding author, e-mail: fponz@inia.es

Viral nanoparticles (VNPs), a generic term including virions and virus-like particles, are biological structures increasingly becoming subjects for nanobiotechnological developments. Plant-derived VNPS present specific characteristics suiting them well in this field. Thus, in addition to the general biocompatibility associated to their biological nature and their potency as presenting nanoscaffolds, plant VNPs are not infectious to higher animals, can be obtained in medium-to-high amounts with a reasonable cost/benefit ratio, and usually do not bear epitopes towards which a previous immune response has been raised. Several platforms based on different specific VNPs are being actively developed.

We have working recently on the development of the platform based on *Turnip mosaic virus* (TuMV), a plant virus that generates long, thin flexuous VNPs. Aims directed towards applications such as immunization, antibiosis, and nanocatalysis are actively being pursued. Another field of application, relatively overlooked in the VNP area, has been antibody detection. However, antibody detection is a critical point in several areas of biotechnology, such as autoimmune or infectious diseases, for example. As a way of example of the potential of plant VNPs in antibody detection, our current results will be presented.

In relation with autoimmune diseases, an epitope derived from HSP60, a human heat-shock protein, has been expressed on the external surface of TuMV VNPS, and used to screen for circulating antibodies in patients' sera. Serum antibodies to HSP60 have been proposed to serve as biomarkers to monitor the immune status of an individual, and HSP60 itself is an immune modulator proposed to play a relevant role in cancer and autoimmune disorders. As to antibodies to infectious agents, there are several examples of viral cattle diseases in which the ability to detect antibodies at a high sensitivity are determinants of slaughtering decisions. One of these is the diseases caused by the Maedi-Visna virus, a lentivirus affecting sheep and goats. As above, epitopes from the lentivirus have been expressed on TuMV VNPs, and used to detect antibodies in sheep sera. The results obtained in both instances indicate the high potential of this approach in the design of antibody-detection tests.

# Influence of E7 epitope from *Human papillomavirus* type 16 oncoprotein inserted in loops of PVX coat protein on virus particles formation and their expression level in plants

Plchova, H. [1]*; Vaculik, P. [1,2]; Moravec, T[1].; Cerovska, N. [1]

[1]Institute of Experimental Botany AS CR, v.v.i., Rozvojova 313, 165 02 Prague 6, Czech Republic

[2] Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, 128 40 Prague 2, Czech Republic

*Corresponding author, e-mail: plchova@ueb.cas.cz

Plants are widely used for heterologous protein expression. The plant virus-based transient expression system could produce large quantities of desired proteins in a relatively short time. Chimeric plant viruses carrying foreign genes at a suitable position in the viral genome are promising peptid-presentating systems for use in vaccination strategies. Viral multiplication rapidly amplifies also foreign genes and thus allows high expression of heterologous proteins.

The *Potato virus X* (PVX) belongs to the order *Tymovirales*, family *Alphaflexiviridae* and genus *Potexvirus*. It is a filamentous, positive single stranded RNA (+ssRNA) virus with diameter about 13 nm and length about 515 nm.

The “high risk” *Human papillomaviruses* (HPVs) are the primary etiologic agents of cervical cancer, which represents the second most common cancer among women worldwide. The availability of prophylactic vaccines against HPVs (based on HPV-16, -18, -6, and -11 L1 structural proteins assembled in virus-like particles - VLPs) represents a milestone in the prevention of this infection, establishing the base for a significant reduction of the rate of cervical cancer in future. However, due to prohibitive costs, the use of these vaccines in developing countries is problematic. Thus, a therapeutic vaccine based on nonstructural viral proteins such as oncoproteins E6 or E7, targeting already infected individuals, is also required.

In our study we use the PVX-based expression vector pGR106. In our previous experiments we used this system to present mutated oncoproteins E6gt and E7ggg or epitopes derived from E7 and L2 proteins of HPV-16 as fusions with the N- or C-terminus of PVX coat protein (PVX CP), with or without amino acid linkers. Although the three-dimensional structure of PVX CP in the virion is unknown we produce the immunodominant HPV-16 $E7_{44-60}$ epitope situated in four different loops which are assumed to be located on the exterior part of PVX CP.

Here we present the cloning strategy of chimeric constructs and first data concerning the expression level and virus particles formation in *Nicotiana benthamiana* plants.

**Acknowledgements**

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## Molecular Farming and the Bioeconomy

Aguilar, A.*

Chairman Task Force Biotechnology

European Federation of Biotechnology

*Until the end of 2012, Head of Unit Biotechnologies, European Commission*

*Corresponding author, e-mail: alfredo.aguilar@efb-central.org

Molecular Farming in Europe has made substantial scientific and technological progresses in the last 10 years. It has already reached a maturity level able to demonstrate at pilot and industrial scale the achievements already obtained in the bench. The advances made in the science base and in the technologies for expressing bioactive compounds (proteins, peptides, etc.), and extracting them from plants have allowed several plan-made products with therapeutic potential to be assessed for safety and efficacy.

The European Union, through the different Framework Programmes, has supported over the last 15 years a substantial number of projects in this area. The projects, targeted aspects such as the expression and production of antiviral and antitumor substances, vaccines, secondary metabolites, etc. Some projects aimed at bringing the advances obtained in Synthetic Biology to develop more efficient “cell factories”, including standardization issues.

The challenges for Molecular Farming in the near future are important: they are not only scientific and technological, but also of IPR and regulatory nature. In that sense, the 2012 Bioeconomy Communication of the European Commission and Horizon 2020 offer new opportunities to Molecular Farming to demonstrate its full potential, bringing to society the benefits of new bioactive compounds and for the socio-economic actors involved economic growth, new skilled jobs, and leadership of European Molecular Farming.

## Plant-derived biopharmaceuticals: what's next?

Fischer, R.*

Institut für Biologie VII, RWTH Aachen University & Fraunhofer IME, Worringerweg 1, D-52074 Aachen, Germany

*Corresponding author, e-mail: fischer@molbiotech.rwth-aachen.de

The development of recombinant antibodies and vaccines has prevented and/or cured many life-threatening diseases. But as things stand in 2013, the restricted capacity and scalability of current production systems is holding back this crucial technology. The large-scale production of antibodies, vaccines and other recombinant pharmaceutical proteins reflects the industry's preference and indeed reliance on fermenter technology, particularly mammalian cell cultures. This expensive and time-consuming production platform is preventing the distribution of recombinant protein drugs to those most in need. One way to address these challenges is the use of plants and plant-based expression systems for the production of recombinant pharmaceutical proteins.

The inexpensive production of plant-derived pharmaceuticals depends on our ability to achieve satisfactory yields and product quality. This presentation will discuss the latest developments in antibody and vaccine production by molecular farming, focusing particularly on strategies to maximize protein yields during upstream production and optimize protein recovery in the downstream processing steps. The expression strategy, including the targeting of recombinant proteins to particular subcellular compartments, can affect yield, stability, quality and purification. Our long-term objective is to ensure that the next generation of plant-based production systems promotes the deployment of novel recombinant antibodies, vaccines and other biopharmaceuticals in the developing world rather than being restricted to the industrialized nations. Two case studies will be presented: one considers HIV-neutralizing antibodies selected for fast-track development, including risk assessment, expression in tobacco and maize, scale-up, downstream processing and regulatory development, with the intention to carry out clinical testing; other considered engineered plant cells producing vaccines for medical and veterinary use.

Pharma-Planta was an EU Sixth Framework Integrated Project, whose primary goal was to

develop an approved production pipeline and regulatory framework for plant-derived pharmaceutical proteins, moving beyond the proof of concept achieved in earlier projects. Pharma-Planta aimed to develop an entire production chain by taking candidate pharmaceutical molecules from the expression platform through all stages of production and processing, ultimately to complete a phase I human trials in Europe. At the beginning of the project, eight target molecules were chosen representing four key indication areas, but two HIV antibodies were chosen for fast-track development, including risk

assessment, cloning, optimized production in plants, scale-up, downstream processing and regulatory development, with the aim of submitting at least one of them for clinical trials during the program. The antibodies were produced successfully in maize and tobacco, the two major production crops considered in the project, but antibody 2G12 was the most promising. The expression levels in tobacco exceeding 100 mg per kg of leaf material and the recovered antibody remained stable and functional in neutralization assays. Scale-up was achieved during upstream production and downstream processing eventually providing grams clinical-grade antibody material for preclinical and clinical testing. A phase I clinical trial was completed successfully in the UK and the work is now moving towards a phase IIa clinical trial funded by an Advanced ERC grant.

The extraordinary success of the Pharma-Planta project has left the field wide open and full of additional possibilities, so where do we go next? To capitalize on the progress, Fraunhofer IME has developed a promising multi-stage malaria vaccine candidate that has developed from concept to a candidate project over several years, based on the optimization of performance and the improvement of manufacturing methods taking into account the many different plant-based platforms now available. This project has advanced with such success that we aim to begin translational research with the candidate products in the next 18 months. We are also developing a state-of-the-art facility that will combine a new concept for the high-throughput testing of plants and plant cells with a strategy for rapid scale up to commercial production using a fully automated vertical farm concept currently under construction in Aachen. Ultimately we plan to implement such concepts by developing simpler version for developing countries.

**Field trials with plants making pharmaceuticals: a case study in Germany**

Broer, I.*

University of Rostock, Agricultural and Environmental faculty

Justus-von-Liebig-Weg 8, D-18059 Rostock, Germany

*Corresponding author, e-mail: inge.broer@uni-rostock.de

In October 2008, the University of Rostock applied for the permission to release transgenic potatoes, each carrying one of three different transgenes. The field trial was planned from 2009 to 2011 at two sites (Thulendorf in Mecklenburg Pomerania and Üplingen in Saxony Anhalt). The genes coded for 1. a viral antigen (VP60), 2. the choleratoxin subunit B and 3. a cyanophycin synthetase, leading to non-ribosomal production of a biodegradable polymer.

The aim of the release was to identify potential effects of the three transgenes on the environment, to analyse the variability of transgene expression in comparison to greenhouse trials and to produce material for feeding studies. Although similar trials have been carried out by the same lab in three previous years without any detectable harm for consumers or the environment, the application induced massive public and political concern. This delayed the permission drastically and was accompanied by severe field destructions. The talk describes the process, the difficulties and the necessities of such trials in Germany.

**Risk assessment and regulation of molecular farming**

*Schiemann, J. [1]; Sparrow, P. [2]; Broer, I[3].

[1]Julius Kuehn Institute, Federal Research Centre for Cultivated Plants (JKI), Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany

[2]John Innes Centre, Norwich Research Park, Norwich, UK

[3]University of Rostock, Rostock, Germany

*Corresponding author, e-mail: joachim.schiemann@jki.bund.de

The general principles of genetically modified (GM) plant risk assessment and the regulatory framework for contained use and open field production of plant-made pharmaceuticals / plant-made industrials (PMP/PMI) will be described. While significant progress has been made for the containment grown production of PMPs, the commercialization of medicinal or industrial products produced in the field has yet to emerge. Suggestions for reducing the regulatory burden for GM plants will be discussed, also in light of the emerging new technologies to modify the genetics of plants. Since regulations surrounding the commercialization of GM crops are very costly and not appropriate for most of the PMP/PMI applications in Europe, we propose that amendments to the EU Directive 2001/18/EC are necessary to allow for the commercialization of products from GM plants without the need of an 'authorization'. To fully acknowledge the overall outcome of adopting plants to produce PMP/PMI, the conclusion is that broader and more balanced legislative oversight is needed. To fully explore the advances of GM technology a paradigm shift in the risk assessment of GM plants is needed. Currently the risk assessment is based on the process, not on the new trait. The experience gained from more than 25 years of experimental field releases and more than 15 years of commercial use of GM plants and derived products has demonstrated that the GM technology is not inherently more risky than other classical or new breeding technologies. The risk assessment should be based on the new trait, rather than on the technology used to introduce it.

## Success and Failure in Commercial Molecular Farming Development

Paul, M.; Thangaraj, H. and Ma, J.*

Research Centre for Infection and Immunity, Division of Clinical Sciences,

St. George's University of London, UK.

*Corresponding author, e-mail: jma@sgul.ac.uk

The development of molecular farming technologies in the 1990's and early 2000's coincided with the boom in biotech and university spin-out companies. Opportunities were seemingly available for anyone with a good idea and the spirit of entrepreneurship. This should have been an ideal time to capitalise on general interest in biotechnology and to push forward the new and exciting concepts of producing recombinant medicines in plants.

What actually happened? In this study, we interviewed representatives from 16 molecular farming biotech companies to gain insight into how and why their companies were formed, their development strategies, key hurdles and major successes, what they did right and what they would change. This survey will give a unique understanding of how (and possibly how not) to develop a biotech idea.

In this talk, we will present the methodology and preliminary results from the work. We will also discuss briefly how these commercial developments are relevant to molecular farming perspectives in developing countries.

# Access to essential medicines in low and middle income countries

Thangaraj, H.*

Infections and Immunity Research Centre, St. George's University of London, UK

*Corresponding author, e-mail: hthangar@sgul.ac.uk

The traditional model for drug development has almost exclusively relied on the enterprise and resources of the commercially driven pharmaceutical industry. The return on investment for drugs that consume very large development costs result in product development being skewed towards more lucrative markets. It also necessitates the zealous protection of intellectual property making it difficult for low and middle income countries to access essential health technologies. Attention in recent years has therefore been directed to new models or mechanisms of addressing the needs of impoverished populations. This will be illustrated through a set of case studies as a result of research within the Access to Pharmaceuticals Project.

## Lysosomal storage diseases: therapeutic applications for plant products

Aerts, J.M. *

Academic Medical Center, University of Amsterdam, The Netherlands

*Corresponding author, e-mail: j.m.aerts@amc.uva.nl

Lysosomal storage disorders are inherited diseases in man due to genetic defects resulting in lysosome dysfunction. Lysosomes are the intracellular compartments involved in recycling of macromolecules among others. Defects in hydrolases involved in lysosomal degradation of macromolecules result in their accumulation ('storage'). The most common inherited lysosomal storage disorders are Gaucher disease and Fabry disease, two disorders in which glycosphingolipids accumulate in lysosomes as the result of deficiencies in glucocerebrosidase and alfa-galactosidase A activity. The features of the two disorders are described, including new insights in pathophysiology. New research tools (activity-based labelling probes) and biomarkers for these disorders are introduced. Finally, the potential of plant derived iminosugars as therapeutic agents is discussed as well as the use of plant-produced recombinant human enzymes for the treatment of Gaucher disease and Fabry disease.

## Application of undifferentiated plant cells for the reliable large scale production of natural substances

Gorr, G.*; Leibold, T.; Pump, M.; Schütte, K.; Ullisch, D.
Phyton Biotech GmbH, Alter Postweg 1, 22926 Ahrensburg, Germany
*Corresponding author, e-mail: gilbert.gorr@phytonbiotech.com

Plants have always been considered as an important source of natural actives. Many of these compounds have been successfully produced by chemical synthesis. However, there is an increasing demand for naturally produced ingredients. Although the production of plant-derived compounds with microbial systems has been realized in some cases, plants or plant cells would be the best resources for the supply of their endogenous substances as seen for paclitaxel - a secondary metabolite isolated from *Taxus species*. In fact it has been shown that the production of secondary metabolites with plant cell fermentation not only can be economically attractive but at the same time allows the supply of the substance of interest at high quality.

Moreover, plant cell cultures are an excellent tool to enrich specific structures out of a number of closely related compounds thus reducing impurities in a significant manner.

Independently from the natural environment a plant cell fermentation process can fulfil the supply needs for active compounds – Reliability, Consistency, Quality and Quantity. However, there is no "one-for-all" plant host and production technology which can be applied to the different kinds of complex plant-derived secondary metabolites.

A thoroughly accomplished development process comprises the identification of a high producing cell line, optimization of growth and production conditions, the development of a robust and reliable production process and its scale-up.

Here the successfully executed development of the most prominent plant cell fermentation process – the production of paclitaxel – will be presented.

## BryoTechnology™: Moss-made products on their way to clinic and market

Schaaf, A. *

Greenovation Biotech GmbH, Freiburg, Germany

*Corresponding author, e-mail: aschaaf@greenovation.com

*Physcomitrella*-based BryoTechnology™ is a cGMP-compliant, eukaryotic production system for demanding pharmaceutical proteins. The system offers sustainable and economically attractive API –production whilst exploiting the mosses unique characteristics such as straightforward genetic engineering and effective photoautotrophic production. These features and the (post-) translational machinery of a higher eukaryote position the moss in the enabling niche of toxic, glyco-designed, difficult to fold and otherwise demanding proteins.

Protein production with *Physcomitrella* relies on stably transgenic BryoMaster™-strains. To assure high yields, the final strain is selected in productivity-based screens from a minimum of one thousand producers. Following extensive tests for process performance and –stability, strain material is stored in a cryo Master Cell Bank (MCB). Upstream production is realized in a fully disposable, wave-based process employing out-of-the-shelf, non-customized components. This configuration allows for a rapid transfer of the moss production process to any production facility, be that of a licensee or a CMO.

greenovations current efforts aim to demonstrate the clinical viability of moss-produced APIs. Therefore, enzyme candidates for replacement therapies have been identified as lead candidates. Strains and processes have been set up and products are under preclinical investigation. Besides these results, the talk will share data and first impressions from a technical transfer of a moss production process to the GMP-environment of a CMO. This activity with all associated developments like an HCP-assay and a cryo-banking system are the results of greenovations strong and focussed dedication to achieving a clinical proof of concept.

In a nonclinical program, greenovation develops moss-made cytokines (Bryokine™) for cell-culture applications. The first product (FGF7) is on the market since mid 2012 and a full pipeline of strains under development will fill the portfolio constantly. Data and specifications of Bryokine™-products will be presented in the talk.

## Moss-based production of asialo-erythropoietin devoid of Lewis A epitopes

Parsons, J. [1]; Lorenz, T. [1]; Altmann, F. [2]; Arrenberg, C. [1]; Koprivova, A. [1]; Beike, A. [1]; Stemmer, C. [3]; Gorr, G. [3]; Reski, R [1,4,5] and Decker, E.L. [1]*

[1]Plant Biotechnology, Faculty of Biology, University of Freiburg, Freiburg, Germany

[2]Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria

[3]Greenovation Biotech, Freiburg, Germany

[4]BIOSS Centre for Biological Signalling Studies, Freiburg, Germany

[5]FRIAS Freiburg Institute for Advanced Studies, Freiburg, Germany

* Corresponding author, e-mail: eva.decker@biologie.uni-freiburg.de

It is well known that the posttranslational modifications displayed on biopharmaceuticals can affect product stability, biological activity and immunogenicity. Considering that the majority of the biopharmaceuticals are glycoproteins, engineering of the plant N-glycosylation patterns is indispensable. While on many human N-glycans β1,4 galactose is linked to the terminal GlcNAc residue, β1,3 galactose (Gal) and α1,4 fucose (Fuc) are common on plant N-glycans, giving rise to the Fucα1-4(Galβ1-3)GlcNAc trisaccharide. This structure, originally described for Lewis-positive histo-blood groups in humans, is known as Lewis A epitope (Lea). Although regarded as human-type glycosylation, this structure occurs only rarely on glycoproteins of healthy adult humans. However, Lea and Sialyl-Lea were shown to be highly increased in the sera from patients with colon cancer. Therefore, the repression of Lea attachment to recombinant plant-made biopharmaceuticals is desirable. The Lea epitope was shown to be enriched on complex-type N-glycans of moss-produced recombinant human erythropoietin, while unknown from the native human protein. Via gene targeting of moss galactosyltransferase and fucosyltransferase genes, we identified the gene responsible for terminal glycosylation and were able to completely abolish the formation of Lea residues on the recombinant biopharmaceutical.

**Structure-based design and experimental engineering of a plant virus nanoparticle for the presentation of immunogenic epitopes and as a drug carrier**

Arcangeli, C. [1]; Circelli, P.[§]; Aljabali, A. [¶‡]; Benvenuto, E. [§]; Lomonossoff, GP. [¶]; Donini, M. [§]* and Marusic, C. [§]

[§] ENEA, Laboratorio Biotecnologie, UTBIORAD, C.R. Casaccia, Via Anguillarese 301, 00123 Roma, Italy.

[1] ENEA, Laboratorio Diagnostico, UTTMAT, C.R. Casaccia, Via Anguillarese 301, 00123 Roma, Italy

[¶] Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, U.K.

[‡] Present address: Department of Cardiovascular Medicine, University of Oxford, Headington, Oxford OX3 9DU, U.K.

*Corresponding author: marcello.donini@enea.it

Biomaterials research for the discovery of new generation nanoparticles is one of the most active areas of biotechnology, known as bio-nanotechnology. This multidisciplinary research area includes the exploitation of biomaterials such as viral capsids for the production of new generation nanoparticles. Plant viruses are robust precisely defined nanometer-sized objects that can be formed by protein self-assembly. In the past twenty years a considerable number of scientific papers described the use of plant viral cages to produce vaccine components and nanoparticles for drug delivery. Recently, an increasing number of studies demonstrated the possibility of modifying both outer surface and inner cavity of capsids to produce multifunctional nanoparticles for targeted imaging or drug delivery (Young et al. 2008, Phytopathology, 46, 361–384; Lomonossoff and Evans 2011, Curr Top Microbiol Immunol.). In this context, we have investigated on the use of the Artichoke Mottled Crinkle virus (AMCV) capsid both as a carrier of immunogenic epitopes or for the delivery of anticancer molecules. A dual approach that combines both *in silico* tools and experimental virology was applied for the rational design of immunologically active chimeric virus-like particles (VLPs) carrying immunogenic peptides. The atomic structures of wild type (wt) and chimeric VLPs were obtained by homology modeling. The effects of insertion of the HIV-1 2F5 neutralizing epitope on the structural stability of chimeric VLPs were predicted and assessed by detailed inspection of the nanoparticle intersubunit interactions at atomic level. Wt and chimeric VLPs, exposing on their surface the 2F5 epitope, were successfully produced in plants. In addition, we demonstrated that AMCV capsids could also function as drug delivery vehicles able to load the chemotherapeutic drug doxorubicin (Arcangeli et al. 2013, J. Biomol. Struct. Dyn. in press). Future efforts will be directed towards the functionalization of such particles with the tumour-targeting human anti-tenascin-C antibody (mAb H10) (Lombardi et al. Transgenic Res. 2010;19:1083-97) in order to obtain cell-specific drug delivery.

**Russell-like bodies sequestering recombinant proteins**

Arcalís, E. [1]; Ibl, V. [1]; Rademacher, T. [2]; Avesani, L. [3]; Pezzotti, M. [3]; Stoger, E. [1]*

[1]University of Natural Resources and Applied Life Sciences, Vienna, Austria;

[2]Institute for Molecular Biotechnology, RWTH Aachen, 52074 Aachen, Germany,

[3]Department of Biotechnology. University of Verona, 37134 Verona, Italy.1st

* Corresponding author, e-mail: Eva.stoger@boku.ac.at

Most molecular farming applications have in common the need for high yields of recombinant protein which can be achieved by increasing expression and ensuring the stable accumulation of functional proteins in the most appropriate subcellular locations. Targeting strategies can be used to enhance the accumulation of a protein, and to control posttranslational modifications such as glycosylation. The success of such strategies is variable and appears to depend on the plant species and tissue, but also on the protein under study. In some cases, targeting strategies have to be adapted to specialized tissues to achieve the desired effect, e.g. the deposition in native storage organelles such as protein bodies or protein storage vacuoles of seeds. In other cases, aberrant localization can be attributed to inherent properties of the protein. For example, the unintended accumulation in ER-derived compartments may arise from covalent interactions with endogenous ER-resident proteins. Thus the partial localization of a recombinant antibody in the periphery of prolamin storage organelles was observed, most likely due an interaction between the heavy chain of the antibody and gamma-zein, an endogenous storage protein of maize.

Even in the absence of endogenous ER-derived storage organelles, recombinant proteins are occasionally sequestered in ER-derived bodies. In Arabidopsis seeds we have identified an insoluble fraction of recombinant murine interleukin-10 and localized this fraction within ER-derived protein accretions. Electron tomography demonstrates that they are detached from the ER and appear very similar to Russell bodies, which occur in connection with human ER storage diseases. Another example was observed in antibody-producing tobacco leaves, where an insoluble proportion of the recombinant protein was detected in ER-derived vesicular structures. We speculate that the content of these ER-bodies corresponds to a transport-incompetent fraction of the recombinant protein, and that, similar to mammalian plasma cells, plant cells are able to form Russell bodies as a self-protection mechanism to avoid blockage of the secretory pathway.

## Characterization of a putative substrate for the tobacco matrix-metalloproteinase NtMMP1

Santos, R. B.[1]*; Abranches, R.[1], Mandal, M. K.[2], Schiermeyer, A.[2]

[1]Plant Cell Biology Laboratory, Instituto de Tecnologia Química e Biológica, UNL, Av. República,

2780-157 Oeiras, Portugal

[2]Department of Plant Biotechnology, Fraunhofer Institute for Molecular Biology and Applied Ecology (IME),

Forckenbeckstrasse 6, 52074 Aachen, Germany

*Corresponding author, e-mail: ritabsantos@itqb.unl.pt

Plant matrix metalloproteinases (MMPs) are conserved proteolytic enzymes found in a wide range of plant species. These enzymes act in the extracellular matrix of the cell and play important roles in many aspects of the plant physiology such as growth and senescence (Marino and Funk, 2012). It is already known that NtMMP1, a MMP from *Nicotiana tabacum*, is localized in the plasma membrane of BY-2 cells and that its level of expression is higher after exposure to bacterial pathogens (Schiermeyer *et al.*, 2009). Furthermore, NtMMP1 is thought to be responsible for the degradation of recombinant proteins secreted into the culture supernatant (Mandal *et al.*, 2010), which is a matter of great importance in the Molecular Farming field, since endogenous proteases from plants act also on heterologous proteins, thus limiting the yields that could be achieved.

We have identified a potential target protein for NtMMP1, a 70 kDa homolog of the tomato β-D-xylosidase (GenBank JQ740833.1), which might be involved in the cell wall metabolism, related to senescence.

Since previous observations only provided indirect evidence, we were aiming for a direct biochemical proof using purified enzymes.

To achieve this goal, a xylosidase cDNA has been cloned from BY-2 cells, cloned into a plant expression vector and transiently expressed in *Nicotiana benthamiana* plants. The recombinant enzyme has been affinity purified via a C-terminally fused Strep-Tag. The final yield of recombinant xylosidase was insufficient for conducting further biochemical assays maybe due to inaccessibility or cleavage of the tag. Therefore alternative purification strategies need to be developed to increase final enzyme yields.

Knowledge about endogenous protease substrates will facilitate the rational design of protease depleted plant cell lines in order to optimize this platform for the production of valuable recombinant proteins.

## Fighting randomness: flow cytometric sorting of tobacco BY2 protoplasts

Reuter, L. [1]*; Kirchhoff, J. [2]; Schinkel, H. [2]; Schiermeyer, A. [2]; Joensuu, J.J. [1]; Ritala, A. [1]; Schillberg, S. [2]

[1] VTT Technical Research Centre of Finland

[2] Fraunhofer Institute for Molecular Biology and Applied Ecology (IME)

* Corresponding author, e-mail: lauri.reuter@vtt.fi

VTT-developed hydrophobin fusion technology has been shown to improve yields and facilitate purification of recombinant proteins in *Nicotiana benthamiana* and in *Trichoderma reesei*. Recently, the technology has been applied to tobacco BY2 suspension cells in order to gain full benefit of the contained and scalable eukaryotic production platform in combination with efficient capturing of the fusion protein from the cell lysate using aqueous two phase separation system.

Still several issues need to be addressed before the production system can compete in feasibility with other developed plant platforms or mammalian cell cultures. Thus far, inconsistent and gradually decreasing expression levels have been a major factor hindering the yields. In order to reach high yields and batch to batch consistency on the level of industrial demand, the inherent problems of somaclonal variation and genetic heterogeneity in the cell line need to be tackled.

A recent study of Kirchhoff et al. (2012) described a flow cytometric sorting -based method for generation of monoclonal BY2 cell lines. The monoclonal lines showed 10-fold increase in yields and also remained stable for long periods of time. A short term scientific mission (STSM) funded by the COST-action Molecular Farming was carried out at Fraunhofer IME in order to obtain hands on experience on protoplast preparation and flow sorting to be applied at VTT.

GFP-hydrophobin expressing BY2 protoplasts were isolated successfully using various protocols. Subsequently, the recovered protoplasts were subjected to flow cytometric analyses. As expected, a degree of heterogeneity in the population was confirmed as variable levels of GFP accumulation in the protoplasts. Individual protoplasts with high fluorescence intensity were sorted on 96-well plate and supplied with wild type feeder protoplasts. The sorting protocol was found to be very efficient: approximately 30% of wells contained a single fluorescent protoplast. Recovery of monoclonal calli has been demonstrated previously, but was not succeeded during the period of the research exchange.

All in all the STSM provided good capability to establish the flow sorting techniques at VTT, thus acting as a valuable platform for transfer of technology and knowledge between the institutes.

**Purification and characterization of the Plant-produced human Surfactant Protein D (hSP-D)**

Salgado, D[1]*

[1]Fraunhofer Institute for Molecular Biology and Applied Ecology

*Corresponding autor, e-mail: Daniela.Salgado@molbiotech.rwth-aachen.de

Surfactant protein D (SP-D) belongs to the family of collectins and was initially identified in the lung as a participant of surfactant innate immunity. Its deficiency cause serious pulmonary diseases, such as respiratory distress syndrome and asthma.

Nowadays, commercial surfactants are administered during the treatment of pulmonary diseases; however these products do not contain SP-D because this hydrophilic protein is lost in the procedure of surfactant extraction from animal lungs. Alternative strategies for SP-D recombinant production have been used, such as mammalian systems and synthetics surfactants; however these preparations are expensive and complicated.

Today, plants represent an alternative for production of recombinant proteins. As bioreactors, they offer the possibility of large-scale production with increased safety concerning pathogenic contaminations. Moreover, proteins can be accumulated to higher levels and secreted into intracellular compartments, affecting post-translational modifications and yields.

Based on the factors discussed above, the focuses of this research are the production, purification and characterization of a recombinant human SP-D (rhSP-D) in plants. To achieve this, an expression construct with a signal peptide for apoplast secretion was created, which demonstrated the best conditions in terms of production and functionality of SP-D in in tobacco leaves.

We were able to purify plant rhSP-D using *N*-acetyl mannosamine chromatography, confirming its natural lectin binding activity and detecting the sizes corresponding to monomeric and trimeric form. In addition to the characterization, as a preliminary result, a bacterial agglutination assay and a competition binding assay were done, which demonstrated the carbohydrate binding and the antimicrobial activity signals of plant rhSP-D.

Despite the *N*-acetyl mannosamine chromatography worked in our first results, the weak binding characteristics of rhSP-D avoids having to scale up, therefore the search of a suitable purification strategy is considered for the next experiments.

## Generation of Nanobodies® for Plant-Made Measures Against Malaria

Pennekamp, H.[1]; Magez, S.[2]; Pradel, G[3] and Warzecha, H* [1]

[1]Faculty of Biology, Plant Biotechnology and Metabolic Engineering, Technische Universitat Darmstadt, Schnittspahnstraße 3-5, 64287 Darmstadt, Germany

[2]Research Unit of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

[3]Institute for Molecular Biotechnology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

* Corresponding author, e-mail: warzecha@bio.tu-darmstadt.de

Malaria is the most frequent infectious disease of humans and other animals worldwide, with the most related deaths occurring in Africa among children under the age of five. This mosquito-borne infection is caused by eukaryotic protists of the genus *Plasmodium*.

Besides conventional methods of controlling malaria, our approach aims to add a novel and easily applicable tool to the array of existing measures. The rationale for our idea is that female *Anopheles* mosquitoes - beside their blood meal - frequently need to take up sugar from natural sources, like plant nectar. Generating plants producing anti-malarial Nanobodies® targeting the *Plasmodium* infection cycle would offer a unique possibility of delivering a remedy to the mosquitoes via the transgenic nectar. Moreover, such “anti-malaria” ornamental plants can be cultivated around mosquito breeding areas and next to human housings, without the need for extensive care. This would make it a cost-effective and easy-to-apply method of reducing malaria transmission.

The objectives of the STSM included the generation of a phage display library using cDNA obtained from the serum of an alpaca, and becoming acquainted with the technique of phage display, allowing the generation of anti-malarial Nanobodies® interfering with the *Plasmodium* life cycle, to ultimately prevent the infection of humans.

**Towards Sustainable Human existence through Plant Synthetic Biology.**

Medford, J.*

Department of Biology, Colorado State University, Fort Collins, CO 80523, USA

*Corresponding author, e-mail: June.Medford@ColoState.EDU

Plant synthetic biology has the potential to enable sustainable human existence on earth. That is an audacious statement. Yet, prior to the late 1800's most humans derived their food, materials and shelters from biological organisms. In the early 1900's chemist produced materials such as plastics that revolutionized societies. A revolution to produce sustainable products and materials is upon us. Plants are outstanding platforms for synthetic biology and can be designed to serve humanity and produced renewable and environmentally friendly materials and shelters. For example, we have designed a synthetic biological input -output system that enables external control of a trait of interest in plants. It is orthogonal to plant function allowing widespread adaptation. Our circuits are highly modular and can be used to control production of materials or produce plants that enable ordinary people to know about dangerous substances such as pollutants in their environments. We have tuned our traits with 'logic circuits' to provide amplification, memory and digital-like controls to transcriptional traits. These logic circuits are also being applied to bioenergy traits to enable at-will production of energy materials. To develop the promise of plant synthetic biology and produce sustainable existence on earth will require efforts of many and willingness to see beyond current limits.

## The use of a non-replicating transient expression system for the rapid production of virus-like particles in plants

Lomonossoff, GP[1]*.; Thuenemann, EC[1].; Lenzi, P[1].; Love, AJ[2].; Taliansky, M[2].; Zahmanova, GG[3].; Minkov, IN[4].; Matić, S[5].; Noris, E[5].; Verwey, J[6].; Meyers, A[7].; Rybicki, EP[7].

[1]Department of Biological Chemistry, John Innes Centre, Norwich, NR4 7UH, UK

[2]Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK

[3]Department of Plant Physiology and Molecular Biology, Plovdiv University, 24 Tsar Assen Str. 4000 Plovdiv, Bulgaria.

[4]Genomics Research Center Ltd., 105 Ruski Boulevard, 4000 Plovdiv, Bulgaria.

[5]Istituto di Virologia Vegetale, Consiglio Nazionale delle Ricerche, Strada delle Cacce 73, 10135 Torino, Italy

[6]Onderstepoort Biological Products SOC Ltd, Onderstepoort 0110, South Africa

[7]Department of Molecular and Cell Biology, University of Cape Town, Rondebosch 7700, South Africa

* Corresponding author, e-mail: george.lomonossoff@jic.ac.uk

Advances in transient expression technologies have allowed the production of milligram quantities of proteins within a matter of days using only small amounts (tens of grams) of plant tissue. Among the proteins that have been produced using this approach are the structural proteins of viruses which are capable of forming virus-like particles (VLPs). These particulate structures are potent stimulators of the immune system and, as such, are excellent vaccine candidates both in their own right and as carriers of additional immunogenic sequences. This presentation will describe some of the work undertaken by the EC FP7 Plant Production of Vaccines (PLAPROVA) consortium which investigated the use of a non-replicative transient expression technology to produce VLPs of both human and veterinary pathogens. VLPs of varying complexity derived from a variety of animal viruses were successfully produced in plants using this approach and their immunological properties assessed. Generally, the plant-produced VLPs were found to have the expected antigenicity and immunogenicity. In the case of bluetongue virus (BTV), the plant-expressed VLPs were shown to be capable of stimulating protective immunity in target animals (sheep). These findings raise the prospect that low-cost plant-produced vaccines could be developed for both veterinary and human use.

**GoldenBraid2.0: A comprehensive DNA assembly framework for multigene engineering in Molecular Farming and Plant Synthetic Biology.**

Sarrión-Perdigones, A. †[1]; Vazquez-Vilar, M. †[1]; Juarez, P.[1]; Forment, J.[1]; Ziarsolo, P.[2]; Blanca, J.[2]; Granell, A.[1] and Orzaez, D. *[1]

†These two authors contributed equally to this work

[1] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia, Spain.

[2]Centro de Conservación y Mejora de la Agrodiversidad (COMAV), Universidad Politécnica de Valencia. Camino de Vera s/n, 46022 Valencia, Spain.

* Corresponding author, e-mail: dorzaez@ibmcp.upv.es

Multigene engineering is turning a prerequisite for the efficient production of high value proteins in plants. Regrettably, there is no a standard DNA assembly methodology used by plant genetic engineers. To enable the exchange of genetic building blocks and to overcome the existing limitations on the design and construction of new genetic combinations, we propose GoldenBraid2.0 (GB2.0), a comprehensive technological framework that aims to foster the exchange of standard DNA parts for Plant Synthetic Biology. GB2.0 relies on the use of TypeIIS restriction enzymes for DNA assembly and proposes a modular cloning schema with positional notation that resembles the grammar of natural languages. Apart from providing an optimized cloning strategy that generates fully exchangeable genetic elements for multigene engineering, the GB2.0 toolkit offers an ever-growing open collection of DNA parts, including a strategically chosen group of functionally-tested, pre-made genetic elements to build frequently-used modules like constitutive and inducible expression cassettes, endogenous gene silencing and protein-protein interaction tools, etc.

GoldenBraid2.0 also offers the GBtool-kit, a set of three computational tools that guide the user through the GB assembly process. The first of them, named 'GBDomesticator', adapts the input DNA sequence provided by the user to the GBstandard. The second tool, named 'GBTUAssembler', performs the *in silico* multipartite assembly of GBparts to create a transcriptional unit (TU). Finally, the last tool is named 'GB Binary Assembler', and carries out the binary assembly between preformed TUs over the GoldenBraid loop to produce multigenic structures.

The GBdatabase and the GBtool-kit are available for the Molecular Farming community at www.gbcloning.org, and can be used for fast GB-engineering of genetic devices including antibody constructs, identity preservation modules, etc.

**Production of Human Apolipoprotein-AI in seeds: expression, purification and use as a cardiovascular therapeutic**

Maurice Moloney[1] and Cory Nykiforuk[2] , SemBioSys Genetics Inc, Calgary Alberta, Canada

[1] Current address: Rothamsted Research, Harpenden, Herts UK

[2] Current address: Cangene Corporation, Winnipeg, Manitoba, Canada

*Corresponding author, email: Maurice.moloney@rothamsted.ac.uk

In recent years, transgenic plants have been widely investigated as potential hosts for the production of therapeutic biomolecules, which traditionally have relied upon fermentation or mammalian cell culture. The most obvious products for which plant-production might offer a solution are those that incur some kind of manufacturing problem e.g. instability or aberrant folding in conventional hosts, those that are required in bulk quantities and those whose production costs are a factor in market development. For this reason, we have focused our efforts on diabetes and cardiovascular disease. These are both widespread problems throughout the world and the therapeutics needed to target these diseases will be required in large quantities. In the case of cardiovascular diseases, a major target is the control of arterial plaque, which is responsible for several life-threatening conditions. Recent work on reverse cholesterol transport as a therapeutic target for the reduction of plaque deposition and possible plaque regression has highlighted the potential therapeutic value of Apolipoprotein AI (ApoA1) and its naturally-occurring variant Apo AI $_{Milano}$. These proteins act in a stoichiometric manner *in vivo* and thus would be required in fairly large amounts if they were used as therapeutics. In this presentation, we show how ApoAI $_{Milano}$ can be produced efficiently in plants to provide a molecule with potent biological activity in the mobilization of cholesterol in model systems, demonstrating the unique capabilities of plant systems for the production of biotherapeutic candidate molecules.

# ABSTRACTS – Poster Sessions

**P1.**

**Recombinant jacalin-like plant lectins are produced at high levels in Nicotiana benthamiana and retain agglutination activity and sugar specificity**

Fernandez-del-Carmen, A.; Juarez, P.; Presa, S.; Granell, A.; Orzaez, D.*

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia. Avda Tarongers SN, Valencia 46022, Spain.

* Corresponding author, e-mail: dorzaez@ibmcp.upv.es

The plant kingdom is an underexplored source of valuable proteins which, like plant lectins, display unique interacting specificities. Furthermore, plant protein diversity remains under-exploited due to the low availability and heterogeneity of native sources. All these hurdles could be overcome with recombinant production. A narrow phylogenetic gap between the native source and the recombinant platform is likely to facilitate proper protein processing and stability; therefore, the plant cell chassis should be specially suited for the recombinant production of many plant native proteins. This is illustrated herein with the recombinant production of two representatives of the plant jacalin-related lectin (JRLs) protein family in *Nicotiana benthamiana* using state-of-the-art magnICON technology. Mannose-specific Banlec JRL was produced at very high levels in leaves, reaching 1.0 mg of purified protein per gram of fresh weight and showing strong agglutination activity. Galactose-specific jacalin JRL, with its complicated processing requirements, was also successfully produced in *N. benthamiana* at levels of 0.25 mg of purified protein per gram of fresh weight. Recombinant Jacalin proved efficient in the purification of human IgA1, and was able to discriminate between plant-made and native IgA1 due to their differential glycosylation status. Together, these results show that the plant cell factory should be considered a primary option in the recombinant production of valuable plant proteins.

**P2.**

# Transient expression of monomeric and secretory forms of Small Immunoproteins (SIPs) produced in *Nicotiana benthamiana*.

Huet-Trujillo, E.; Juarez, P.; Sarrion-Perdigones, A.; Vazquez-Vilar, M.; Granell, A. and Orzaez, D.*

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia. Avda Tarongers SN, Valencia 46022, Spain.

* Corresponding author, e-mail: dorzaez@ibmcp.upv.es

Engineered Antibody fragments are particularly attractive for the treatment of both infectious diseases, such as enteric infections, and autoimmmune disorders, such as inflammatory bowel disease (IBD). Small Immune Proteins (SIPs) are antibody-based structures comprising a scFv fragment fused to a constant immunoglobulin domain. SIPs combine the bivalency of full-length antibodies with the small size of scFv and prevent the shuffling of the variable regions in multigenic (cocktail) combinations. Here, we describe the construction and the expression in *N. benthamiana* of SIPs incorporating either the CH3 or the CH2CH3 domain of the human immunoglobulin HCα1 and containing the variable regions against the human tumor necrosis factor (TNF-α) or the VP8* peptide of rotavirus strain SA11. Assembly of DNA parts was carried out with the GoldenBraid (GB) assembly system. The correct expression of the SIPs was confirmed by Western blot and their functionality by antigen ELISA test. Moreover, we showed that SIPs can form secretory complexes in combination with the secretory component (SC) and joining chain (JC).

**P3.**

## Combinatorial Analysis of Secretory Immunoglobulin A (sIgA) Expression in Plants

Juarez, P.; Huet-Trujillo, E.; Sarrion-Perdigones, A.; Falconi, E-E.; Granell, A and Orzaez, D*

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia. Avda Tarongers SN, Valencia 46022, Spain.

* Corresponding author, e-mail: dorzaez@ibmcp.upv.es

Delivery of secretory immunoglobulin A (sIgA) to mucosal surfaces as a passive immunotherapy agent is a promising strategy to prevent infectious diseases. Recombinant sIgA production in plants requires the co-expression of four transcriptional units encoding the light chain (LC), heavy chain (HC), joining chain (JC) and secretory component (SC). As a way to optimize sIgA production in plants, we tested the combinatorial expression of 16 versions of a human sIgA against the VP8* rotavirus antigen in *Nicotiana benthamiana*, using the recently developed GoldenBraid multigene assembly system. Each sIgA version was obtained by combining one of the two types of HC (α1 and α2) with one of the two LC types (k and λ) and linking or not a KDEL peptide to the HC and/or SC. From the analysis of the anti-VP8* activity, it was concluded that those sIgA versions carrying HCα1 and LCλ provided the highest yields. Moreover, ER retention significantly increased antibody production, particularly when the KDEL signal was linked to the SC. Maximum expression levels of 32.5 µg IgA/g fresh weight (FW) were obtained in the best performing combination, with an estimated 33% of it in the form of a secretory complex.

**P4.**

## Social perception of edible vaccines: food versus medicine.

Cebadera, E.; Cámara, M.*

ALIMNOVA Research Group. Dpto. Nutrición y Bromatología II. Facultad de Farmacia.

Universidad Complutense de Madrid.

*Corresponding author, e-mail: mcamara@ucm.es

The increased role of biotechnology in society, and the development of research in two different fields such as nutrition and health, has created the right framework to think about its public perception. The social debate generated around GMOs resulting from modern biotechnology application is an example of the confrontation between the inescapable of scientific and technological progress and the fears that generates uncontrolled progress in advanced societies.

Food biotechnology it is taken with some scepticism, for the vast majority of social actors, due to an overestimation of the risks associated to its consumption.

Edible vaccines can be understood from two conceptual alternatives (food vs. medicine) which result is two different conceptions. When there is the possibility of eating a GM food, taking to account other nutritional alternatives exist, the new food is automatically rejected. However, if we look at this issue from a therapeutic point of view, it will always be more socially acceptable.

We believe that in the case of edible vaccines, its acceptance is conditioned by the trinomial (risk / benefit / geographic factor). In the case of edible vaccines and genetically modified foods, consumers in developed countries perceive a lack of benefit and potential risks, especially if the geographical area in which to develop the technology is close to its environment. However, if consumers consider edible vaccines as a therapeutic alternative for people who have no access to a solid health system, and its use is done in a very remote geographical environment, the result would be significantly different, finding more benefits than risks.

Taking into account the different the various developments of agro biotechnology related to human health, we consider the production of antigens in GM plants (edible vaccines) may be one of the most promising because of its importance both economically and for human and animals health.

**P5.**

## Slovenian contribution to molecular “pharming”

Morisset, D [1,2]*.; Gutierrez-Aguirre, I [1,2].; Ravnikar, M [1,2].; Peterka, M [2].;

[1]National Institute of Biology, Department of Biotechnology and System Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

[2]Centre of Excellence for Biosensors, Instrumentation and Process Control, Laboratory for Bio-Analytics, Velika pot 22, SI-5250 Ljubljana, Solkan

* Corresponding author, e-mail: dany.morisset@nib.si

The Department of Biotechnology and Systems Biology at the National Institute of Biology has been working on plant biotechnology for more than ten years, focusing on understanding the molecular mechanisms of plant-pathogens (bacteria, phytoplasma and viruses) interactions and on the development of virus resistant potato plants obtained by genetic engineering. More recently, the department has also initiated programs for the development of drought and salinity resistant plants and plants expressing new insecticidal toxins.

The interest in the field of molecular “pharming”, has motivated the department to join the COST FA0804 action in order to gain knowledge on the possible applications and bottlenecks of the plant platforms for the production of molecules of therapeutic or commercial interest.

In collaboration with the Laboratory for Bio-Analytics (LBA) from the Centre of Excellence for Biosensors, Instrumentation and Process Control (COBIK), a new R&D program has been recently initiated focussing on the plant-based production of virus-like particles for therapeutic applications.

The R&D program encompasses all the processes of molecular “pharming”. The long experience of the Department of Biotechnology and Systems Biology in plant biotechnology and virology is preeminent in the initial step, including the selection of the best combination of plant platform / plant virus vector, cloning and plant transformation, plant event selection and growth. The substantial expertise of LBA in developing upstream and downstream processes for biomolecules and viruses is of high value. LBA will use new convective interaction-based supports such as inorganic porous support, hollow fibres and macroporous chromatographic monoliths. The processes developed by the Laboratory for Bio-Analytics are GMP-compliant and scalable from the laboratory level, through the pilot level, and up to production level.

In addition, to improve the downstream processes and future QC assessment, we are developing biosensors for viruses and virus-like particle analysis. Biosensors will be applicable in production processes of viral vaccines and viral vectors, where fast methods for accurate and reliable in-process control are needed.

**P6.**

**Chimeric Hepatitis B virus-like particles produced in plants and their immunogenicity in mice**

Mazalovska, M.; Minkov, I. N.; Zahmanova, G. G.*

Department of Plant Physiology and Molecular Biology, University of Plovdiv, Plovdiv, Bulgaria

*Corresponding author, email: gerganaz@uni-plovdiv.bg

The immunogenicity of small proteins can be significantly increased by their presentation on particulate carriers such as the core antigen of Hepatitis B virus. The core antigen of Hepatitis B virus is highly immunogenic and spontaneously assembles into virus-like particles (VLPs). These VLPs offer the possibility of displaying foreign peptides at several different positions on their surface. Here, we investigated whether Hepatitis B can be used as a vaccine carrier of M2e avian influenza peptide. Chimeric VLPs have been successfully transiently expressed in plants using CPMV-HT expression system (Sainsbury and Lomonossoff, Plant Physiol.148, 1212-1218,2008) and their immunological properties assessed. Plant chimeric HBcM2e VLPs were expressed at level 68 µg per gram of fresh *Nicotiana benthamiana* infiltrated leaves. Generally, the chimeric plant-expressed HBcM2e VLPs have been shown to be capable to induce immune response in BALB/c mice. This study demonstrates efficiency of plant produced VLPs and we believe that plant-produced vaccines could be find application for human and veterinary vaccine design.

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**P7.**

## New topology for peptide or protein presentation in *Potato virus* X capsid protein

Vaculik, P. [1,2] *; Plchova, H. [1]; Moravec, T. [1]; Cerovska, N. [1,2]

[1] Institute of Experimental Botany, v. v. i., Academy of Science of the Czech Republic, Rozvojova 313, 165 02 Prague 6, Czech Republic

[2] Faculty of Science, Charles University in Prague, Albertov 6, 128 43 Prague 2, Czech Republic

* Corresponding author, e-mail: vaculik@ueb.cas.cz

*Potato virus X* (PVX) is widely used as a peptide or protein presentation system in plant biotechnology, despite the fact that three-dimensional structure of its capsid protein (CP) is unknown. It has been shown experimentally that PVX CP can be modified for transient expression of desired peptides or proteins by fusing them to the N- or C-terminus of CP. In our previous experiments we used this system to present oncoproteins E7 and E6 or epitopes derived from E7 and L2 proteins of *Human papillomavirus* (HPV) type 16. The E6 and E7 oncoproteins or other HPV epitopes were fused to N-terminus or C-terminus of PVX CP, with or without amino acid linkers. To find out other suitable locations for heterologous epitopes presentation in PVX CP leading to their better expression we have focused on the latest model of the three-dimensional organization of PVX CP in virion.

The latest model of the PVX CP assembled in virion predicts four surface located loops, which could be promising for peptides or proteins presentation. To examine this hypothesis, we decided to insert 17 AA epitope derived from E7 oncoprotein of HPV 16 fused with 6xHis tag into these loops.

Here we present the first part of our project: design of cloning strategy for four different fusions of E7 epitope with PVX CP, their expression in bacterial expression vector pMPM4-A4Ω and visualization of virus-like particles under transmission electron microscope to assess the characteristics of these recombinant proteins prior to be further produced in plants.

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**P8.**

# Building up a collection of standard DNA parts and modules for the GoldenBraid2.0 cloning framework.

Sarrion-Perdigones, A.; Palací, J.; Vazquez-Vilar, M.; and Orzaez, D.*

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia. Avda Tarongers SN, 46022 Valencia, Spain

* Corresponding author, e-mail: dorzaez@ibmcp.upv.es

The GoldenBraid2.0 (GB2.0) is the last version of the GoldenBraid cloning strategy for Plant Synthetic Biology. In this new version, we defined a common assembly standard and generated a collection of pre-made plug-and-play genetic parts to facilitate the building of frequently used genetic structures. Among other frequently-used standard parts, multigene engineering requires the use of different regulatory regions to avoid silencing effects associated with the repeated use of a DNA sequence in the same construct. Moreover, it is important to have a range of regulatory regions available, and that the expression strength provided by each promoter/terminator combination is properly characterized so that the multigene expression can be adjusted accordingly. To meet these requirements, we incorporated several regulatory 5´ and 3' regions into the GB2.0 collection. 5´ standard regulatory regions comprise a promoter and 5´-UTR, whereas 3´ regulatory regions are standard GBparts comprising 3'-UTR and terminator. As a first approach towards characterization, we finely measured the relative strength of each individual element using the Renilla/Luciferase system in transiently-transformed *N. benthamiana* leaves. Moreover, we studied to what extend the transcriptional levels conferred by a promoter/terminator combination can be inferred from the separated contribution of each region, and concluded that for most combinations the experimental data closely match the theoretical values.

**P9.**

## Recent advances in plant-derived therapeutic vaccines against HPV-related cancers

Franconi, R.[1]*; Illiano, E.[2]; Paolini, F.[3]; Demurtas, O.C.[4]; Radaelli, A.[2], Giuliano, G.[4]; Venuti, A.[3]; Massa, S.[1]

[1]ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, C.R. Casaccia, BIORAD-FARM Unit, Rome, Italy.

[2]Dept. of Pharmacological and Biomolecular Sciences, State University of Milan, Milan, Italy.

[3]Regina Elena National Cancer Institute, Laboratory of Virology, Rome, Italy.

[4]ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, C.R. Casaccia, AGRI-GEN Unit, Rome, Italy.

* Corresponding author, e-mail: rosella.franconi@enea.it

Cervical, anal-penile and a large set of head and neck (H/N) tumors are serious health problems caused by high-risk Human Papilloma Viruses (hr-HPV) for which there are no specific and effective pharmacological treatments. The available preventive HPV vaccines are expensive and do not provide a therapy for already infected/tumor-affected subjects. A therapeutic vaccine for early-stage cancers should reduce the incidence of the disease by stimulating cell-mediated responses. Low-cost and safer production platforms (DNA, plants, microalgae) can be used to obtain high-quality therapeutics. As a source of safe 'activating bio-molecules', plants can also provide bio-molecules with immunomodulating and anti-cancer properties able to enhance the immunogenicity of antigens.

We will present data about the recent advances in the generation of novel hr-HPV E7-based genetic/protein/combined vaccines for HPV-related cancers, exploiting plants and microalgae as biofactories and as a source of immunoenhancers.

These innovative products efficaciously treat HPV infections/tumors, at least in animal models, lower the production costs, and might be used in combination with surgery/radio/chemotherapy. To this purpose, since pre-clinical tests and models are crucial to foresee efficacy of immunotherapy before clinical trials, we created new pre-clinical orthotopic mouse models for HPV-associated H/N and cervical tumors. These models, by allowing *in vivo* total body imaging, will provide a more reliable evaluation of the new plant-derived formulations.

**P10.**

# Transgenic tobacco plants as platform for production of chimeric proteins containing granulocyte-macrophage colony-stimulating factor (GM-CSF).

Redkiewicz, P. ; Góra-Sochacka, A. *; Sirko, A.

[1]Institute of Biochemistry and Biophysics Polish Academy of Sciences, Warsaw, Poland

* Corresponding author, e-mail: annag@ibb.waw.pl

Our studies are focused on the efficient production of biologically active recombinant cytokines in plant expression systems. In an attempt to improve the stability of recombinant cytokines we decided to produce them as chimeric proteins in a fusion with two selected proteinase inhibitors. The first one is the trypsin inhibitor, CMTI from *Cucurbita maxima*. The second one, SPI2 from *Galleria mellonella* inhibits variety of serine proteases. This approach led to demonstration that human interleukin 2 (hIL-2) produced as a chimeric protein was protected by its fusion partner, CMTI against trypsin digestion.

In this study the similar strategy of improved production of another pharmaceutically important cytokine, GM-CSF (granulocyte-macrophage colony-stimulating factor) was applied. Aside from proteinase inhibitors, elastin-like polipeptide (ELP) was used as a fusion partner of GM-CSF.

Finally, five plant expression cassettes were obtained. Two of them contained the translational fusion, *SPI2::GM-CSF* and *CMTI::GM-CSF*, where the cDNA of GM-CSF was followed by the sequence encoding endoplasmic reticulum retention signal, KDEL. Three other constructs are translational fusions of *GM-CSF::ELP* with signals targeting recombinant proteins into either apoplast, ER or cytoplasm. The ELP examined in our study was composed of 30 pentapeptide (VGVPG) repeats. The transgenes were under control of RbcS1 promoter and terminator. The obtained expression cassettes were used to transform *Nicotiana tabacum* plants. Subsequently, the level of expression of recombinant chimeric proteins in leaf tissue of transgenic plants was estimated by ELISA assay. The high specific activity of plant produced GM-CSF was confirmed using the mammalian cells proliferation assay. Moreover, plant-produced CMTI was shown to protect its fusion partner, GM-CSF, against digestion with proteases.

**P11.**

## Purification and characterization of the barley (*Hordeum vulgare* L.) endoprotease B2 expressed in *Pichia pastoris*

Rosenkilde, AL.; Dionisio, G.; Brinch-Pedersen H.*

Aarhus University, Faculty of Science and Technology, Dept. of Molecular Biology and Genetics, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark.

*Corresponding author, e-mail: henrik.brinchpedersen@agrsci.dk

Barley (*Hordeum vulgare* L.) cysteine proteases are of fundamental biological importance during germination but may also have a large potential as industrial enzyme in a market where ~60% of all commercialized enzymes in the world are proteases. Barley cysteine endoprotease B2 (HvEPB2) was expressed in *Pichia pastoris* from a pPICZαA based construct encoding a HvEPB2 C-terminal truncated version (HvEPB2ΔC) and a new proteolytic resistant $His_6$ tag. Maximum yield was obtained after 4 days of induction. Recombinant HvEPB2ΔC (r-HvEPB2ΔC) was purified using a single step of $Ni^{2+}$-affinity chromatography. Purified protein was evaluated by SDS-PAGE, western blotting and activity assays. A purification yield of 4.26 mg r-HvEPB2ΔC per l supernatant was obtained. r-HvEPB2ΔC follows first order kinetics ($K_m$ = 12.37 µM) for the substrate Z-Phe-Arg-pNA and the activity is significantly inhibited by the cysteine protease specific inhibitors E64 and leupeptin. The temperature optimum for r-HvEPB2ΔC is 55˚C, thermal stability $T_{50}$ value is 44˚C and the pH optimum is 4.5. r-HvEPB2ΔC was incubated with native purified barley seed storage proteins for up to 48 h. After 12 h, r-HvEPB2ΔC efficiently reduced the C and D hordeins, as evaluated by SDS-PAGE. C and D intensities were decreased to close to nothing after 24 h. The intensities of the B and γ hordein bands decreased marginally over 48 h. No degradation occurred in the presence of E64. Recombinant hordeins (B1, B3 and γ1) was expressed in *E. coli*, and after 2 h of incubation with r-HvEPB2ΔC, significant degradation was observed.

**P12.**

# Production of the high quality, natural-like recombinant allergens needed for high efficiency of allergy treatment

Gomord, V.; Fitchette, A-C.; Catala, V. and Faye, L.*

ANGANY Genetics 70 route de Lyons la Foret, 76000 Rouen, France

* Corresponding author, e-mail: loic.faye@anganygenetics.com

Since the 1960's, the prevalence of allergy has increased sufficiently to become a major public health concern. The WHO estimates that by 2030, 50% of the world's population will suffer from this disease. Surprisingly, when almost a quarter of the world's population already suffers from allergy, allergy –related diseases are still clearly both undertreated and underdiagnosed. This is mainly due to the use of allergen extracts in common allergy diagnostic tests, as well as in immunotherapy.

Allergen extracts are heterogeneous mixtures of allergenic and non- allergenic molecules contained in crude extracts prepared from sources of allergens, such as pollen or house dust mites. These products are difficult to standardize and their composition is only partially known. Due to the complexity and poor allergenic activity of natural allergen extracts, allergy diagnostic is not specific and the clinical success of immunotherapy is low, with only 30% allergic patients having less symptoms after several years of desensitization.

In this context, recombinant allergens represent a valid alternative for allergy treatment with some obvious advantages over allergen extracts. Indeed, they can be produced in a reproducible way as highly pure and well-defined molecules with a high batch to batch consistency.

Unfortunately, most allergens are complex proteins and their current production in primitive expression systems, such as yeasts or bacteria, often results in recombinant allergen products with immunological properties and structural features different from their natural counterpart. Consequently, most recombinant allergens currently available are not adapted for either diagnostic applications or immunotherapy where allergens in their most natural conformation are needed.

ANGANY Genetics has developed AllergoPur™ for the production of the high quality, natural-like recombinant allergens needed for high efficiency of allergy treatment. AllergoPur™ technology is a rapid, flexible, high-yielding and robust allergen production system. With AllergoPur™, ANGANY Genetics has already produced the 8 recombinant allergens required for an accurate diagnosis and personalized immunotherapy of dust mite allergy, the most common respiratory allergy found in children.

**P13.**

**A coat-independent superinfection exclusion rapidly imposed in Nicotiana benthamiana cells by tobacco mosaic virus is not prevented by depletion of the movement protein.**

Julve, JM.; Gandía, A.; Fernández del Carmen, A.; Sarrion-Perdigones, A.; Castelijns, B.; Granell, A.; Orzaez, D.
Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas (CSIC),Universidad Politécnica de Valencia, Avda Tarongers SN, 46022, Valencia, Spain
*Corresponding author, e-mail: dorzaez@ibmcp.upv.es

New evidence is emerging which indicates that population variants in plant virus infections are not uniformly distributed along the plant, but structured in a mosaic-like pattern due to limitation to the superinfection imposed by resident viral clones. The mechanisms that prevent the infection of a challenge virus into a previously infected cell, a phenomenon known as superinfection exclusion (SE) or Homologous Interference, are only partially understood. SE is relevant to molecular farming because impedes the co-expression of two or more recombinant proteins in the same cell when cloned in separate viral replicons. By taking advantage of ICON´s deconstructed tobacco mosaic virus (TMV) system, where the capsid protein (CP) gene is replaced by fluorescent proteins, an exclusion mechanism independent of CP was unveiled. Time-course superinfection experiments provided insights into SE dynamics. Initial infection levels affecting less than 10 % of cells led to full immunization against secondary infections in only 48 h, and measurable immunization levels were detected as early as 6 h post-primary infection. Depletion of a functional movement protein (MP) was also seen to slow down, but not to prevent, the SE mechanism. These observations suggest a CP-independent mechanism based on competition for a host-limiting factor, which operates at very low virus concentration. The possible involvement of host factors in SE has interesting implications as it would enable the host to influence the process. The possible uses of SE for recombinant protein production will be discussed.

**List of Participants:**

Abranches Rita
Instituto de Tecnologia Quimica e Biologica, Portugal
ritaa@itqb.unl.pt

Aerts Johannes
Academic Medical Center, The Netherlands
j.m.aerts@amc.uva.nl

Aguilar Alfredo
European Federation of Biotechnology, Belgium.
alfredo.aguilar@efb-central.org
www.efb-central.org

Bellucci Michele
Institute of Plant Genetics, CNR, Italy
michele.bellucci@igv.cnr.it

Bosch Dirk
Plant Research International
PO Box 619, Wageningen, The Netherlands
dirk.bosch@wur.nl

Brinch Pedersen Henrik
Aarhus University, Denmark
henrik.brinchpedersen@agrsci.dk

Broer Inge
University of Rostock, Germany
Inge.Broer@Uni-rostock.de

Cámara Hurtado Montaña
Universidad Complutense de Madrid, Spain
mcamara@ucm.es

Capell Maria Teresa
Universitat de Lleida, Spain
teresa.capell@pvcf.udl.cat

Castilho Alexandra
University of Natural Resources and Life Sciences, Austria
alexandra.castilho@boku.ac.at,

Christou Paul
Universitat de Lleida, Spain
christou@pvcf.udl.cat

Conrad Udo
IPK Gatersleben, Germany
conradu@IPK-gatersleben.de

Cusidó Rosa M.
Faculty of Pharmacy. University of Barcelona, Spain
rcusido@ub.edu

Depicker Anna
Ghent University – VIB, Belgium
anpic@psb.ugent.be

Donini Marcello
ENEA, Italy
marcello.donini@enea.it

Faye Loïc
ANGANY Genetics, France
loic.faye@anganygenetics.com

Fernández del Carmen Asun
IBMCP (CSIC-UPV), Spain
maferde3@ibmcp.upv.es

Fischer Rainer
Fraunhofer IME, Germany
rainer.fischer@ime.fraunhofer.de

Franconi Rosella
Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Italy
rosella.franconi@enea.it

Gal-On Amit
Volcani Center, ARO Plant pathology Department, Israel
amitg@agri.gov.il

Gomord Véronique
ANGANY Genetics, France
veronique.gomord@anganygenetics.com

Gora-Sochacka Anna
Institute of Biochemistry and Biophysics, Poland
annag@ibb.waw.pl

Gorr Gilbert
Phyton Biotech GmbH, Germany
gilbert.gorr@phytonbiotech.com

Granell Antonio
IBMCP-CSIC-UPV, Sapin
agranell@ibmcp.upv.es

Girich Anatoli
Nomad Bioscience GmbH, Germany
giritch@nomadbioscience.com

Hakkinen Suvi
VTT Technical Research Centre of Finland, Finland
suvi.hakkinen@vtt.fi

Hood Elizabeth
Arkansas State University, USA
ehood@astate.edu

Huet Trujillo Estefanía
IBMCP-UPV-CSIC, Spain
eshuetru@upvnet.upv.es

Iannacone Rina
ALSIA- Centro Ricerche Metapontum Agrobios, Italy
riannacone@agrobios.it

Jané Font Albert
Phyture Biotech S.L.
a.jane@phyturebiotech.com

Juárez Paloma
IBMCP-UPV-CSIC, Spain
pjuarez@ibmcp.upv.es

Krczal-Gehring Gabriele
RLP AgroScience GmbH, Germany
gabi.krczal@agroscience.rlp.de

Kvarnheden Anders
Swedish University of Agricultural Sciences, Sweden
anders.kvarnheden@slu.se

Lainez Andres Manuel
INIA, Spain
Dirección.general@inia.es

Liu Clarke Jihong
Norwegian Institute for Agricultural & Environmental Research, Norway
jihong.liu-clarke@bioforsk.no

Lomonossoff George
John Innes Centre, UK
george.lomonossoff@jic.ac.uk

Lorenz Timo
University of Freiburg, Germany
timo.lorenz@biologie.uni-freiburg.de

Ma Julian
St George's University of London, UK
jma@sgul.ac.uk

Mäkinen Kristina
University of Helsinki, Finland
kristiina.makinen@helsinki.fi

Medford June
Colorado State University, USA
June.Medford@ColoState.EDU

Meeusen Els
Monash University, Australia
els.meeusen@monash.edu

Mentag Rachid
National Institute of Agricultural Research (INRA), Morocco
rachidmentag@yahoo.ca

Moloney Maurice
Rothamsted Research, UK
maurice.moloney@rothamsted.ac.uk

Moravec Tomas
Institute of Experimental Botany, Czech Republic
moravec@ueb.cas.cz

Morisset Dany
National Institute of Biology, Slovenia
dany.morisset@nib.si

Oksman-Caldentey Kirsi-Marja
VTT Technical Research Centre of Finland, Finland
kirsi-marja.oksman@vtt.fi

Orzaez Diego
IBMCP-CSIC-UPV, Spain
dorzaez@ibmcp.upv.es

Parsons-Widmaier Juliana
Plant Biotechnology, Faculty of Biology, University of Freiburg, Germany
juliana.parsons@biologie.uni-freiburg.de

Pennekamp Henning
TU-Darmstadt, Germany
pennekamp@bio.tu-darmstadt.de

Plchova Helena
Institute of Experimental Botanym, Czech Republic
plchova@ueb.cas.cz

Ponz Fernando
INIA, Spain
fponz@inia.es

Reuter Lauri
VTT Technical Research Centre of Finland, Finland
lauri.reuter@vtt.fi

Ritala Anneli
VTT Technical Research Centre of Finland, Finland
anneli.ritala@vtt.fi

Salgado Daniela
Fraunhofer IME, Germany
Daniela.Salgado@molbiotech.rwth-aachen.de

Santos Ana Rita
Instituto de Tecnologia Química e Biológica – ITQB, Portugal
ritabsantos@itqb.unl.ptl

Sarrión Perdigones Alejandro
IBMCP-UPV-CSIC, Spain
asarrion@ibmcp.upv.es

Schaaf Andreas
Greenovation Biotech GmbH, Germany
aschaaf@greenovation.com

Schiemann Joachim
Julius Kuehn Institute, Germany
joachim.schiemann@jki.bund.de

Schillberg Stefan
Fraunhofer IME, Germany
stefan.schillberg@ime.fraunhofer.de

Schots Arjen
Wageningen University - Plant Sciences, The Netherlands
arjen.schots@wur.nl

Shaaltiel Yoseph
PROTALIX LTD., Israel
shaaltiel@protalix.com

Sirko Agnieszka
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland
asirko@ibb.waw.pl

Stavridou Ioanna
Science Officer Food and Agriculture, Belgium
Ioanna.Stavridou@cost.eu

Stoger Eva
University of Natural Resources and Life Sciences
eva.stoger@boku.ac.at

Thangaraj Harry
St George's University of London, UK
hthangar@sgul.ac.uk

Tomasson Thorsteinn
MATIS Food &Biotech Institute of Iceland, Iceland
tht@matis.is

Torregrosa Rubén
Axeb Biotech, Spain
r.torregrosa@axeb.net

Ullisch David
Phyton Biotech, Germany
david.ullisch@phytonbiotech.com

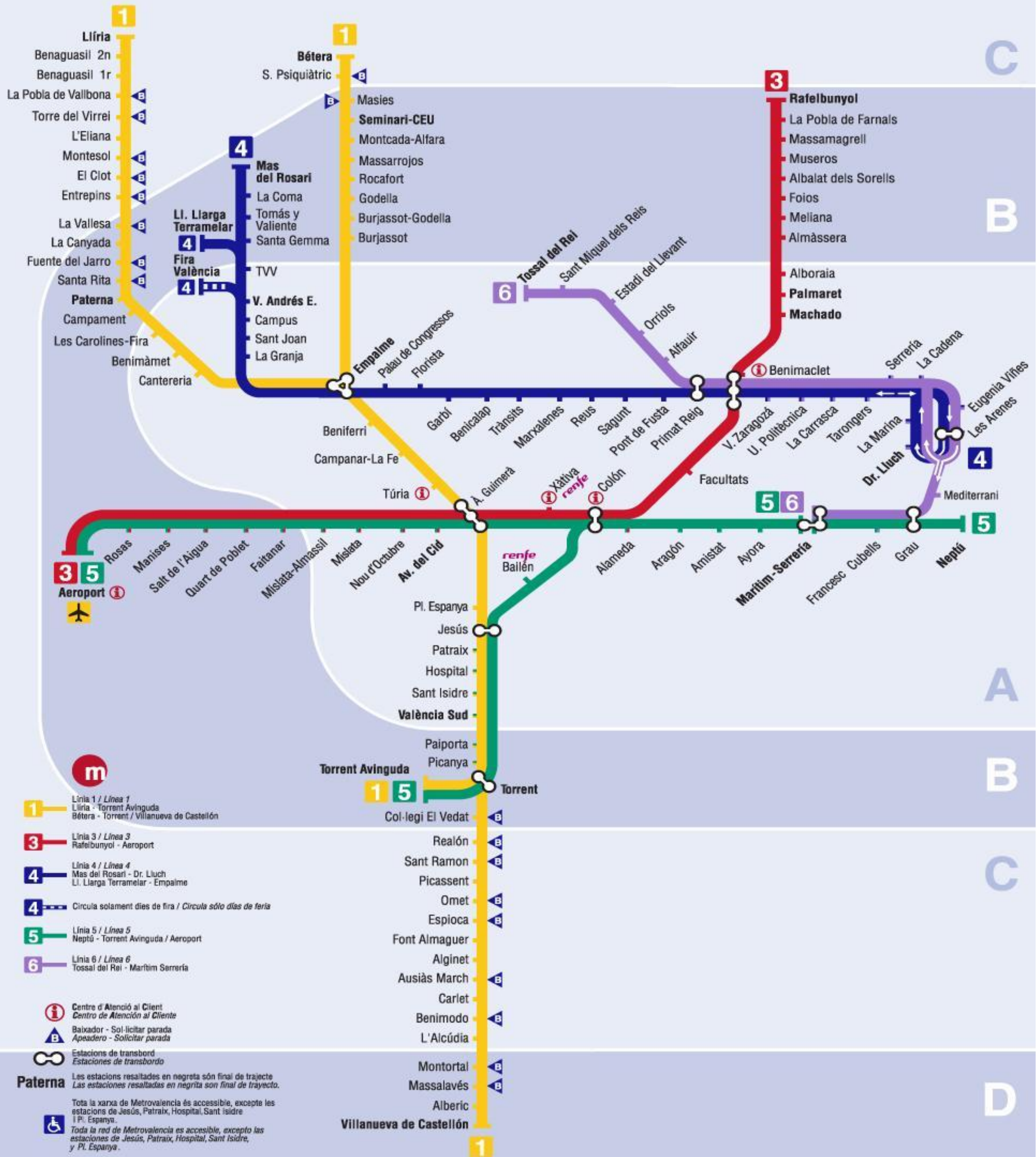
Vaculik Petr
Institute of Experimental Botany, Czech Republic
vaculik@ueb.cas.cz

Vázquez Vilar Marta
IBMCP-UPV-CSIC, Spain
marvzvi@upvnet.upv.es

Warzecha Heribert
Technische Universitaet Darmstadt, Germany
warzecha@bio.tu-darmstadt.de

Zahmanova Gergana
Plovdiv University, Bulgaria
gerganaz@gmail.com

# SUBWAY MAP: METRO VALENCIA



Dinner May 6th: Hotel NH Ciudad de Valencia at 21:00 (Av. Del Puerto 214)
Dinner May 7th: Restaurant 39º 27N at 21:00 (Marina Real Juan Carlos I)