



**The First Conference of the
International Society for Plant
Molecular Farming**



Berlin-Dahlem, 17-19 June, 2014

Final Programme and Abstract Book



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Lilya Kopertekh

Joachim Schiemann

Tobias Wittig

Address:

Julius Kühn-Institut

Institute for Biosafety in Plant Biotechnology

Erwin-Baur-Str. 27

06484 Quedlinburg, Germany

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June 17, 2014

11:00-13:45	Registration and Poster Mounting Lunch can be taken in the JKI Canteen (not included in registration fee)
13:45-14:00	Welcome and Conference Opening Joachim Schiemann, Julius Kuehn Institute, Germany Julian Ma, St George's, University of London, UK
14:00-14:40	Key Note 1 Immunogenic and immunomodulatory glycoproteins of helminth parasites, and the potential of plants as recombinant production platform C.H. Hokke, Leiden University Medical Center, The Netherlands
Session 1: Glycosylation of plant-derived recombinant proteins Chair: Arjen Shots	
14:40-15:00	Expression of Schistosoma-derived Omega-1 with diantennary glycans carrying Lewis X motifs in Nicotiana benthamiana plants L. Westerhof, Wageningen University, The Netherlands
15:00-15:20	Expression and glycomodification of therapeutic proteins using heterologous biosystems K. Ko, Chung-Ang University, Korea
15:20-15:40	Interaction of plant-derived anti-HIV mAb with human receptors S. Stelter, St George's, University of London, UK
15:40-16:00	Production of plant-derived recombinant proteins with engineered human-like n-glycans: a review A. Castilho, University of Natural Resources and Life Sciences, Austria
16:00-16:30	Coffee Break

Session 2: Downstream processing Chair: Stefan Schillberg	
16:30-16:50	Assessing the quantity and quality of Plant-made Pharmaceuticals by Surface Plasmon Resonance M. Sack, RWTH Aachen University, Germany
16:50-17:10	Protein quality control – from protein recognition and degradation to conditional protein expression N. Dissmeyer, Leibniz Institute of Plant Biochemistry, Germany
17:10-17:30	Production of ELPylated haemagglutinin in plants HT. Phan, IPK Gatersleben, Germany
17:30-17:50	Spider silk proteins from plants: Multimerization strategies and characterization tools N. Weichert, IPK Gatersleben, Germany
17:50-18:10	Hydrophobin as a fusion partner to produce a soluble influenza vaccine in plants N. Jacquet, Institut des Sciences de la Vie, Université catholique de Louvain, Belgium
18:30-19:45	Reception at the JKI building (separate registration)
20:00-22:00	Guided Walk through the Botanical Garden (separate registration)

June 18, 2014

09:00-09:40	<p>Key Note 2 Plant-made idiotypic vaccines for non-Hodgkin's lymphoma: techno-economic feasibility study V. Klimyuk, Icon Genetics GmbH, Germany</p>
<p>Session 3: Virus-mediated transient expression systems Chair: Inge Broer</p>	
09:40-10:00	<p>HPV pseudovirion production in plants I. Hitzeroth, University of Cape Town, South Africa</p>
10:00-10:20	<p>Exploiting Virus Superinfection Exclusion for the Production of Recombinant Polyclonal Antibodies in Plants D. Orzáez, IBMCP-CSIC, Spain</p>
10:20-10:40	<p>Plant virus nanoparticles: toxicity and teratogenicity evaluation using in vitro and in vivo models C. Lico, ENEA Casaccia Research Center, Italy</p>
<p>Session 4: Seed expression systems Chair: Kirsi-Marja Oksman-Caldentey</p>	
10:40-11:00	<p>Seeds for delivery of edible vaccine L. Rossi, University of Milan, Italy</p>
11:00-11:30	<p>Coffee Break</p>
11:30-11:50	<p>Bulking up antibody production, translation from a model plant to crops V. Virdi, Ghent University, Belgium</p>
11:50-12:10	<p>Molecular farming in plant seeds: a comparative analysis I. Broer, University of Rostock, Germany</p>
12:10-12:30	<p>Comparison of VHH-Fc antibody production in <i>Arabidopsis thaliana</i>, <i>Nicotiana benthamiana</i> and <i>Pichia pastoris</i> A. Depicker, Ghent University, Belgium</p>

Session 5: Moss expression systems Chair: Kirsi-Marja Oksman-Caldentey	
12:30-12:50	Gene targeting for precision glyco-engineering: Production of biopharmaceuticals devoid of plant-typical glycosylation in moss bioreactors R. Reski, University of Freiburg, Freiburg
12:50-13:10	Moss-aGal: preclinical results of the first moss-made biopharmaceutical A. Schaaf, Greenovation Biotech GmbH, Germany
13:10-14:20	Lunch Break
Session 6: PlantEngine - Plant Molecular Farming with small molecules Chair: Heribert Warzecha	
14:20-14:30	Introduction of the COST Action PlantEngine Heribert Warzecha, TU Darmstadt, Germany
14:30-15:10	Key Note 3 Metabolic engineering of diterpenoids in plants Alain Tissier, Leibniz Institute of Plant Biochemistry, Germany
15:10-15:30	Production of cosmetic ingredients by cell cultures of Nordic berry species R. Puupponen-Pimiä, VTT Technical Research Centre of Finland, Finland
15:30-15:50	GoldenBraid2.0: A comprehensive DNA assembly framework for multigene engineering in Molecular Farming and Plant Metabolic Engineering M. Vazquez-Vilar, Universidad Politécnica de Valencia, Spain
15:50-16:10	Metabolic engineering of rosmarinic acid and icetaxanes diterpenoids biosynthetic pathways in <i>Nicotiana tabacum</i> L. Bassolino, CRA-FSO, Italy
16:10-16:30	From bench to application: ANTHOPLUS – ANTHOCyanin production PLatform Using plant Suspension cultures as green factories (ERA-IB) S. Martens, TransMIT Project Division for Plant Metabolites and Chemicals (PlantMetaChem), Germany

16:30-17:30	Coffee Break and Poster Session
16:35-17:00	<i>Odd numbers</i>
17:00-17:25	<i>Even numbers</i>
Session 7: Plant cell production systems Chair: Dirk Bosch	
17:30-17:50	The model legume <i>Medicago truncatula</i> expression system: Towards high-yield production of recombinant proteins in cell suspension cultures R. Abranches, Instituto de Tecnologia Quimica e Biologica, Portugal
17:50-18:10	The challenge of proteolysis in plant molecular farming: tobacco BY-2 cells as a case study A. Schiermeyer, Fraunhofer IME, Germany
18:10-18:30	Plant-cell packs: a new tool for molecular farming T. Rademacher, Fraunhofer IME, Germany
19:00-22:00	Barbecue Party at the JKI building

June 19, 2014

09:00-10:30	ISPMF Meeting
10:30-11:00	Coffee Break
11:00-11:40	Key Note 4 Plant-derived Non-Hodgkins lymphoma vaccine: results of clinical evaluation J.E. Butler-Ransohoff, Bayer HealthCare AG, Germany
Session 8: Plant-derived recombinant proteins for medical applications Chair: Julian Ma	
11:40-12:00	Generation of an anti-HIV microbicide cocktail plant A.Y.H. Teh, St. George's, Univeristy of London, UK
12:00-12:20	Polymeric IgG-dengue fusion proteins as vaccine candidates M. Kim, St George's, University of London, UK
12:20-12:40	Production of an active anti-CD20-hIL-2 immunocytokine in <i>N. benthamiana</i> M. Donini, ENEA, Italy
12:40-13:40	Lunch Break

Session 9: Different topics Chair: Joachim Schiemann	
13:40-14:20	Key Note 5 Metabolic Engineering for Omega-6 and Omega-3 oils on oilseeds Maurice M Moloney, CSIRO, Australia
14:20-14:40	Plant roots as a useful vaccine production system using E.coli LTB as a model antigen J.D. Hamill, Deakin University, Australia
14:40-15:00	Technoeconomics of transient production of high-yield horseradish peroxidase in <i>Nicotiana benthamiana</i> E.P. Rybicki, University of Cape Town, South Africa
15:00-15:10	Final Remarks and Closure Joachim Schiemann, Julius Kuehn Institute, Germany Julian Ma, St George's, University of London, UK

Abstracts: Oral Sessions

Key Note 1

Immunogenic and immunomodulatory glycoproteins of helminth parasites, and the potential of plants as recombinant production platform

C.H. Hokke

Leiden University Medical Center, Department of Parasitology, PO Box 9600, 2300 RC
Leiden, The Netherlands

Email of corresponding author: c.h.hokke@lumc.nl

Helminth parasites cause chronic infections in a large proportion of the world's population. In the various stages of infection helminths expose and/or secrete immunomodulatory glycoproteins that promote long term survival by modulation and regulation of host immune responses. These glycoproteins are promising targets for vaccination against helminth infections as well as potential therapeutic biologicals for the treatment of a range of inflammatory and immune-related disorders such as diabetes, allergies and Crohn's disease. Specific glycan motifs in helminth glycoproteins are associated with their antigenicity and/or immunological effects. For instance, terminal glycan motifs such as the LeX antigen, mannosyl residues or GalNAc β 1-4GlcNAc contribute to interactions with C-type lectin receptors that mediate or induce endocytosis, cytokine responses and signalling by immune cells, parasite-specific glycan elements such as Fucal α 1-2Fuc in schistosomes are strong targets of the antibody response, and N-glycan core modifications shared between helminth and plant glycoproteins form cross-reactive carbohydrate determinants with various allergens. Recently we identified an immunomodulatory molecule from eggs of the human blood fluke *S. mansoni*. Omega-1 is a glycoprotein that imprints a characteristic Th2-phenotype on dendritic cells via mannose receptor (MR) by its RNase activity. The glycans of natural omega-1 are important for initial binding to MR, and a recombinant molecule without the proper glycosylation is not active. Current research is aimed at tailoring and optimising glycosylation of omega-1 produced in *N. benthamiana* with different glycosylation repertoires. The results in relation to the immunological activity of omega-1 will be presented. The potential of glyco-engineered plants for the production of immunologically active helminth glycoproteins will be discussed.

Key Note 2

Plant-made idiotype vaccines for non-Hodgkin's lymphoma: techno-economic feasibility study

V. Klimyuk

Icon Genetics GmbH

Weinbergweg 22, Halle (Saale), 06120, Germany

Email of corresponding author: klimyuk@icongenetics.de

Cancer vaccines that conceived as alternative/additional therapeutic tool for cancer treatments are expected to improve the treatment efficacy, patient's quality of life and provide cost-effective alternative to existing monoclonal antibody-based immunotherapies. The most advanced in this field are idiotype cancer vaccines that can serve as an example of personalized medicine, extending its borders beyond predominantly diagnostics field. However, personalized strategies present an extraordinary challenge to the cGMP production process. In its strictest definition, individualized medicine requires a unique drug product to be developed, produced and released for each patient. This is in drastic contrast with existing manufacturing and release processes of biopharmaceuticals destined for large populations of patients. As an example of individualized therapy is the experimental treatment of indolent Non-Hodgkin's Lymphoma (NHL) patients with idiotype vaccine. In this presentation technical, regulatory and economic challenges of plant-made idiotype vaccine for treating patients with follicular NHL will be discussed.

Key Note 3

Metabolic engineering of diterpenoids in plants

A. Tissier

Leibniz Institute of Plant Biochemistry, Weinberg 3, 06120 Halle (Saale), Germany

Email of corresponding author: atissier@ipb-halle.de

Diterpenoids are C-20 terpenoids, which include a number of industrially relevant compounds such as Taxol, an anti-cancer pharmaceutical ingredient, sclareol, a precursor for the synthesis of Ambrox, or carnosic acid, an anti-oxidant compound. Engineering of diterpenoid biosynthesis in plants represents a potential solution to reduce production costs and to augment availability of rare compounds. Different systems have been explored, both by transient expression in *Nicotiana benthamiana*, or by stable transformation of various plant species. We have focused on tobacco glandular trichomes, which are specialized organs secreting large amounts of diterpenoids. Our metabolic engineering strategy relies on the use of trichome specific promoters and on knocking-out or reducing the expression of endogenous tobacco diterpene synthases. This can be done either by gene silencing, or by a genetics approach via crossing of tobacco cultivars with distinct diterpene profiles. In parallel, we are using *N. benthamiana* to explore the co-expression of isoprenoid precursor pathway genes for enhanced output. Finally, we have developed a Transcription Activator-Like Effector based system for the regulation of multiple pathway genes by a set of synthetic promoters. This has general applicability beyond the field of terpenoid metabolic engineering.

Key Note 4

Plant-derived Non-Hodgkins lymphoma vaccine: results of clinical evaluation

J.E. Butler-Ransohoff

Bayer HealthCare AG, Aprather Weg 18, Wuppertal, 42113, Germany

Email of corresponding author: john.butler@bayer.com

The vast majority of B-cell lymphomas derive from the clonal expansion of neoplastic B-cells, all featuring the same surface antibody and consequently the same idiotype. We have used a plant-based protein expression system (magnICON[®]) to generate patient- and tumor-specific idiotype-bearing vaccine antigen.

The NHL Vaccine clinical study aimed at assessing:

1. Safety and efficacy of magnICON[®] -derived vaccines;
2. Regulatory viability of magnICON[®] for the production of proteins under GMPs.

Fifteen patients received at least one dose of study drug. A total of 135 doses of study drug were given in the trial. Eleven of the 15 patients who received at least one dose of study drug are evaluable for the trial's primary endpoint having completed 6 vaccinations. Among the vaccinated patients, there were NO TREATMENT RELATED severe adverse events. Eight patients had detectable immune response (all cellular, 3 also humoral). 7 of 10 patients analyzed for minimal residual disease (MRD) by next generation sequencing of the B-cell receptor also mounted a detectable immune response. Four of these seven remained MRD negative.

Key Note 5

Metabolic Engineering for Omega-6 and Omega-3 oils on oilseeds

M. Moloney

CSIRO, 5 Julius Ave, North Ryde, NSW 2113, Australia
Email of corresponding author: maurice.moloney@csiro.au

There has been much attention on the beneficial properties of omega-6 fatty acids such as gamma-linolenic acid and also of long chain omega-3 fatty acids such as those found predominantly in fish oils. Both classes of fatty acids present significant supply constraints either because of cost, quality or source. Metabolic engineering of fatty acid metabolism offers a solution to these problems, but will require a GM approach to provide a high specification product. This can involve significant regulatory complications en route to commercialization. In this presentation, we shall discuss the metabolic engineering required to make higher value oils of this type and describe a pathway through the regulatory framework. This approach has allowed for commercialization in a relatively short period at modest cost compared to many GM crops currently used in agriculture.

Session 1: Glycosylation of plant-derived recombinant proteins

Expression of Schistosome-derived Omega-1 with diantennary glycans carrying Lewis X motifs in *Nicotiana benthamiana* plants

L. Westerhof, R. Wilbers, D. van Raaij, E. Capuder, A. van der Schuren, D. Nguyen, L. Hussaarts, N. Driessen, C. Hokke, M. Yazdanbakhsh, D. Bosch and A. Schots
Wageningen University, Droevendaalsesteeg 1, Wageningen, 6708 PB, The Netherlands
Email of corresponding author: lotte.westerhof@wur.nl

Plants are an excellent platform for the heterologous production of (glyco)proteins. The surprising tolerance of plants to glyco-engineering allows the production of many different N-glycans. N-glycans play a key role in the interaction between helminth secreted proteins and the host immune system. The exact role of N-glycans on the immunomodulatory properties of helminth secreted glycoproteins remains to be elucidated. Studying the biological function of helminth secreted glycoproteins is challenging, as the purification of a single glycoprotein from helminths or their eggs is relatively inefficient. Plants are an appropriate platform to express these proteins in large quantities with their native N-glycan structures.

Omega-1 is a T2 ribonuclease (RNase) secreted by *Schistosoma mansoni* eggs and is a key factor for the induction of T-helper 2 (Th2) cell differentiation. Induction of Th2 responses by Omega-1 depends on its RNase activity as well as on N-glycan mediated internalisation by antigen presenting cells. Wild type Omega-1 carries two core-difucosylated diantennary N-glycans containing terminal Lewis X motifs. Lewis X is known to be an immunomodulatory glycan due to its interaction with DC-SIGN and the mannose receptor.

In this study we efficiently produced Schistosome-derived Omega-1 in *Nicotiana benthamiana* by means of agroinfiltration. The protein was purified from the intercellular space (apoplast) of *Nicotiana benthamiana* leaves and was shown to have RNase activity. Omega-1 produced in wild-type plants carried diantennary N-glycans containing typical plant β 1,2-xylose and core α 1,3-fucose, but lacked terminal GlcNAc's. By the controlled co-expression of two glycosyltransferases, Omega-1 could be engineered to carry terminal Lewis X motifs. Furthermore, both wild-type Omega-1 and Omega-1 carrying Lewis X motifs were able to induce Th2 cell polarisation. All-in-all our results demonstrate that plants are a promising platform for the expression of helminth glycoproteins carrying engineered N-glycans, which opens up a new field of research.

Expression and glycomodification of therapeutic proteins using heterologous biosystems

K. Ko

Department of Medicine, Medical Research Institute, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

Email of corresponding author: ksko@cau.ac.kr

Therapeutic proteins can prevent or treat wide ranges of diseases from cancer and viral or bacterial infections. Plants have advantages which include the lack of animal pathogenic contaminants, low cost of production, and ease of agricultural scale-up compared to other currently available systems. However, plants are not ideal expression systems for the production of biopharmaceutical proteins, due to incapability of the authentic human N-glycosylation process. The majority of therapeutic proteins are glycoproteins which harbor N-glycans, which are often essential for their stability, folding, and biological activity. Thus, the effective plant production system for recombinant therapeutics requires the appropriate plant expression machinery with optimal combination of transgene expression regulatory conditions such as control of transcriptional and post transcriptional events. We are interested in developing a plant system to express a large amount of therapeutic proteins in plant cells using so called cell-reprogramming and several glyco-engineering strategies in plants, particularly including glycoprotein subcellular targeting, inhibition of plant specific glycosyltransferases and addition of human specific glycosyltransferases. Among the variety of available heterologous expression systems, the baculovirus-based insect cell expression system also has been utilized frequently for the high-level production of therapeutic recombinant proteins and harbors glycosylation processing pathways, which constitute an advantage over other prokaryotic systems that lack glycosylation. Currently, we have successfully developed both plant and insect expression systems for production of monoclonal antibodies for immunotherapy.

Interaction of plant-derived anti-HIV mAb with human receptors

S. Stelter, M.J. Paul, A.Y.H. Teh, H. Steinkellner, R. Allen, J.K. Ma
St George's, University of London, Cranmer Terrace, SW17 0RE London, UK
Email of corresponding author: p1205234@sgul.ac.uk

Optimising interaction of antibodies with human receptors is an important consideration in the design of antibody-based therapeutics. Because receptor binding properties are influenced by glycan structure present in the antibody Fc region, distinct plant glycosylation, which can be effectively engineered, opens up new opportunities for modulation of effector functions. Here we have used *Nicotiana benthamiana* to generate three different glycovariants of VRC01, a broadly-neutralising anti-HIV antibody, to assess the impact of glycosylation on binding properties to human receptors. Flow cytometry assays with THP-1 monocytic cell line showed that VRC01 bearing a uniform plant-typical glycan structure (GnGnXF) has limited ability to interact with cellular receptors. Removal of core fucose and xylose can however significantly restore binding capacity. To further investigate binding to particular Fc receptors we have developed a surface plasmon resonance assay. Full kinetic and affinity analyses revealed that plant-derived VRC01 has significantly reduced affinity to both human FcγRI and FcγRIIIa than the HEK-cell-expressed version. Nevertheless, a plant glycovariant deprived of core fucose and xylose not only effectively interacts with FcγRI, but also is able to engage FcγRIIIa with high affinity, raising the prospect for more effective antibody-dependent cell-mediated cytotoxicity against HIV-infected cells.

Production of plant-derived recombinant proteins with engineered human-like n-glycans: a review

A. Castilho

University of Natural Resources and Life Sciences, Department of Applied Genetics and Cell Biology, Muthgasse 18, 1190, Vienna, Austria

Email of corresponding author: alexandra.castilho@boku.ac.at

Plants are being developed as inexpensive, large-scale production systems to meet the demand for complex biopharmaceuticals. *Nicotiana benthamiana* is a commonly used plant for the production of recombinant proteins. The great majority of therapeutic products in the market are glycoproteins. Glycosylation is considered to be an important quality attribute due to its strong effect on quality, immunogenicity, pharmacokinetics and potency. Plants carry out complex protein glycosylation however their N-glycans differ to some extent from the mammalian profiles. Therefore, one challenge that needs to be addressed before plant-derived therapeutics can be produced commercially is their glycan chain structure.

Here we review some of the most outstanding results from our group on the transient expression of therapeutically relevant proteins simultaneously modulating their glycosylation pattern. This requires the coordinated co-expression of several genes acting in different subcellular compartments at different stages of the glycosylation pathway. Our results provide a proof-of-concept that plants can express correctly folded and assembled multimeric human proteins (e.g. IgG, Butyrylcholinesterase and IgM), with glycosylation resembling their plasma-derived orthologues.

The *in planta* generation of functionally active highly complex human proteins discloses new potentials of plant-derived proteins for pharmaceutical applications. Moreover, target glycoengineering allows these proteins to be produced with different glycosylation profiles providing an important tool for structure-function studies which in turn has the potential to jump start research into the structures that are most appropriate for clinical development.

Session 2: Downstream processing

Assessing the quantity and quality of Plant-made Pharmaceuticals by Surface Plasmon Resonance

M. Sack, R. Fischer
RWTH Aachen University, Department of molecular Biotechnology,
Worringer Weg 1, Aachen, 52074, Germany
Email of corresponding author: sack@molbiotech.rwth-aachen.de

Biophysical assays, particularly surface plasmon resonance (SPR) spectroscopy offer significant advantages for characterising plant-derived recombinant proteins and are useful in supporting the early development of product candidates and up- and downstream processes. The SPR based assays are very robust and reproducible and often provide high precision and accuracy, which makes them an attractive-choice for quality control in GMP setting. Upon identification of suitable molecular interaction pairs, and establishment of coupling and regeneration conditions, different assays can be implemented to obtain various molecular properties such as standard-dependent and independent active concentration, absolute and relative single binding site activities, sample homogeneity and the kinetic rate and equilibrium constants etc. By employing multiple surfaces, binding signal ratios can be used to assess protein assembly and binding site activities and modifications. Ligands suited for the SPR measurements vary from small molecules to synthetic peptides and recombinant proteins, and their binding affinities can be in the milli- to picomolar range. The SPR measurements, and especially the use of multiple Ligands, increase the overall power of the analytics and provide additional opportunities for identifying deviations from expected or specified parameters for a manufacturing process. The use of native Ligands, such as antibody-Fc receptors, offers the potential to establish and utilize important structure-function relationships of therapeutic relevance. Soluble Fc-receptors are also particularly interesting as their binding is sensitive to variations in N glycosylation and the conformation of the Fc-region, and thus may be indicative for in vivo effector functions of therapeutic antibodies. Several examples will be presented to illustrate the analytical power of the SPR based assays for characterizing plant-derived products and plant-based processes.

Protein quality control – from protein recognition and degradation to conditional protein expression

N. Dissmeyer

Leibniz Institute of Plant Biochemistry (IPB) & ScienceCampus Halle - Plant-Based Bioeconomy, Independent Junior Research Group on Protein Recognition and Degradation, Weinberg 3, D-06120 Halle (Saale), Germany

Email of corresponding author: nico.dissmeyer@ipb-halle.de

The ON/OFF status of functional proteins within a cell's proteome must be precisely controlled to ensure its proper life by checkpoint-like protein quality control (PQC) mechanisms. PQC also kicks in if proteins are “used up” after their action and thus need to be removed from the cell. In plants, PQC is important for breakdown of storage reserves in seeds, germination, leaf and shoot development, flower induction, cell division, and possibly plant-pathogen interaction. Functional plant proteins as one of the premier storage units for energy are hallmarks of plant development and their environmental stress tolerance, but also in the light of producing plant-made pharmaceuticals. We functionally analyze novel enzymatic components and substrates with special emphasis on the N-end rule (NERP). In plants, NERP is poorly understood. We have developed an in vivo transgenic protein stability reporter system. Our laboratory work is mainly focused on studies of enzymatic NERP components (E3 Ubiquitin ligases, arginyl-transferases, and amidases), their substrate proteins as well as on the use of protein expression “on demand” as biotechnological applications in plants. Therefore, we are establishing conditional stabilization/destabilization assays of diverse functional classes of proteins such as enzymes, transcription factors, storage and reserve proteins but also toxic and large proteins with difficult folds which might be used in molecular farming.

Production of ELPylated haemagglutinin in plants

HT. Phan and U. Conrad

IPK Gatersleben, Corrensstrasse 3, Seeland OT Gatersleben, 06466, Germany

Email of corresponding author hoang@IPK-gatersleben.de

Fusion protein strategies are useful tools to enhance expression and to support the development of purification technologies. The capacity of fusion protein strategies to enhance expression was explored in tobacco. C-terminal fusion of elastin-like polypeptides (ELP) to influenza hemagglutinin under the control of either the constitutive CaMV 35S or the seed-specific USP promoter resulted in increased accumulation in both leaves and seeds compared to the unfused hemagglutinin. We show here that, depending on the target protein, both hydrophobin fusion and ELPylation combined with endoplasmic reticulum (ER) targeting induced protein bodies in leaves as well as in seeds. The *N*-glycosylation pattern indicated that KDEL sequence-mediated retention of leaf-derived hemagglutinins and hemagglutinin-hydrophobin fusions were not completely retained in the ER. In contrast, hemagglutinin-ELP from leaves contained only the oligomannose form, suggesting complete ER retention. In seeds, ER retention seems to be nearly complete for all three constructs. An easy and scalable purification method for ELPylated proteins using membrane-based inverse transition cycling could be applied to both leaf- and seed-expressed hemagglutinins. Reducing the cost of vaccine production is a key priority for veterinary research, and the possibility of heterologously expressing antigen in plants provides a particularly attractive means of achieving this. Avian influenza virus haemagglutinin (AIV HA) is produced in tobacco, both as a monomer and as a trimer in its native and its ELPylated form. The trimeric form of AIV HA was found to enhance the HA-specific immune response compared with 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. ELPylation did not influence the functionality and the antigenicity of the stabilized H5 trimers.

Spider silk proteins from plants: Multimerization strategies and characterization tools

N. Weichert, V. Hauptmann and U. Conrad

IPK Gatersleben, Corrensstrasse 3, Seeland OT Gatersleben, 06466, Germany

Email of corresponding author: conradu@IPK-Gatersleben.de

Spider silk combines outstanding mechanical properties with biocompatibility, low inflammatory potential, antimicrobial activity, and slow biodegradation, thus enabling new options for a growing number of biomedical and nanotechnological applications. Silk threads from spiders exhibit extraordinary mechanical properties, such as superior toughness and tensile strength. They consist of several different large repetitive silk proteins up to several hundred of kilodaltons. To date, all spider silk proteins are of such a high molecular weight. For this reason it was concluded that the extended size of spider silk proteins is a key factor for their outstanding features. Other essential characteristics are the high frequency of motifs conducive for inter- and intra-chain interactions and the small number of chain end defects that leads to an improved mechanical stability of the resulting fibers. Here, we present three different successful strategies to produce native-sized recombinant spidroins in planta: (1) with the help of non-repetitive C-terminal domains *in vivo* which suggest a crucial role for the formation and structure of spider silk filaments; (2) *in vitro* cross-linking mediated by transglutamination to generate non-linear multimers by intercrossing of peptide chains and (3) the formation of linear multimers triggered by posttranslational splicing activity of terminal C- and N-terminal intein sequences. We also show first nanomechanical analyses of the produced multimerized recombinant spider silk proteins using atomic force microscopy techniques.

Hydrophobin as a fusion partner to produce a soluble influenza vaccine in plants

N. Jacquet, C. Navarre and M. Boutry

Institut des Sciences de la Vie, Université catholique de Louvain, ISV - Croix du sud 4-5 bte
L7.07.14, Louvain-la-Neuve, 1348, Belgium

Email of corresponding author: marc.boutry@uclouvain.be

Different strategies have been reported for the production of recombinant hemagglutinin (HA) vaccines in plants. Expression of full-length HA was first demonstrated (Shoji et al., 2008). A soluble version of the protein was then developed by Fraunhofer USA (Shoji et al., 2011). Another approach consisted of producing virus-like particles by transient expression (D'Aoust et al., 2008). In order to simplify the purification procedure as well as the scaling-up, a fusion of soluble HA with an elastin-like polypeptide (ELP) was recently investigated (Phan et al., 2013). In our study, we tested the properties of HA fused to Hydrophobin I. This is a small and amphiphilic fungal protein that facilitates purification of the fusion partner.

We replaced the sequence encoding the membrane span and the C-terminal region of HA (H1N1 strain A/Texas/05/2009) with the hydrophobin (HFBI) sequence from the filamentous fungus *Trichoderma reesei*. The chimeric gene encoding HA-HFBI was inserted into the binary vector pEAQ-HT (Peyret and Lomonosoff, 2013) and expressed either transiently in *Nicotiana benthamiana* leaves or stably in *Nicotiana tabacum* BY-2 cells.

HA-HFBI was localized in ER-derived protein body structures. It was purified (>90%) by the surfactant-based aqueous two-phase system (Joensuu et al., 2010) followed by ammonium sulfate precipitation. This two-step procedure was validated for volumes ranging from 1 ml to 1 L of plant leaf extract. The purified protein was used to immunize mice. An anti-HA immune response was identified and the neutralizing capacity of the induced antibodies was demonstrated by an hemagglutination inhibition assay.

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Session 3: Virus-mediated transient expression systems

HPV pseudovirion production in plants

I. Hitzeroth, P. Kennedy and E.P. Rybicki
Biopharming Research Unit, Dept MCB, University of Cape Town, Private Bag X3,
Rondebosch, 7701, Cape Town, South Africa
Email of corresponding author: inga.hitzeroth@uct.ac.za

There are two virus-like particle-based HPV vaccines on the market - against HPV types 16 and 18, or against HPVs 6, 11, 16 and 18 - which show good efficacy, but are expensive and type-specific – thus, second generation HPV vaccines that are cheap, stable and elicit cross-neutralising antibodies against a broader range of HPV types are needed. These need to elicit antibodies that neutralize HPV pseudovirions (PsVs) in a PsV-based neutralisation assay (PBNA). HPV PsVs consist of HPV L1 and L2 capsid proteins encapsidating pseudogenomes encoding reporter genes. Current PsV production utilises mammalian cell culture, which is both expensive and difficult. Transient L1 and L2 expression in plants coupled with a novel self-replicating plant viral DNA construct offers a viable alternative to the current method.

In this study, we used the circular ssDNA Bean yellow dwarf geminivirus (BeYDV) genome to create a vector (pRIC) which self-replicates to high copy numbers when introduced into plants via *Agrobacterium tumefaciens* infiltration. We further modified the pRIC3 vector to include one of the reporter genes required for the PBNA. Autonomous replication of the pRIC3 vector resulted in replicons of approximately 5 - 7.5 Kbp, a size efficiently packaged by assembling HPV virions. The replicating vector was co-infiltrated into plant leaves with non-replicating vectors expressing L1 and L2, and candidate PsVs were purified and tested for DNA encapsidation to confirm their production in plants. These PsVs were then further tested for their ability to express the reporter gene in mammalian cells and were used in PBNA. To our knowledge, this is the first demonstration of the production of viable HPV PsVs in plants, and their use in a PBNA. The success of the technique also opens the way to making encapsidated DNA vaccines in plants, for use in mammals.

Exploiting Virus Superinfection Exclusion for the production of Recombinant Polyclonal Antibodies in Plants

J.M. Julve-Parreño, E. Huet-Trujillo, A. Fernández-del-Carmen, P. Wei-Song , A. Segura, D. Pla, C. Gutierrez, J.J. Calvete, J.M. Gutierrez, A. Granell, D. Orzáez
IBMCP-CSIC, Avda Tarongers SN, Valencia, 46022, Spain
Email of corresponding author: 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 mechanism that prevents the infection of a challenge virus into a previously infected cell is known as Superinfection Exclusion (SE) or Homologous Interference and it is only partially understood. The segregated distribution of viral clones imposed by Superinfection Exclusion offers a competition-free microenvironment that ensures the survival of low-fitness clones which would be otherwise displaced by high fitness competitors in an environment without Superinfection Exclusion. This highly-structured low-competition environment can be biotechnologically exploited for the production of complex mixes of recombinant proteins in a reproducible manner and without comprising the diversity of the final product. We will discuss examples where SE is exploited for the production of large repertoires of recombinant immunoglobulins.

Plant virus nanoparticles: toxicity and teratogenicity evaluation using in vitro and in vivo models

C. Lico, A. Blandino, L. Barberini, C. Cirotto, P. Giardullo, M. Mancuso, E. Benvenuto, P. Blasi, L. Santi, S. Baschieri

Laboratory of Biotechnology, ENEA Casaccia Research Center, Via Anguillarese 301,
00123, Rome, Italy

Email of corresponding author: chiara.lico@enea.it

The use of nanometer-sized carriers in biomedicine, especially in vaccine/drug delivery and diagnostic is a rapidly emerging field of research with enormous potentials. Both organic (lipids, polymers) and inorganic (metal, metal oxide, carbon) materials have been used to construct nanoparticles, but none of these components is immune from drawbacks (i.e. difficult large-scale manufacturing, poor long-term stability, in vivo toxicity). In this scenario, the use of biological or bio-inspired materials, and of plant virus nanoparticles (pVNP) in particular, appears very intriguing for the wide diversity they offer concerning symmetry and dimensions and the ease of chemical and biological engineering of both the surface and/or the internal cavity. Moreover, these self-assembling nanostructures can be produced in plants easily, safely and rapidly, and might represent an ideal delivery tool in terms of biocompatibility and biodegradability. In this perspective, we tried to define the safety profile of two structurally different plant viruses produced in *Nicotiana benthamiana* plants, the filamentous Potato Virus X (PVX) and the icosahedral Tomato Bushy Stunt Virus (TBSV). To this aim, we performed experiments to test their possible effects on human erythrocytes (to evaluate general cytotoxic effects in vitro) and on chicken embryo development (a well-established assay to assess in vivo toxicity and teratogenicity). The data obtained indicate that these structurally robust particles maintain the ability to infect plants after incubation in serum up to 24 hours, possessing neither toxic nor teratogenic effects on in vitro animal cells and in vivo animal models. To get the whole picture of the performances of pVNP in vivo, a mouse model is being used to test their immunogenicity and biodistribution following intravenous delivery.

Session 4: Seed expression systems

Seeds for delivery of edible vaccine

L. Rossi, A. Lombardi, C. Giromini, A. Baldi

Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10,
Milan 20133, Italy

Email of corresponding author: luciana.rossi@unimi.it

The oral delivery of plant-made vaccines is particularly attractive since the low costs, the heat stability, the avoidance of the injections and for the production of specific antibodies in the mucosa, where the major pathogens gain access to the body. In pig industry novel strategies are required to control enteric infections, in particular caused by verocytotoxic *Escherichia coli* (VTEC) strains and *Salmonella thyphimurium*, the two most important bacterial pathogens in the weaning period.

The general aim of this study was to produce three different lines of transgenic tobacco plants expressing in the seeds the F18 fimbriae adhesive, the VT2e-B toxin (both from O138 VTEC strain) and the flgK flagellin (from *Salmonella thyphimurium*) respectively, as a model of edible vaccine. We also preliminary evaluated the immunogenic properties of transformed tobacco seeds in animal model.

Tobacco leaf disks were transformed via *Agrobacterium tumefaciens* EHA105 with chimeric constructs containing structural parts of the major subunit FedA of the F18 adhesive fimbriae, VT2e B-subunit and flgK flagellin genes under control of a seed specific GLOB promoter: pBIpGLOB-F18, pBIpGLOB-VT2eB, pBIpGLOB-flgK. The GLOB promoter is the soybean basic 7S globulin promoter (DDBJ no. AX006477) and was used for the seed-specific protein expression.

We showed that the foreign genes were stably accumulated and expressed in storage tissue by the immunostaining method. Obtained data showed the inheritance of transgenes in the R0, R1, R2 generations and the stable integration of VT2e-B, F18 and flgK genes into tobacco genome. Balb-C mice receiving transgenic tobacco seeds via the oral route showed a significant increase in IgA-positive plasma cell presence in tunica propria when compared to the control group with no observed adverse effects. Moreover oral administration of recombinant tobacco seeds expressing the antigenic proteins against VTEC strains can induce a protective effect against challenger strain in piglets.

Bulking up antibody production, translation from a model plant to crops

V. Viridi^{1,2}, H. Mikschofsky³, I. Broer³ and A. Depicker^{1,2}

¹Department of Plant Systems Biology, VIB, Gent, Belgium,

²Department of Plant Biotechnology and Genetics, Ghent University, Belgium,

³Department of Agrobiotechnology, Agricultural and Environmental Faculty, University of Rostock, Germany

Email of corresponding author: ann.depicker@psb.vib-ugent.be

In feed delivery of antibodies produced in seeds can be a convenient solution to large-scale passive immunisation of farm animals against infectious diseases. We demonstrated this in our proof of concept study in a piglet model, wherein feeding milled Arabidopsis seeds producing pathogen specific antibodies protected from the infection [1]. The model plant—Arabidopsis can be transformed more easily than many other plants; several generations can be grown in greenhouse in a short period of time and thus suffice for screening a battery of candidate antibodies for feed based passive immunisation of animals. However for further phases of animal trials with the efficacious antibodies, typically in an experimental farm with hundreds of animals, and for eventually developing a prophylactic product, the antibodies have to be transformed in a crop plant that allows for bulk seed production. To this end, we investigated the production of three antibodies (expressing up to 3%, 2% and 0.2% of seed weight and exhibiting differential protease stability in Arabidopsis) in soybean and pea seeds. Here we give a comparative account of the expression levels and molecular integrity of antibodies produced in these different plant species, so as to make a more informed choice of suitable plant species for feed based oral passive immunisation of farm animals.

Viridi, V., et al., Orally fed seeds producing designer IgAs protect weaned piglets against enterotoxigenic Escherichia coli infection. Proc Natl Acad Sci U S A, 2013.

Molecular farming in plant seeds: a comparative analysis

J. Huckauf¹, S. Baars¹, N. Qu¹, S. De Buck², J. Nolf², V. Viridi², A. Depicker² and I. Broer¹

¹ Agrobiotechnology, University of Rostock, Justus-von Liebig-Weg 8, 18059 Rostock, Germany

² Department Plant Biotechnology and Bioinformatics in Ghent University, Department Plant Systems Biologie in VIB, Technologiepark 927 Gent 9052, Belgium

Email of corresponding author: inge.broer@uni-rostock.de

Plant seeds are considered as perfect storage organ for plant made products. We have analyzed the expression of two different transgenes, one coding for the plant made vaccine CTB-VP60 and one coding for cyanophycin synthetase resulting in production of the biopolymer cyanophycin, in seeds of tobacco, pea and Arabidopsis using up to five different promoters. Strong differences between plant species, the promoters and the effect of the transgene encoded protein on plant fitness, germination capacity and yield were observed. It has to be concluded that there is no general optimal seed production system; the choice being dependent on the transgene used and the characteristics and application of the recombinant protein.

Comparison of VHH-Fc antibody production in *Arabidopsis thaliana*, *Nicotiana benthamiana* and *Pichia pastoris*

T. De Meyer, B. Laukens, J. Nolf, R. De Rycke, A. Debeuckelaer, E. Van Lerberge, S. De Buck, N. Callewaert and A. Depicker
Ghent University and VIB, Technologiepark 927, Gent, 9052, Belgium
Email of corresponding author: ann.depicker@psb.ugent.be

Since their discovery almost 20 years ago, nanobodies (VHHs) have acquired wide acceptance as single-domain antibody molecules with unique protein binding characteristics. They have been generated for diagnostic and therapeutic purposes, and for various applications in research.

We have made fusions between existing diagnostic VHHs and an Fc domain to create dimeric VHH-Fcs and evaluated three platforms for VHH-Fc production at laboratory scale: transient expression in *Nicotiana benthamiana* leaves, and stable expression in *Arabidopsis thaliana* seeds and *Pichia pastoris*. By analyzing accumulation levels, degradation, VHH-Fc dimerization and N-glycan composition, a clear overview will be provided of the properties and peculiarities of the different expression systems. The functionality of the VHH-Fc proteins produced by the three different platforms was also evaluated: they were applied as primary antibodies in ELISA and electron microscopy. In this way, we could demonstrate that several VHH-Fc antibodies outperformed their respective VHHs in terms of sensitivity due to the Fc fusion. These results should encourage researchers to explore VHH-Fcs as possibly better research tools than the already existing VHHs, and to apply Fc-based avidity to other antibody fragments.

Session 5: Moss expression systems

Gene targeting for precision glyco-engineering: Production of biopharmaceuticals devoid of plant-typical glycosylation in moss bioreactors

E.L. Decker and R. Reski

Plant Biotechnology, Faculty of Biology, University of Freiburg
Schaenzlestrasse 1, 79104 Freiburg

Email of corresponding author: ralf.reski@biologie.uni-freiburg.de

The moss *Physcomitrella patens* has been employed for the recombinant production of high-value therapeutic target proteins (e.g. VEGF, Complement Factor H, IgG's, EPO). As a higher eukaryote, moss is able to synthesise multimeric complex proteins with posttranslational modifications as complex-type Asparagine (N)-linked glycosylation resembling those of mammalian cells. However, differences exist in the final processing of N-glycans which may result in immunogenic sugar moieties.

Being genetically well characterised and exceptionally amenable for precise gene targeting via homologous recombination, essential steps for optimisation of moss as a bioreactor for recombinant protein production have been undertaken: different types of photo-bioreactors for highly standardised cultivation were realised and conservation of strains as master cell banks was enabled via the International Moss Stock Center (IMSC). Synthetic expression modules were established (chimeric vectors carrying moss promoters and secretory signals as well as mammalian multicistronic, tunable systems (autoregulation, inducible on/off systems)).

In order to avoid immunogenic sugar structures on the recombinant biopharmaceuticals we characterised the moss's protein N- and O-glycosylation machinery. Transgenic moss strains synthesising human therapeutic glycoproteins were engineered for non-immunogenic glycosylation via gene targeting of the relevant Golgi membrane-located glycosyltransferases.

Our future challenge for providing fully human glycosylation will be the establishment of a synthetic scaffold of human glycosylation enzymes in precise positions within the Golgi apparatus moss.

Moss-aGal: preclinical results of the first moss-made biopharmaceutical

A. Schaaf

Greenovation biotech GmbH

Hans-Bunte-Straße 19, 79107 Freiburg, Germany

Email of corresponding author: aschaaf@greenovation.com

Physcomitrella-based BryoTechnology™ is a cGMP-compliant, eukaryotic production platform for demanding protein biopharmaceuticals. The technology exploits unique characteristics of the moss for the manufacture of customized product versions in a biosafe and disposable-based process.

Recent developmental efforts of greenovation aimed to demonstrate the clinical viability of moss-produced biopharmaceuticals. Therefore, two human lysosomal enzymes have been identified as clinical candidate products. With human alpha-Galactosidase (aGal), greenovations current lead product, a pivotal clinical study in Germany is scheduled for 2014. The successful implementation of this ambitious project is based on extensive developmental efforts. Creating a safe and well-documented high producer strain and the corresponding cGMP-compliant up- and downstream parts of its production process, designing a clean-room environment with moss-specific production equipment and transferring the production process into the latter are only part of the story.

Besides some highlights from this moss-process development, the presentation will have its main focus on the preclinical performance of moss-aGal. Designed as comparisons to the marketed products for Fabry-disease, both in vitro- and in vivo-studies clearly demonstrate the superiority of the plant-made enzyme over the mammalian-cell derived products. As all three protein versions have identical amino-acid sequences, the presented data will nicely demonstrate the importance of homogenous N-glycosylation and the potential of its targeted design.

Session 6: PlantEngine - Plant Molecular Farming with small molecules

Production of cosmetic ingredients by cell cultures of Nordic berry species

R. Puupponen-Pimiä, L. Nohynek, H. Rischer, K.M. Oksman-Caldentey
VTT Technical Research Centre of Finland, Tietotie 2, Espoo, 02044, Finland
Email of corresponding author: riitta.puupponen-pimia@vtt.fi

Berries are rich in compounds with proven beneficial effects on human health and well-being. Due to the Nordic latitude and harsh arctic growth conditions our berries accumulate exceptionally high amounts of secondary metabolites, such as phenolic compounds. In addition Nordic berries are well known for valuable fatty acids, highly aromatic taste and flavour and intensive colour. These benefits of berries have also attracted cosmetic industry, and thus certain cosmetic preparations contain berry-derived ingredients. However, field grown or wild collected berries are often available only during certain seasons and the harvest amounts and the quality of the material fluctuate considerably year by year. Nowadays the berries and plants can also be polluted, and some wild species are endangered and getting more rare in the nature. There is a general trend towards sustainable production of natural ingredients in cosmetic products.

Cell cultures of berry plants constitute a sustainable alternative to intact berries. Cell cultures of even rare species can be generated year-round at different scales with consistent quality and yield. They consist of totipotent cells and produce valuable metabolites beneficial for the well-being of the skin. We have developed *in vitro* cell culture systems of Nordic berry plants, e.g. species of *Vaccinium*, *Rubus* and *Empetrum*. Our unique cell cultures exhibit numerous bioactivities, such as antimicrobial and antioxidant activities. They also possess other characteristics such as high protein and lipid content, and fruity fragrance and bright colour, and therefore they are an outstanding choice for sustainable production of raw material for cosmetic preparations.

Our berry cell cultures were initiated from sterile berry seed or ex-plant, originating from the pure Nordic nature. Primary callus was first induced on growth media with proper hormone combination. Stable suspension cultures were finally established from selected callus through continuous selection and optimization process. Scale-up and down-stream processes were developed for the cell cultures for controlled production and harvesting of material of interest. Our industrial scale bioreactors (up to 1200 litres) guarantee continuous production of cell cultures for the needs of the cosmetic industry.

GoldenBraid2.0: A comprehensive DNA assembly framework for multigene engineering in Molecular Farming and Plant Metabolic Engineering

M. Vazquez-Vilar¹, A. Sarrion-Perdigones¹, E. Huet-Trujillo¹, P. Ziarsolo², J. Blanca² and D. Orzaez¹

¹Instituto 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; ²Centro de Conservación y Mejora de la Agrodiversidad (COMAV), UPV, Camino de Vera s/n, 46022 Valencia, Spain

Email of corresponding author: dorzaez@ibmcp.upv.es

In recent years, DNA assembly tools are becoming relevant for Molecular Farming and Plant Metabolic Engineering because of the applicability of multigene engineering in these fields. To overcome the existing limitations on the design and construction of new genetic combinations, the GoldenBraid (GB) assembly system was improved into a new version, GB2.0, which has strengthened its grammar and has created a collection of standard DNA parts and genetic modules. GB is an iterative cloning system based on type IIS restriction enzymes consisting of eight destination plasmids (pDGBs) designed to host multipartite assemblies and to combine them binarily. The GB assembly process, though simple, implies multiple steps to build a multigenic module. To facilitate multigene engineering with GB, GB2.0 offers a set of online tools that guide the user through the GBassembly process. The first of them, named GBDomesticator, adapts the input DNA sequence provided by the user to the GBstandard. The second tool, named GBMultiAssembler, software-assists the multipartite assembly of GBparts to create transcriptional units (TUs). Finally, the last tool is named GBinAssembler, and allows the binary assembly of preformed TUs or modules over the GB loop to produce multigenic structures. All these tools are linked with the GBdatabase that hosts a collection of DNA parts, including a group of functionally tested, premade genetic modules to build constitutive and inducible expression cassettes, endogenous gene silencing, protein-protein interaction tools, etc. 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 high-value protein constructs, identity preservation modules, etc.

Metabolic engineering of rosmarinic acid and icetaxanes diterpenoids biosynthetic pathways in *Nicotiana tabacum*

L. Bassolino, L. De Benedetti, C. Alberto G. Infante, B. Ruffoni
CRA-FSO, Corso Inglesi, 508, 18038, Sanremo, Italy
Email of corresponding author: barbara.ruffoni@entecra.it

Plant exudates, derived from the secretory structures of different aromatic plant species, are a valuable source of secondary metabolites such as polyphenols and diterpenoids. Those biologically active compounds have been widely adopted in traditional medicine due to their diverse phytochemicals properties. Rosmarinic acid (RA) is a polyphenolic acid well-known for its antioxidant and antiinflammatory properties. Icetaxanes, belonging to diterpenoids family act as inhibitors of microbial biofilms and cancer chemopreventive. This study aims to increase the relative concentration of these compounds *in planta* through a molecular farming approach. Two genes, the *hydroxyphenylpyruvate reductase* from *Salvia officinalis* (*SoHPPR*) and the *kaurene synthase like-1* from *Salvia miltiorriza* (*SmKSL1*), encoding for the putative enzymes of RA and diterpenoids synthesis respectively, have been cloned into the pK7FWG2 binary vector (Gateway technology) which allow their overexpression using *Agrobacterium tumefaciens*. Further, we transformed the model plant *N. tabacum* which was chosen due to its efficient transformation procedure, with the selected genes. Transformed tobacco plants will be assessed for their RA and diterpenoids content through HPLC and GC-MS analysis. In future, we aim to set up transformation protocols for two selected *Salvia* species, *S. dolomitica* and *S. corrugata*, to enhance the yield of phytochemical compounds. At the same time, we are developing a protocol for *A. rhizogenes* transformation of *Salvia* species to obtain the hairy roots (HR) phenotype. HRs can be exploited as “green farms” for secondary metabolites production.

From bench to application: ANTHOPLUS – ANTHOCyanin production PLatform Using plant Suspension cultures as green factories (ERA-IB)

S. Martens^{1,*}, C. Martin², I. Appelhagen², T. Hvoslef-Eide³, A. Oertel^{1,5}, D. Marko⁴, G. Pahlke⁴, H.P. Mock⁵, A. Matros⁵

¹ TransMIT Project Division for Plant Metabolites and Chemicals (PlantMetaChem), Kerkrader Str. 3, 35394 Gießen, Germany; ² John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK; ³ Norwegian University of Life Sciences, Institutt for plantevitenskap (IPV), Dept of Plant Sciences, 1432 Ås, Norway; ⁴ Institute of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währingerstr. 38, 1090 Vienna, Austria; ⁵ Applied Biochemistry Group, IPK-Gatersleben, Corrensstr. 3, 06466 Gatersleben, Germany
Email of corresponding author: martens@transmit.de

Anthocyanins are water-soluble pigments that colour the fruit and flowers of many plants. More than 635 different anthocyanins have been identified, distinguished by methylation, glycosylation and acylation with both aliphatic and aromatic groups. There is mounting evidence that consumption of anthocyanin-rich food promotes health, supported by many recent studies of anthocyanin-rich fruits such as blueberry, bilberry and cranberry.

Their relative abundance in the diet and their potency against a range of chronic diseases have made anthocyanins the subject of intense research in experimental and preventive medicine and, more recently for formulating natural colours, a fast growing market. However, the limited range of anthocyanins commercially available and the expense of pure preparations mean that most research is done with crude extracts of plants which are not standardised with respect to the particular anthocyanins they contain, nor the amounts of each anthocyanin in the extract. Variations in anthocyanin decoration account for differences in colour stability and hue of anthocyanins and underpin the need for developing production systems for pure anthocyanins for investigating the effects of chemical specificity on uptake, signalling and physiology, toxicity of anthocyanins for medical applications and for developing new formulations in the natural colours industries.

In ANTHOPLUS robust new plant cell cultures will be developed for the stable production of a wide variety of anthocyanins in green factories. These cell cultures, uniquely, allow sustained, high level production of diverse anthocyanins with novel complexity in side chain decoration, or labelled with stable isotopes for assaying the composition of feedstocks for natural colours, for bioavailability, bioefficacy and mechanistic research in experimental medicine and as standards for assaying natural colorant extracts for improved formulations. Enhanced supplies of pure anthocyanins will be highly valuable for the colourants industry to investigate the effects of decorations, co-pigments, pH on colour and stability to provide a robust scientific foundation for developing new plant sources of natural colourants and new formulations for natural colours.

Session 7: Plant cell production systems

The model legume *Medicago truncatula* expression system: Towards high-yield production of recombinant proteins in cell suspension cultures

R. Abranches

Instituto de Tecnologia Quimica e Biologica, ITQB-UNL, Av. Republica, 2780-157 Oeiras, Portugal

Email of corresponding author: ritaa@itqb.unl.pt

Plant cells are significantly more economical and safer than animal cells and unlike bacteria they are able to produce complex proteins with appropriate post-translational modifications. However, until now very few proteins have reached the market, which is largely due to low yields of product accumulation. This bottleneck needs to be removed so that plant cells become truly competitive. Our team has been working mainly on the use of *Medicago truncatula*, the model legume, for the production of recombinant proteins; we have already produced fungal and human proteins with several applications, including Erythropoietin and Prostaglandin D synthase. We are now addressing the issue of low product accumulation by using two complementary approaches. One more upstream in the production process, is to increase the expression levels of recombinant proteins by altering the epigenetic status of the plant cells with the use of small molecule enhancers (SME). Another, more downstream, is to identify *M. truncatula* proteases that may be responsible for degradation of the recombinant product and eliminate them by knock out/down or co-expression of protease inhibitors. The use of epigenetic modulators has been reported with considerable success in animal cells producing recombinant proteins. Such cells when treated with different SMEs have been shown to increase recombinant protein yields. However, these molecules have not been explored in plant cells producing recombinant pharmaceuticals or other valuable molecules. Many of these chemical compounds are inhibitors of histone deacetylases that promote higher levels of histone acetylation, which is generally associated with higher transcriptional levels. I will present our results on the use of commercially available SMEs, such as sodium butyrate, SAHA or Trichostatin A, as well as other in house synthesized SMEs, on our cell lines producing human proteins, and discuss the advantages of adding these molecules to the culture medium for improved production of recombinant proteins.

The challenge of proteolysis in plant molecular farming: tobacco BY-2 cells as a case study

M.K. Mandal, H. Ahvari, S. Schillberg, A. Schiermeyer
Fraunhofer IME, Forckenbeckstrasse 6, 52074, Aachen, Germany
Email of corresponding author: andreas.schiermeyer@ime.fraunhofer.de

Recombinant biopharmaceuticals produced in plant cells are subject to proteolysis by endogenous plant proteases. Degradation of recombinant biopharmaceuticals negatively affects the final yield and quality of the product. Therefore a detailed elucidation of the proteolytic degradation process of different biopharmaceuticals is desirable. A thorough characterization of the involved proteases and their enzymatic action on different biopharmaceutical substrates will enable the targeted engineering of the expression host or the modification of the recombinant protein to achieve increased yield and improved quality of the product. As most biopharmaceuticals are glycoproteins, the analysis of proteolytic enzymes of the secretory pathway is of particular interest.

Recent results on the stability of a variety of biopharmaceutical proteins in tobacco BY-2 suspension cells and spent culture medium will be presented and strategies to improve this production platform will be discussed.

Plant-cell packs: a new tool for molecular farming

T. Rademacher

Fraunhofer IME, Forckenbeckstrasse 6, Aachen, Germany

Email of corresponding author: thomas.rademacher@ime.fraunhofer.de

During the past decades, great efforts have been dedicated to the establishment of plant-based systems for the accumulation and harvesting of native or heterologous proteins and secondary metabolites. In particular, transient expression systems are very attractive for the production of recombinant proteins because of the speed and the possibility to achieve accumulation levels that are much higher than those typically obtained in stably transformed lines. In comparison to intact plants, the use of suspension cells has the advantage that homogeneous material can be reproducibly produced under controlled, aseptic and contained conditions. Although transient expression systems based on the co-cultivation of *Agrobacteria* and plant suspension cells have been described, the current methods suffer from low transformation efficiency and face the problem of the bacteria quickly overgrowing the plant cells unless antibiotic or auxotrophic strains are employed.

We have established a novel transient expression technology based on plant cell biomass in the form of a medium-deprived, porous structured and compact cell pack which can be transfected by *Agrobacteria* with efficiency and reproducibility comparable to those observed for whole plants.

This so called 'Plant-cell Pack technology' uses uniform biomass (which can be supplied through continuous plant cell fermentation), is highly versatile (besides recombinant protein production, it can be used for metabolic engineering and secondary metabolite production or as a synthetic biology platform), and can be applied in several formats and scales (from microtiter plate for high-throughput screening to large disks or big columns for scaled-up production). The use of a compact cell pack instead of a liquid culture also overcomes the problem of handling large volumes of medium and buffers during processing including product removal, extraction and purification

A variety of plant species can be exploited for their production capabilities and genetic backgrounds and the high-throughput format allows the testing of multiple hosts, constructs, combinations of genes and process conditions using statistical experimental designs.

Session 8: Plant-derived recombinant proteins for medical applications

Generation of an anti-HIV microbicide cocktail plant

A.Y.H. Teh, S. Osafo, J.K.C. Ma

St. George's University of London, Cranmer Terrace, SW17 0RE, London, UK

Email of corresponding author: ateh@sgul.ac.uk

HIV microbicides are one of the most promising alternatives to condoms as primary prevention of HIV transmission, particularly in the developing world. Effective HIV control would require a combination of different microbicide components to provide broad neutralizing coverage across different clades and avoid viral escape. However, this is complicated by the potential need to develop multiple manufacturing processes for a single product. The objective of this project is to explore the possibility of co-expressing multiple HIV microbicides components using a transgenic plant system. We have chosen to express three anti-HIV components in a single plant: VRC01, a broadly neutralizing conventional antibody capable of neutralizing over 90% of known HIV-1 isolates; Griffithsin (GRFT), a lectin isolated from the red algae *Griffithsia* sp.; and J3-VHH, a nanobody derived from a llama heavy chain only antibody. Transient expression experiments have shown that VRC01, GRFT and J3-VHH can be expressed simultaneously in tobacco and all molecules bind to HIV-1 epitopes gp120 and gp140 in vitro. The three components were also targeted to different cellular compartments, namely the apoplast and the cytosol, to maximize the protein accumulation capacity of the cell and to avoid potential interactions between the microbicide proteins. Individual homozygous lines, each expressing one of the three anti-HIV molecules, will be generated and crossed to generate a microbicide cocktail plant. By using one plant instead of three separate lines, it may be possible to reduce the cost of manufacture and reduce the amount of space required for plant cultivation. HIV microbicides could thus become more affordable and be manufactured locally in third-world countries.

Polymeric IgG-dengue fusion proteins as vaccine candidates

M. Kim, C.van Dolleweerd, R.Reljic, J. Ma
Institute for Infection and Immunity, St George's University of London, Cranmer Terrace,
London SW17 0RE, UK
Email of corresponding author: mkim@sgul.ac.uk

Dengue fever is one of the most important emerging mosquito-borne viral diseases, primarily of children. Dengue virus infection continues to be prevalent in over 100 tropical and sub-tropical countries which together account for over two-fifths of the world's population. A safe and effective dengue vaccine is needed in order to control the rapid spread of dengue through the changing demographics, urbanization, environment, and global travel. This study sets out to design and test a novel approach for developing a dengue vaccine, based on molecular engineering of adjuvanticity. A gene encoding the consensus dengue virus envelope protein domain III (EDIII) with cross-neutralizing activity against four dengue virus serotypes was used as a target antigen to develop a tetravalent dengue vaccine. To overcome the poor immunogenicity of EDIII, a novel IgG Fc-fusion based polymeric platform was designed to remove the need for adjuvants. The polymeric IgG Fc fusion gene was constructed by N-terminal fusion of consensus EDIII (cEDIII) to mouse IgG2a Fc to which the μ -tail piece of IgM was added. Thus, the recombinant IgM-like polymers of IgG Fc fusion protein bearing an increased number of binding sites is expected to facilitate more effective interactions with the classical complement cascade and low affinity Fc receptors, thereby priming both CD4+ and CD8+ T-cell responses. The construct was successfully expressed in both tobacco plants and CHO mammalian cells and purified to a high degree. The presence of high molecular weight polymers was confirmed by SDS-PAGE and Western blot analysis. The polymers bound to C1q component of the complement, confirming their functionality. We are currently evaluating the immunogenicity of this novel-type dengue vaccine candidate in mice.

Production of an active anti-CD20-hIL-2 immunocytokine in *N. benthamiana*

C. Marusic, F. Novelli², A.M. Salzano¹, A. Scaloni¹, E. Benvenuto, C. Pioli¹ and M. Donini

¹ENEA, UTBIORAD Laboratory of Biotechnologies, Roma, Italy

²Laboratory of Radiation Biology and Biomedicine, Rome, Italy and ISPAAM, National
National Research Council, Napoli, Italy

Email of corresponding author: marcello.donini@enea.it

Anti-CD20 murine or chimeric antibodies (Abs) have been used to treat non-Hodgkin lymphomas (NHLs) and other diseases characterized by overactive or dysfunctional B cells. Anti-CD20 Abs demonstrated to be effective in inducing regression of B-cell lymphomas although in many cases patients relapse following treatment. A promising approach to improve the outcome of mAb therapy is the use of anti-CD20 antibodies to deliver cytokines to the tumor microenvironment. In particular, IL-2-based immunocytokines have shown an enhanced antitumor activity in several preclinical studies. In this work we report the engineering of an anti-CD20-human interleukin 2 (hIL-2) immunocytokine based on the C2B8 (rituximab) mAb and its transient expression in *N. benthamiana*. The engineered immunocytokine is fully assembled in plants with minor degradation products as assessed in SDS-PAGE and gel filtration analyses. Purification yields using protein-A affinity chromatography were in the range of 10mg/kg of fresh leaf weight and glycan analysis confirmed the presence of plant-type typical sugars. The purified immunocytokine was assayed by flow cytometry on Daudi cells revealing a CD20 binding activity comparable to that of rituximab. Moreover, the biological activity of the fused plant-produced hIL-2 was confirmed using a mammalian cell line proliferation assay. This is the first example of a successfully plant-produced immunocytokine based on the therapeutic rituximab antibody that could represent a valuable approach in the treatment of non-Hodgkin lymphomas.

Session 9: Different Topics

Plant roots as a useful vaccine production system using E.coli LTB as a model antigen

G. De Guzman, A Pelosi, R. Shepherd, E Meeusen, D.E. Webster, A M Walmsley and
J.D. Hamill

Deakin University, 1 Pigdons Road, Waurn Ponds, Geelong, Vic, 3216, Australia
Email of corresponding author: john.hamill@deakin.edu.au

In the current study, the β -subunit of the heat-labile toxin (LTB) from E.coli, a widely used model antigen, was expressed in clonal hairy root cultures of various Solanaceous species using the CaMV35S promoter. Concentrations of LTB were assessed throughout the growth cycle and reached levels of $\sim 70 \mu\text{g g}^{-1}$ FW tissue in roots harvested during the latter stages of their growth cycle. Hairy roots cultured in vitro, and also roots of regenerated plants grown hydroponically, were capable of secreting LTB into their respective nutrient media. Plants regenerated from hairy roots were self-fertile. After several months of storage at room temperature, T1 seeds germinated in vitro on medium containing selection agent and readily formed vigorous hairy root cultures capable of producing high levels of LTB antigen. Oral immunogenic efficacy of hairy roots producing LTB was tested by feeding freeze-dried tissues to mice as a model monogastric system and also to sheep as a model ruminant system. In both cases, hairy roots containing LTB were able to elicit significant mucosal (IgA) and systemic (IgG) immune responses. Further studies utilized the wound-inducible promoter of the Nicotiana QPT2 gene to compare with the CaMV35S promoter in hairy cultures and regenerated plants. The CaMV35S promoter produced higher levels of LTB in cultured hairy roots than did the QPT2 promoter. In roots of wounded plants however, the QPT2 promoter produced levels of LTB that were 5-6 fold higher than was achieved using the CaMV35S promoter, attaining levels of $300 \mu\text{g LTB g}^{-1}$ FW tissue. Together, our results demonstrate that transgenic hairy roots, cultured in vitro and/or from regenerated plants grown hydroponically, have significant potential as stable systems for production of therapeutic proteins.

Technoeconomics of transient production of high-yield horseradish peroxidase in *Nicotiana benthamiana*

S. Huddy, D. Walwyn, [E.P. Rybicki](#)

Biopharming Research Unit, Department of Molecular and Cell Biology, University of Cape Town, Cape Town, 7701 South Africa

Email of corresponding author: ed.rybicki@uct.ac.za

Despite a number of advantages of plant expression systems relative to microbial or mammalian cell culture, plant-made biopharmaceuticals have yet to be approved by regulatory agencies in any quantity. While the technology is also suited to the production of less regulated products, such as reagents for molecular biology, one of the challenges for biofarming has been the lack of accurate data on costs of manufacture. Here we report a technoeconomic analysis of the transient production of a recombinant “gold standard” commercial enzyme, horseradish peroxidase (HRP).

N benthamiana codon-adapted full-length, C-, and N- and C-terminally truncated versions of the HRP C gene HRP C were synthesized. The constructs were expressed in *N. benthamiana* via an *Agrobacterium tumefaciens*-mediated transient expression system, with the effects of both the NSs silencing suppressor and protein targeting to various cellular compartments being investigated. Maximum expression was achieved using either full length or C-terminally truncated HRP C with the native HRP C N-terminal ER-targeting signal peptide. HRP C levels of 40-60 U.g⁻¹ fresh weight were consistently achieved. HRP C was purified using ammonium sulphate precipitation and ion exchange chromatography. The preparation gave an overall yield of 54% and the purified HRP C had a Reinheitszahl value of >3 and a specific activity of 458 U.mg⁻¹.

Based on the yield and productivity demonstrated (130 mg/kg of pure protein and 15 kg biomass/m²/year respectively), HRP could be manufactured economically in a small to medium scale facility (<3.5 kg HRP/year). The process would be highly competitive compared to the extraction of the enzyme from horseradish, with further improvements in yield and productivity and differentiation relative to the existing product being possible. Production scale, protein yield and biomass productivity were found to be the most important determinants of overall viability.

Abstracts: Poster Session

P1

Antibody sensing with plant-made elongated flexuous Viral NanoParticles (VNPs) presenting specific peptides

F. Ponz, F. Sánchez, C. Yuste, C. Mansilla, C. Cruz, L. Zurita
Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Autopista M40, km 38.
Campus Montegancedo, Spain
Email of corresponding author: fponz@inia.es

Turnip mosaic virus (TuMV) is a potyvirus, a group of plant viruses whose virions are elongated flexuous particles with helical symmetry, formed by a single virus-encoded protein, the coat protein (CP). Transiently expressed TuMV CP is able to form virion-like particles (VLPs) in plants. Collectively, virions and VLPs are referred to as VNPs.

We have previously shown that TuMV virions genetically modified to express a specific peptide derived from human Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3), can be used as sensors for the presence of VEGFR-3 antibodies in animal serum, log-increasing sensing sensitivity in immunodiagnostic techniques as compared with similar amounts of the peptide alone. We have now adapted this ability of recombinant TuMV virions to recombinant TuMV VLPs expressing peptides with a diagnostic potential. Results will be presented and discussed in relation with the detection of autoantibodies related to human autoimmune diseases and to induced inflammatory diseases in animal models. Antibodies in infectious diseases will also be considered.

P2

Plant-produced Hepatitis B Virus core particles can be modified to act as generic scaffolds for the display of whole-protein antigens of interest

H. Peyret, E.C. Thuenemann, G.P. Lomonosoff
The John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK
Email of corresponding author: hadrien.peyret@jic.ac.uk

In order to create a plant-based antigen-presentation system, the core antigen (HBcAg) of Hepatitis B Virus (HBV) was used. This protein is known to readily assemble into stable core-like particles (CLPs), with a highly immunogenic e1 loop region which studs the surface of the particles and is readily modified with small peptide inserts. Moreover, it has been known for many years that it is possible to produce HBcAg particles in the solanaceous plant *Nicotiana benthamiana*, and production of HBcAg CLPs using the Cowpea Mosaic Virus-based pEAQ-HT expression vector allows a recovered yield of up to 200-500 mg of purified CLPs per kg fresh weight tissue (FWT).

We have demonstrated that it is possible to use the pEAQ-HT vector to direct in planta production of HBcAg particles which display functional antibody on the surface. Initial experiments were aimed at displaying a single-chain variable fragment (scFv) antibody in the e1 loop of HBcAg. This proved problematic, so an even simpler antibody was sought. The variable regions of single-domain heavy chain antibodies of Camelids offered an interesting alternative due to their small, simple structure. By using these antibody fragments, we have managed to produce different HBcAg-based CLPs which display antibodies against GFP, HIV surface glycoprotein gp120, or HIV capsule protein p24. This is, to our knowledge, the first example of antibody display using HBcAg, as well as the first example of antibody display on an icosahedral virus capsid in a plant expression system. The constructs and production methods will be presented, along with characterisation of the resulting antibody-bearing CLPs (termed “tandibodies”) and the implication for vaccine design and production.

P3

Stability-improved expression vectors based on Potato virus X

C. Dickmeis, U. Commandeur
RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
Email of corresponding author: christina.dickmeis@rwth-aachen.de

Plus-strand RNA viruses such as Potato virus X (PVX) are often used as high-yielding expression vectors in plants because they can tolerate extra subgenomic promoter-like sequences (SGPs) that allow transgene expression without disrupting normal virus functions. However, vectors with duplicated SGPs often suffer from genetic instability that can cause the complete loss of any inserted sequences. Although heterologous SGPs have been successfully used in Tobacco mosaic virus vectors, only homologous SGP duplications have been used in vectors based on PVX. We therefore stabilized PVX-based vectors expressing green fluorescent protein (GFP) by combining heterologous SGPs from related potexviruses with an N-terminal coat protein (CP) deletion. We selected two SGPs with core sequences homologous to PVX, from Bamboo mosaic virus (BaMV) and Cassava common mosaic virus (CsCMV), as well as a heterologous core sequence from Foxtail mosaic virus (FoMV). We found that the BaMV and CsCMV SGPs were both recognized by the PVX replicase but the transgene remained unstable, probably reflecting the presence of an additional region with strong sequence similarity apparently introduced during initial vector construction. A revised version of the vector lacking this region was more stable. The BaMV SGP combined with the N-terminal CP deletion mutant did not affect systemic movement but achieved much higher vector stability and improved reporter gene expression.

We constructed a suitable expression vector for an easy cloning procedure and expressed proteins with different molecular weights to confirm our vector improvement. We successfully expressed the light chain of the 2G12 antibody with a molecular mass of 25 kDa, as well as the heavy chain with 50 kDa, and the β -glucuronidase (GUS) with 70 kDa. RNA analysis showed an improved genomic stability compared to PVX vectors with SGP duplication or fusions via the 2A sequence of the Foot and mouth disease virus (FDMV).

P4

In vivo and in vitro encapsidation of RNA molecules with TMV coat protein

T. Moravec, L. Dlabalova, P. Vaculik, H. Plchova and N. Cerovska
Institute of Experimental Botany, Rozvojova 313 , 16502, Prague 6, Czech Republic
Email of corresponding author: moravec@ueb.cas.cz

We decided to use the well documented ability of Tobacco mosaic virus coat protein to specifically encapsidate RNAs containing Origin of assembly (OAS) sequence to produce encapsidated RNA molecules both in vitro and in vivo. Chimeric VLPs will then be used for RNA delivery to eukaryotic cells. Also as the length of the particle is set by the length of the encapsidated RNA, such chimeric VLPs will be useful for various nanotechnology applications. Both the outer surface and inner channel of these VLPs can be modified either by genetic fusion or by chemical linkage to increase the flexibility of the system.

P5

Transient expression of Hepatitis C virus (HCV) truncated Core Antigen in Jafarabadi Tobacco Leaves

S. Mohammadzadeh, F. Roohvand and P. Ehsani
Pasteur Institute of Iran, no 69,12 Farvardin , Tehran, Iran
Email of corresponding author: P_ehsani@yahoo.com

Plants have gained considerable attention as an alternative expression system for producing safer and less expensive biopharmaceutical proteins. We studied possibility of transient-expression of HCV core protein (HCVcp) in tobacco (Iranian Jafarabadi cultivar)leaves for development of a plant-based system to produce HCVcp, using agroinfiltration technique. To this end, a codon optimized gene encoding the Kozak sequence, hexa-histidine (6×His)-tag peptide, HCVcp (aminoacids: 1-122) and KDEL (Lys-Asp-Glu-Leu) as ER retention peptide were situated in tandem. The designed fusion construct was chemically synthesized and inserted into Potato virus X (PVX)-based (PVX-GW) or pBI121-binary vectors under control of duplicated PVX coat protein (CPP) and CaMV 35S promoters respectively. The resulted recombinant plasmids (after confirmation by restriction and sequencing analyses) were transferred into *Agrobacterium tumefaciens* strain LB4404 and vacuum infiltrated into tobacco leaves. The effect of gene silencing suppressor P19 protein originated from tomato bushy stunt virus was tested on expression yield of HCVcp expression w/o co-infiltration of P19 carrying agrobacterium. The expression of the HCVcp was confirmed by ELISA and dot blot analysis. Results of optimization design showed that codon optimized gene had increased adaptation index (CAI) value (from 0.65 to 0.85) in tobacco and removed the possible deleterious effect of “GGTAAG” splice site in 358-363 regions. Moreover, the expression level of HCVcp was also enhanced 2.8 fold in P19 co-agroinfiltrated plants. The tobacco-derived HCVcp could properly be identified by commercially available HCVcp-antibody. The HCV-infected human sera could recognize the purified plant produced HCVcp by direct ELISA. The results provide potential for diagnostic/immunization applications

P6

Viral vector-mediated transient gene expression using detached leaves: Drying process immediately after agro-infiltration is essential to a high accumulation level of hemagglutinin

N. Fujiuchi¹, R. Matsuda², N. Matoba^{2,3}, K. Fujiwara¹

¹The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan; ²Owensboro Cancer Research Program, 1020 Breckenridge Street, Suite 201, Owensboro, KY 42303, USA;

³University of Louisville School of Medicine, 323 East Chestnut Street, Louisville, KY 40202, USA

Email of corresponding author: naofujiuchi@gmail.com

Detached plant leaves are potentially a cost-effective host for transient gene expression compared to intact whole plants in plant-based large-scale protein production systems. Here we report that drying process immediately after vacuum infiltration with agrobacterium suspension (ago-infiltration) is essential to a high accumulation level of hemagglutinin (HA), an influenza vaccine antigen, in a viral vector-mediated transient expression system using detached leaves. Detached *Nicotiana benthamiana* leaves were inoculated with a deconstructed tobamoviral vector (magnICON) via agro-infiltration, and were placed in a dehumidified chamber into which dried air at a relative humidity of 10% was introduced. The drying duration was varied between 40 and 262 min to obtain leaves with different relative fresh weight (RFW; the ratio of fresh weight immediately after the drying process to that immediately before agro-infiltration), resulted from different levels of total transpiration. The leaves were then incubated in humidified chambers at a relative humidity of over 80% for 6 d. There was a negative correlation between HA content in leaves on 6 d post infiltration (dpi) and the RFW on 0 dpi. The maximum HA content per leaf fresh weight of 800 $\mu\text{g g}^{-1}$ was recorded in a detached leaf at a RFW of 1.03, which was 95% of HA content in intact plant leaves. Averaged HA content in detached leaves at RFW of 1.0–1.1 was 2.2-fold as much as that in detached leaves at RFW of 1.2–1.3. Total soluble protein content on 6 dpi in detached leaves at different RFW was comparable, suggesting that insufficient transpiration specifically interfered with HA synthesis. On the other hand, growth rates of detached leaves during the 6-d incubation period were lower than those of intact plant leaves. Thus, required detached leaf biomass on 0 dpi for unit of unpurified HA was at least 1.5-fold as much as intact. We will next clarify environmental conditions that promote growth of detached leaves.

P7

Transient expression of scFc-Fc fusions in *N.benthamiana*

E. Huet-Trujillo, A. Puchol-Tarazona, A. Sarrión-Perdigones, D. Orzáez
IBMCP-CSIC, Avda Tarongers SN, 46022, Valencia, Spain
Email of corresponding author: dorzaez@upv.es

Engineered Antibody fragments are particularly attractive for the treatment of infectious diseases, such as enteric infections. scFv-Fc fusions are antibody-based structures comprising a scFv fragment fused to a constant immunoglobulin domain. scFv-Fc fusions 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 four scFv-Fc fusions incorporating the HingeCH2 CH3 domain of the human immunoglobulin HC_v1 that differ in the variable regions against the VP8* peptide of rotavirus strain SA11. scFv-Fc fusions were expressed in plants using plant virus vector based on tobacco mosaic virus (TMV) MagnIcon system. The expression of four scFv-Fc fusions was detected by western blot. The activity was determined by indirect ELISA, where the different scFv-Fc fusions were compared. The recombinant protein was purified by affinity chromatography. The accumulation of protein was found up to 25% of the total soluble protein (TSP) in the apoplast of agroinfiltrated leaves.

P8

PVX a powerful tool for autoimmune diseases diagnosis

L. Avesani¹, E. Tinazzi², M. Merlin¹, R. Zampier¹, E. Gecchele¹, C. Lico³, M. Pezzotti¹
Department of Biotechnology, University of Verona¹; Department of Internal Medicine,
University of Verona², ENEA, Rome³
Email of corresponding author: <mailto:linda.avesani@univr.it>

We explored the use of Potato Virus X as a scaffold for the expression of an immunodominant peptide associated to Sjogren's syndrome.

Sjögren's syndrome (SjS) is a chronic autoimmune disease in which the body's white cells destroy the exocrine glands, specifically the salivary and lacrimal glands that produce saliva and tears, respectively.

Human lipocalin was recently identified as a primary autoantigen associated to the disease and the immunodominant peptide associated to the protein was discovered using the sera of SjS patients. Autoantibodies directed to the peptide allow to identify in the population patients whose sera was negative to anti nuclear antibodies and to the rheumatoid factor.

The lipocalin peptide was cloned into a PVX-based vector which mediates the peptide display on the virus coat protein, yielding Chimeric Virus Particles (CVPs). CVPs allow a high-density exposure of the peptide on the viral surface. The CVPs were used for coating an ELISA plate giving an assay which reproducibility, stability and sensitive was compared to the use of the peptide alone.

P9

Production of different human antibodies in plant suspension cells: impact of culture conditions

J. Tollet, B. Magy, C. Navarre and M. Boutry

Institut des Sciences de la Vie, Université catholique de Louvain, ISV - Croix du sud 4-5 bte
L7.07.14, Louvain-la-Neuve, 1348, Belgium

Email of corresponding author: marc.boutry@uclouvain.be

Plant suspension cells have been widely studied as an expression system for monoclonal antibodies. However, a major limitation is the low amount of antibody that accumulates in the culture medium compared to the high titers obtained using mammalian expression systems. We tested the impact of the cultivation system on the accumulation of antibodies in the extracellular medium of suspension cell cultures. We analyzed the production of three human isotypes (IgG1, 2 and 4) provided with the same variable regions in the extracellular medium of *Nicotiana tabacum* BY2 and *Arabidopsis thaliana* Col-0 suspension cells. Firstly, we compared the accumulation of IgG when cells were cultivated in 5 mL six-well plates, 50 mL Erlenmeyer flasks or a 4 L stirred-tank bioreactor. Although all tested isotypes were detected in the extracellular medium using SDS-PAGE and a functional ELISA, up to 10-fold differences in the level of intact antibody were found according to the isotype expressed, to the host species and to the culture conditions. In the best combination (BY-2 cells secreting human IgG1 cultivated in six-well plate), we reported accumulation of more than 30 mg/L of intact antibody in culture medium. However, these differences were reduced when cells were grown in the optimized D11b culture medium designed by Vasilev et al. (Plant Biotechnol J (2013) Vol 11, pp 867-874). We then analyzed the growth parameters of cells cultivated in three different bioreactors (WAVE Bioreactor, airlift reactor and stirred-tank bioreactor) and measured the IgG concentration in the culture medium. Results of this comparison will be reported.

Magy, B., Tollet, J., Laterre, R., Boutry, M., Navarre, C. (2013) Accumulation of secreted antibodies in plant cell cultures varies according to the isotype, host species and culture conditions. Plant Biotechnol J doi: 10.1111/pbi.12152

P10

A Design of Experiments approach to development a production medium for *Nicotiana tabacum* BY-2 derived recombinant proteins

T. Holland, D. Blessing, R. Fischer, M. Sack
Fraunhofer IME, Forckenbeckstr 6, 52074, Aachen, Germany
Email of corresponding author: tanja.holland@ime.fraunhofer.de

Plant cell cultures have been demonstrated to be an attractive expression host for recombinant protein production for over two decades. The quality of plant cell culture products has been shown to be excellent in terms of protein folding and binding activity, but the protein yields are often significantly lower than those reported in mammalian cell cultures. To overcome this bottleneck, we carried out an extensive media analysis and optimization for the production of the full-size human anti-HIV antibody 2G12 in *N. tabacum* cv. BY-2 using a combination of a full factorial design (FFD) and response surface methodology (RSM). The investigation of the extra cellular 2G12 concentration of a cultivation in 14 different plant media showed that six nutrients (KH_2PO_4 , $\text{Ca}(\text{NO}_3)_2$, CaCl_2 , KNO_3 , NH_4NO_3 , MgSO_4) can have an impact on the production yields. The effect of these six nutrient and the c-source (sucrose) in terms of cell growth and protein yields were analysed in a full factorial design followed by more detailed analysis with RSM. The nutrient concentration of KH_2PO_4 , KNO_3 and sucrose showed the strongest effects on cell growth and the protein yield. We found that not only the nutrient concentrations but also their interactions were important for cell growth and protein yield. We developed predictive models for the cell growth and extra cellular 2G12 concentration to optimize the medium composition. The new medium increased the yield 20-fold compared to the MS-medium and 2-fold compared to the optimised production medium MSN. The results were scalable from shake flasks to stirred-tank bioreactors, in which the maximum yield per cultivation volume was 18 mg/l over 12 days. The new optimised medium did not affect antibody quality and activity, as determined by SDS-PAGE, Western blots, surface plasmon resonance binding assays and N-glycan analysis.

P11

Purification of a synthetic spider silk by acetone precipitation

R. Heppner and M. Pietzsch

Martin-Luther-University Halle-Wittenberg, Weinbergweg 22, Halle (Saale), 06120, Germany

Email of corresponding author: markus.pietzsch@pharmazie.uni-halle.de

Spider silk is a class of natural fibres which show highly interesting properties, e. g. high tensile strength and elasticity, self-healing and enhancement of cell adhesion. Recently progress has been made in the production of recombinant spider silk. The purification of a recombinant protein generates up to 80 % of the overall production costs, mainly due to expensive chromatography material. We show a simple and scaleable way to purify a spider silk analogue, produced in *Nicotiana tabacum* leaves, by acetone precipitation and membrane assisted inverse transition cycling.

P12

VH domain chimeras for mapping yield-limiting regions

J. Zischewski, M. Sack, L. Bortesi, R. Fischer
RWTH Aachen, Worringer Weg 1, 52074, Aachen, Germany
Email of corresponding author: julia.zischewski@rwth-aachen.de

A recurring challenge for the development of commercially viable plant-made-pharmaceuticals is the varying and at times low yield of the product. Sufficient recombinant antibody accumulation in the plant, for instance, is crucial for the downstream processing, which in turn drives the majority of manufacturing cost. Reasons why antibodies comprising different variable regions but identical constant regions and regulatory elements show different yields in planta are not yet fully understood. Here we describe a general protein engineering strategy to identify yield-limiting regions within the antibody variable domain. Parts of the VH domain of a high-yielding and a low-yielding antibody were exchanged at highly conserved and structurally-identical positions. The resulting chimeric IgG1 heavy chains were expressed transiently in *Nicotiana benthamiana* using agro-infiltration. We analyzed the mRNA and protein levels by qRT-PCR and surface plasmon resonance spectroscopy. Our results show that post-transcriptional gene silencing has only small impacts on the antibody yield. In contrast, co-expression of the antibody light chain caused a substantial yield increase (1.7 to 200-fold), clearly demonstrating that protein assembly and degradation are critical yield determinants. Notably, in the small-scale expression experiments some VH chimeras accumulated up to 2000 µg/g fresh leaf tissue four days after infiltration. Interestingly, two “inverted” VH chimeras both showed higher accumulation levels than 4E10. In conjunction with the expression data of the other VH chimeras this suggests that there is no single consecutive region within the 4E10 VH that is significantly yield-limiting. Instead it appears that 4E10 VH may contain two separate regions that are yield-limiting when both present. We have employed a general strategy that relies on standard molecular biology methods, and we believe that this approach can easily be applied to other antibodies to assess yield improvement. The mapping of yield-limiting regions and a more detailed understanding of the underlying factors is a powerful auxiliary technology to make antibody production *in planta* commercially more viable.

P13

Rational design of a host cell protein heat precipitation step simplifies the subsequent purification of recombinant proteins from tobacco

J. F. Buyel and R. Fischer

RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

Email of corresponding author: johannes.buyel@rwth-aachen.de

The benefits of plants for the production of biopharmaceutical proteins include the ease of scale-up and the low pathogen burden, but the purification of target proteins is often affected by low expression levels and the abundance of host cell proteins in leaf extracts. These factors can significantly diminish the competitiveness of plant-based expression systems. We therefore compared three different heat precipitation steps that remove most host cell proteins while leaving a heat-stable candidate malaria vaccine protein in solution. A design of experiments approach was used to test each method and analyze the resulting data, allowing us to model and optimize the corresponding process. The heat treatment of leaves was superior to both methods for the heat treatment of extracts in terms of process-scale implementation, energy consumption and speed. We discuss additional benefits and drawbacks of these methods compared to current strategies for the purification of biopharmaceutical proteins produced in plants.

P14

Plant-made vaccines for spaying

A. Maslobov, J. Yousef, J. Huckauf and I. Broer
Agrobiotechnology, University of Rostock, Faculty of Agricultural and Environmental
Sciences, Justus-von-Liebig-Weg 8, 18059 Rostock, Germany
Email of Corresponding Author: axel.maslobov@uni-rostock.de

Contraception for small animals and pets is often realized via surgical sterilization or hormone preparation. The first option is not always save and very stressful for the patient and the second is very expensive over long time and charged with side effects. Spaying via an autoimmune response initiated by a vaccine seems to be a promising alternative. E.g. such vaccines purified from pig oocytes are used for regulation of wild animal population with a great success. Unfortunately, the effectivity depends on the combination with a strong pro inflammatory adjuvant (CFA) which is leading to inflammations and subsequently permanent sterility. For pets, this is disadvantageous if stress should be minimized and transient sterility is envisaged. Recombinant expression of the antigens that could bypass these effects is not commercially established. Having the choice between different recombinant production systems like E.coli, yeast or animal cells, plants might be preferred due to the following reasons: (I) They might be more cost effective in production, (II) The plant specific glycosylation might increase the immunogenicity, (III) The antigen can be stored in seeds at room temperature, (IV) The plant-system offers the possibility to insert animal specific cleavage sites into the vaccine. This can improve antigen processing in the patient and increase the immune reaction.

P15

Polio VLPs expressed in plants: potential vaccines for the post-eradication era

J. Marsian

The John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK

Email of corresponding author: johanna.marsian@jic.ac.uk

The overall aim of this project is to produce a novel vaccine against poliovirus using a plant expression system. Precisely, I want to generate non-infectious poliovirus-like particles that have the capability to become a powerful vaccine that elicit an immune response against polio. To execute this plan, virus-like particles (VLPs) of polio will be transiently expressed in *Nicotiana benthamiana*. Transient expression in plants allows for production of high titres of recombinant proteins in a matter of days without biocontainment or genetic drift concerns. The intercellular space within the plant leaves is flooded with a suspension of bacteria in a process known as agro-infiltration (Bechtold & Pelletier, 1998). The pEAQ vector system is based on a deleted version of Cowpea Mosaic Virus (CPMV) and has been successfully used for a wide range of protein products (Sainsbury et al., 2009). It permits extremely high-level and rapid production of proteins in plants without viral replication. Using a plant-based platform for the production of pharmaceutical proteins such as vaccines comes with many advantages like: low cost, safety and a protein yield of over 1g per kg fresh weight plant tissue (Sainsbury & Lomonosoff, 2008). The CPMV expression system has been used to produce proteins from a wide range of enveloped and non-enveloped viruses, including Hepatitis B virus (HBV), papillomaviruses and Porcine respiratory and reproductive syndrome virus (PRRSV).

The World Health Organisation (WHO) seeks for novel polio vaccines because the orally administered Sabin vaccine (OPV) and also the Salk vaccine (IPV) have major disadvantages. Despite being designed to prevent poliomyelitis infections, OPV can cause it. It might become the first example where the vaccine itself causes more cases than the disease against which it is meant to protect.

Synthesis of immunogenic but non-infectious poliovirus-like particles has been shown in insect and mammalian cells. However, it has never been done in a plant-expression system and is therefore a novel approach.

P16

A recombinant HIV-neutralizing antibody produced in rice endosperm is mostly present in an aglycosylated form that does not comprise its ability to neutralize the virus.

E. Vamvaka, R.M. Twyman, A.M. Murad, S. Melnik, A.Y.H. Teh, E. Arcalis, F. Altmann, E. Stoger, E. Rech, J. Ma, P. Christou and T. Capell
University of Lleida, Av. Alcalde Rovira Roure, 191, 25198, Lleida, Spain
Email of corresponding author: teresa.capell@pvcf.udl.cat

Protein microbicides against HIV can help to prevent infection but they are required in large, repetitive doses which makes current fermenter-based production systems prohibitively expensive. Plants are advantageous because they offer a safe, economical and scalable alternative, and cereals such as rice are particularly attractive because they allow pharmaceutical proteins to be produced economically and on a large scale in developing countries, and to be stored as seed thus circumventing the need for a cold chain. Here we report the development of transgenic rice plants expressing the HIV-neutralizing antibody 2G12 in the endosperm. Unusually for antibodies expressed in plants, the heavy chain was predominantly aglycosylated. Nevertheless, the heavy and light chains assembled into a functional immunoglobulin with greater in vitro binding activity and more potent HIV-neutralization activity than 2G12 bearing typical high-mannose or plant complex-type glycans. Immunolocalization experiments showed that the assembled antibody accumulated predominantly in PB-II storage vacuoles and also induced the formation of novel, spherical storage compartments surrounded by ribosomes indicating that they originated from the endoplasmic reticulum. The comparison of wild-type and transgenic plants at the transcriptomic and proteomic levels indicated that the expression of several genes is modulated by the accumulation of 2G12. Our data provides further insight into the factors that affect the production and functional efficacy of neutralizing antibodies in plants

P17

Plant based production of natural raspberry ketone

S.T. Häkkinen and H. Rischer

VTT Technical Research Centre of Finland, Tietotie 2, Espoo, 02044, Finland

Email of corresponding author: suvi.hakkinen@vtt.fi

This research deals with the polyketide pathway, one of the central routes of plant secondary metabolism. Type III PKSs are nearly exclusively plant specific, homodimeric enzymes with a scant resemblance to (and an apparent evolutionary relationship with) fatty acid synthase. They are much simpler enzymes than the modular polyketide synthases (Type I PKSs) of microbial origin, but share the capacity to make an extremely diverse array of metabolites of biological and medical importance, and the potential for engineering of novel compounds with valuable applications for the pharmaceutical, fine chemical and flavours & fragrance sector. While many type III PKSs are known there is only a very little information on the accessory reductases involved (potentially, aldo-keto reductases belonging to the AKR4 subfamily). It is thought that these specific reductases play a crucial role in enabling correct folding and structure of polyketide-derived products.

A characteristic aroma in raspberry (*Rubus idaeus*) fruits is designated to 4-(4-hydroxyphenyl)butan-2-one, also called as raspberry ketone. The biosynthesis of this industrially demanded and valuable flavour compound is comparatively well characterised diketide pathway, involving the condensation of 4-coumaryl-CoA and malonyl-CoA by a type III PKS called benzalacetone synthase. The reduction of 4-hydroxybenzalacetone (4-OHBA) to raspberry ketone proceeds by a NADPH-dependent reductase raspberry ketone/zingerone synthase 1 (ZS1). Although this enzyme has been characterized at the enzymatic level, so far it has not been expressed *in planta*. The aim of this work was to perform functional testing of this gene by heterologous expression in tobacco. It was shown that when ZS1 was expressed transiently in *N. benthamiana* or stably in tobacco hairy roots, a correct bioconversion of fed precursor into raspberry ketone was accomplished in substantial yields.

P18

Use of sequence specific nucleases for targeted transgene integration in tobacco

L. Bortesi and R. Fischer

WTH Aachen University - Institute for Molecular Biotechnology, Worringer Weg 1 - 52074

Aachen – Germany

Email of corresponding author: luisa.bortesi@molbiotech.rwth-aachen.de

One of the challenges that remain to be addressed in plant molecular farming is the untargeted nature of current transgene insertion techniques in the nuclear genome of higher plants. As a consequence, lines independently generated with the same constructs are not directly comparable. They often display variable expression levels due to position effects, which imply the use of more or less tedious screening procedures to identify the best producer. Also, uncontrolled integration of transgenes can interfere with the plant's metabolism and lead to potential unintended effects. For these reasons, regulators currently consider every transgenic plant as a completely different event that must be evaluated separately.

Ideally, the establishment of a generic recipient line, characterized in terms of genetic background, in which the transgene of interest could be integrated in a predetermined site that grants high yield of recombinant protein, would accelerate both the development and the approval of new plant production lines.

One method to achieve targeted integration is the use of nucleases to induce a double strand break at a specific genomic sequence, and then exploit the DNA repair mechanisms of the host to introduce the desired transgene at the selected position via homologous recombination (HR). However, this procedure is limited in higher plants by the very low natural frequency of HR as repair mechanism in contrast to non-homologous end joining (NHEJ) on one side, and the very efficient random integration of exogenous DNA on the other side. Therefore, the development of strategies aimed at promoting HR vs. NHEJ, easily discriminating between HR and NHEJ events, and avoiding random integration of incoming DNA is necessary.

Here we present our approaches for achieving nuclease-mediated targeted transgene integration via HR in tobacco and show preliminary results.

P19

IPBO, a VIB-department that wants to bring plant biotechnology to developing countries and emerging economies

S. De Buck, I. Ingelbrecht, I. Pertry, D. De Oliveira, G. Gheysen and M. Van Montagu
IPBO/VIB-UGent, Technologiepark 3, B-9052 Gent, Belgium
Email of corresponding author: sylvie.debuck@vib-ugent.be

Modern plant biotechnology holds many of the potential solutions for sustainable agriculture. However, converting this promise into practice in less developed regions and emerging economies requires a concerted effort in training in--and access to--the latest technological developments and the design of effective biosafety and regulatory mechanisms. While the adoption of GM crop technology in Europe is still facing challenges, there has been a more rapid uptake of GM crop products in developing and emerging countries where, since 2012, more than half of the world's GM crops are cultivated. Therefore, showing the experiences from these countries to relevant stakeholders in Europe, will also illustrate the benefits of this technology.

The mission of the institute of Plant Biotechnology Outreach (IPBO), founded in 2000 by Em. Prof. Marc Van Montagu, is fourfold: (1) improve understanding and create awareness about the importance of green biotechnology applications for sustainable development (communication), (2) empower plant biotechnologists and plant breeders from developing countries and emerging economies through training and capacity building in plant biotechnology (training), (3) act as a focal platform for green biotechnology in Europe and leverage outreach to developing countries and emerging economies via IIBN (networking), and (4) catalyze and coordinate projects between developing countries and emerging economies and Flanders (project development).

P20

Cardiac and hepatic role of recombinant plant HSP70: basal effects and protection against ischemic and sepsis conditions

R. Iannacone³, T. Pasqua¹, E. Filice¹, R. Mazza¹, A.M. Quintieri^{1,2}, M.C. Cerra^{1,2}, D. Melfi³, C. Indiveri¹, Gattuso¹ and T. Angelone^{1,2}

¹Department of Biology, Ecology and Earth Sciences, University of Calabria, Arcavacata di Rende (CS); ²National Institute of Cardiovascular Research, Bologna; ³ALSIA-Research Center Metapontum Agrobios, Metaponto (MT), Italy

Email of corresponding author: rina.iannacone@alsia.it

Heat Shock Proteins (HSPs) are highly conserved in all organisms; they act mainly as molecular chaperones and are activated by environmental stresses. Human HSP70 has been demonstrated to act as general immune-stimulant of both innate and acquired immunity. Due to the high similarity, plant HSP70 may function as mammalian HSP70. The aim of this work was to evaluate whether plant origin HSP70 (rec-AtHSP70) was able to protect rat cardiac and hepatic function under ischemic and sepsis conditions.

We demonstrated for the first time that, in ex vivo isolated and perfused rat heart, exogenous r-AtHSP70 induced negative inotropic and lusitropic effects, significant from 10-12M to 10-8M, that disappeared at the highest dose tested, describing U-shaped curves without modifying Coronary Pressure (CP) and Heart Rate (HR). This effects was dependent by Akt/eNOS pathway. In addition, r-AtHSP70 induced post-conditioning cardioprotection via RISK and SAFE pathways, and did not cause hepatic damage. In vivo administration of LPS plus r-AtHSP70 protected both heart and liver against sepsis, as revealed by the reduced plasma levels of IL-1 β , TNF α , AST, and ALT. In conclusion, we suggest that r-AtHSP70 i) directly modulates normal heart performance, ii) protects against I/R myocardial injuries, iii) counteracts LPS-dependent inflammatory pathways in both heart and liver. Results may propose exogenous r-AtHSP70 as a modulator molecule able to prevent heart and liver dysfunctions during inflammatory, also encouraging the clarification of its clinical potential.

P21

Plant-derived recombinant immune complexes as self-adjuvanting TB immunogens

I. Pepponi, G. Diogo, E. Stylianou, M. Paul, L. Sibley, J. K-C. Ma and R. Reljic
St George's University of London, Cranmer Terrace, London SW17 0RE, UK

Email of corresponding author: mpaul@sgul.ac.uk

Progress with protein-based tuberculosis (TB) vaccines has been limited by poor availability of adjuvants suitable for human application. Recombinant immune complexes (RICs) constitute novel approach to molecular engineering of adjuvanticity that circumvents the need for exogenous adjuvants. We generated and expressed in transgenic tobacco plants RICs incorporating the early secreted Ag85B and the latency-associated Acr antigen of *Mycobacterium tuberculosis*, genetically fused as a single polypeptide to the heavy chain of a monoclonal antibody to Acr (TB-RICs). Self-polymerisation of RIC complexes was driven by the affinity of the antibody to the antigen moiety of a second antigen-antibody monomer. TB-RICs were purified from the plant extracts and shown to be biologically active by demonstrating that they could bind to C1q component of the complement and also to the surface of antigen-presenting cells. Mice immunized with BCG and then boosted with two intranasal immunizations with TB-RICs developed antigen-specific serum IgG antibody responses with mean end-point titres of 1 : 8100 (Acr) and 1 : 24 300 (Ag85B) and their splenocytes responded to in vitro stimulation by producing interferon gamma. 25% of CD4+ proliferating cells simultaneously produced IFN- γ , IL-2 and TNF- α , a phenotype that has been linked with protective immune responses in TB. When employed as a mucosal boost of BCG-primed mice, TB-RICs led to a statistically significant reduction **M. tuberculosis** infection in their lungs from \log_{10} mean = 5.69 ± 0.1 to 5.04 ± 0.2 . We therefore propose that the plant-expressed RICs represent a powerful and flexible molecular platform for developing self-adjuvanting mucosal vaccines.

P22

Improving the plant based expression platform: elucidation of factors that generate paucimannosidic N-glycan structures

T. Radebner, A. Castilho, F. Altmann, M. Windwarder and H. Steinkellner
University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria
Email of corresponding author: herta.steinkellner@boku.ac.at

Plants are increasingly being recognized as expression platform of recombinant proteins. Particularly interesting is the possibility of homogeneous protein glycosylation and in planta glycoengineering towards human like structures (Bosch et al., 2013, *Curr Pharm Des.*19:5503-12). This allows the generation of pharmaceutically relevant proteins with enhanced activities. Notwithstanding, plants exhibit some peculiarities which makes customized glycosylation a challenging issue in some cases. This includes paucimannosidic structures (PMS), a truncated oligosaccharide formation widespread in the plant kingdom. Such structures were also found on recombinantly expressed proteins (Dirnberger et al., 2001, *Eur J Biochem.* 268:4570-9) which might impair functional activities. At the moment it is not predictable when and where PMS are generated which diminishes the usability of the plant based expression system. Here we aim to elucidate factors that are responsible for the generation of PMS.

We show subcellular distribution of PMS structures in *N. benthamiana*, a plant species widely used for recombinant protein expression. We elucidated the glycome present in the apoplast, which represents the secretome (all secreted proteins) of a plant cell. MS based glycosylation profiles of the secretome exhibited the presence of equal amounts of complex and paucimannosidic N-glycans. This was unexpected since PMS are associated with vacuolar proteins (Lerouge et al., 1998, *Plant Mol Biol.* 38:31-48).

In parallel, a series of recombinant proteins (designed for secretion) are being expressed in *N. benthamiana*. MS based analyses show the presence of PMS on some proteins (e.g. FSH, α -galactosidase, A1AT) while others exhibit complex N-glycans (e.g. IgG, EPO). These results indicate that intrinsic factors of the protein contribute to its final glycosylation status.

Additionally computer based modelling and simulation will be carried out to exhibit possible structural features that might determine the final glycosylation status of a given protein. Finally the combination of experimental data with data obtained by PC based methods should give novel insights in glycosylation processes.

P23

Expression systems for the production of pharmaceutical or technical proteins in barley

G. Hensel

IPK Gatersleben, Corrensstrasse 3, Seeland OT Gatersleben, 06466, Germany
Email of corresponding author: hensel@ipk-gatersleben.de

Molecular Farming became an interesting option for the production of complex recombinant proteins. As a consequence it was an increasing demand for highly efficient and cost effective expression systems. Beside transient expression systems for tobacco leaves, seeds and cereal grains are naturally prone for the production and storage of proteins. Since barley belongs to the most important crops worldwide in the last decade numerous genomics tools and resources such as specific cDNA libraries, EST databases, molecular markers as well as physical and genetic maps have been developed for this species. In addition a powerful cereal transformation platform based on the use of *Agrobacterium tumefaciens* has been established in our laboratory. Either immature embryos or isolated microspores stimulated to undergo embryogenic development have been routinely used as gene transfer targets. The employment of these methods has resulted in the transformation of various spring and winter type cultivars of barley. Functional gene analyses and biotechnological approaches further require cell-specific promoters. In this respect, we are facing the general problem that most promoters from dicotyledons are not useful in monocotyledonous plants. For the production of recombinant proteins in the barley grain two systems based on the wheat α -GLIADIN and the oat GLOBULIN1 promoter with specificities for the endosperm were developed. Expression pattern and quantifications with the reporter gene gfp will be presented. In addition examples are presented for the production of recombinant proteins in barley grains.

P24

Biological activity of human interleukin-22 expressed in *Nicotiana benthamiana* is independent of its N-glycosylation

R.H.P. Wilbers, L.B. Westerhof, L.J. Reuter, A. Castilho, D.R. van Raaij¹, G. Smant, J. Bakker, A. Schots

Wageningen University, Laboratory of Nematology, Droevendaalsesteeg 1, Wageningen, 6708 PB, Netherlands

Email of corresponding author: ruud.wilbers@wur.nl

In the last decade plants have emerged as a suitable expression platform for several therapeutic proteins, but the expression of cytokines in plants has been challenging so far. Interleukin-22 (IL-22) is a member of the IL-10 cytokine family and has only recently been shown to have therapeutic potential. IL-22 is an unusual cytokine, as it does not act directly on immune cells, but mainly on epithelial cells. IL-22 controls the differentiation, proliferation and expression of anti-microbial proteins of epithelial cells, thereby maintaining epithelial barrier function. IL-22 activity is mediated via IL-22R1 and IL-10R2, the latter being shared by other members of the IL-10 cytokine family. Furthermore, IL-22 activity is reported to be dependent on the glycosylation of asparagine 54 (Asn54) as core α 1,6-fucose on this N-glycan enables binding to IL-10R2. Surprisingly, bacterial expressed IL-22 is active, while lacking N-glycans. In this study we investigated the possibility to express human IL-22 transiently in *Nicotiana benthamiana* and in more detail the role of N-glycosylation on IL-22 activity. Yield obtained for IL-22 was up to 90 μ g IL-22/gram fresh weight and IL-22 could be efficiently purified from the leaf apoplast fluid. Plant-produced IL-22 was shown to be biologically active, but most strikingly as active as bacterial and human cell expressed IL-22. Further examination of the role of N-glycosylation of IL-22 by mutagenesis of Asn54, in vivo deglycosylation with PNGase F or glyco-engineering by co-expression of human α 1,6-fucosyltransferase (Fut8) revealed that N-glycosylation of IL-22 is not required for biological activity. All in all our data demonstrate that plants are a promising platform for the expression of IL-22, but also offer a great toolbox for studying the role of N-glycans on glycoprotein function.

P25

Mutated storage organelles for biopharmaceutical production

A. Hofbauer, J. Peters, E. Arcalis, E. Stoger
Department of Applied genetics and Cell Biology, University of Natural Resources and
Life Science, Muthgasse 18, 1190 Vienna, Austria
Email of corresponding author: anna.hofbauer@boku.ac.at

Seed storage proteins are produced and stored in a very stable and efficient way within protein bodies (PBs) and protein storage vacuoles. These storage organelles provide a unique opportunity for the generation of new drug delivery systems, combining bio-encapsulation and high protein expression levels. Naturally occurring storage proteins, such as gamma-zein, have been used as fusion partner to induce the packing of the resulting chimeric proteins within storage bodies (1). The first 90 amino acids of gamma-zein are crucial for the normal PB formation, which is facilitated by disulphide bond formation and lateral protein-protein interaction (2). PBs can also be generated outside of the ER by the use of different signal peptides. Moreover modified PBs can be created by the mutation of gamma-zein. The normal PBs as well as the modified ectopic protein bodies are promising platform for the mucosal delivery of pharmaceuticals with novel properties in terms of cell penetration and stability.

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P26

Molecular Farming: Plants as Vehicles for Production of Antimicrobial Peptides

E. Holásková, M. T. Öz, P. Galuszka, I. Frébort
Department of Molecular Biology, CRH, Palacký University in Olomouc
Šlechtitelů 11, 78371 Olomouc, Czech Republic
Email of corresponding author: editaA@seznam.cz

Large varieties of antimicrobial peptides (AMPs) are produced as important components of the innate defence systems of all species of life. As these peptides exhibit broad activity spectra against pathogenic microorganisms including bacteria, protozoa, parasites, enveloped viruses, fungi and cancerous cells, they have drawn an interest for their use as novel anti-infective agents. One of the barriers to the success of AMPs in clinical use is the lack of an economically viable manufacturing platform. Plant bioreactors represent promising expression systems for these peptides as they offer cost effective large scale production and display almost no risk of product contamination by animal or human pathogens. Despite many advantages of plant molecular farming there are various limitations that need to be improved before this technology can be exploited as a feasible platform for AMP production. These limitations can be overcome by optimization of expression parameters for enhanced yield and development of methods for simple downstream processing. Therefore, we have designed various plant transformation constructs and assessed effects of different fusion partners on AMP production in different transient expression systems. Additionally, we have aimed to achieve expression of AMPs in barley grains, as seeds offer many advantages for AMP production such as possibility of long-term stable storage of product and a biochemically inert environment that facilitate purification of heterologously produced AMP. As the choice of seed specific promoter hold the key to match the requirements for high product accumulation, several plant expression vectors that combine different promoters have been designed and used for stable barley transformation. Regenerated barley plants will be analysed in future.

P27

High-Level Production of Recombinant Trypsin by Utilization of an Alternative Carbon Source and Recycling System in Transgenic Rice Cell Culture

M. Yang and T. Kwon

Chonbuk National University, 567 Baekjedae-ro, 561-756, Jeonju, Republic of Korea

Email of corresponding author: mskyang@jbnu.ac.kr

Productivity of recombinant bovine trypsin using a rice amylase 3D promoter has been studied in transgenic rice suspension culture. Alternative carbon sources were added to rice cell suspension cultures in order to improve the production of recombinant bovine trypsin. It was demonstrated that addition of alternative carbon sources such as succinic acid, fumaric acid and malic acid in the culture medium could increase the productivity of recombinant bovine trypsin 3.8 - to 4.3 -fold, compared to that in the control medium without carbon source. The highest accumulated trypsin reached 68.2 mg/L in day 5 in the culture medium with 40 mM fumaric acid.

The feasibility of repeated use of the cells for recombinant trypsin production was tested in transgenic rice cell suspension culture when the culture medium contained the combination of variable sucrose concentration and 40 mM fumaric acid. Among them, the combination of 1% of sucrose and 40 mM fumaric acid could result in production yield up to 53 mg/L 5 days after incubation. It also increased 31% of dry cell weight and improved 43% of cell viability compared to that in control medium without sucrose. Based on these data, recycling of trypsin production process with repeated 1% sucrose and 40 mM fumaric acid supplying-harvesting cycles was developed in flask scale culture. Recombinant bovine trypsin could be stably produced with production yield up to 53-39 mg/L per cycle during five recycling.

P28

Towards the improvement of cereal seeds for heterologous production of glycoproteins through down-regulation of endo-glycosidases: a case study on maize

S. Melnik, F. Altmann, P. Christou, E. Stöger

Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, 1190, Muthgasse 18, Vienna, Austria

Email of corresponding author: stanislav.melnik@boku.ac.at

Highly specialized seed storage tissues naturally provide the attractive capacity for production, processing and stable accumulation of complex proteins, including recombinant glycoproteins of pharmaceutical importance, such as monoclonal antibodies. However, one of the unanticipated features of the seed storage tissues is the accumulation of sometimes significant fractions of glycoproteins with incomplete N-glycan moieties, often comprised of single GlcNAc residues (1). The presence of an endo-N-acetylglucosaminidase (ENGase) is regarded by us as the most probable cause for the observed modifications in the maize endosperm. To gain more insight into the properties and functional role of this deglycosylating plant enzyme, as well as to follow the fate of its substrates, we set out to develop stable maize lines with reduced ENGase activity.

1) Rademacher, T., Sack, M., Arcalis, E., Stadlmann, J., Balzer, S., Altmann, F., Quendler, H., Stiegler, G., Kunert, R., Fischer, R. Stöger, E. (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. *Plant Biotechnol. J.* 6, 189-201

P29

Plant-derived therapeutics for the treatment of a rare disease (glycogen storage disease type III, GSDIII) and of HPV-related tumors

S. Massa¹, M. Rodriguez Hernandez^{1,6}, E. Illiano², F. Paolini³, A. Pasquo¹, D. De Carolis¹, P. Di Micco⁴, V. Morea⁵, C. De Giuli Morghen², A. Radaelli², C. Dionisi-Vici⁶, A. Venuti³,
R. Franconi¹

¹ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Casaccia Res. Ctr., Rome, Italy; ²Department of Pharmacological Sciences, Università di Milano, Milan, Italy; ³Regina Elena National Cancer Institute, Laboratory of Virology – HPV-UNIT; Rome, Italy; ⁴Department of Biochemical Sciences ‘A. Rossi Fanelli’, ‘Sapienza’ University of Rome, Rome, Italy; ⁵CNR-National Research Council of Italy, Institute of Molecular Biology and Pathology, Rome, Italy; ⁶‘Bambino Gesù’ Children’s Hospital, Scientific Institute, IRCCS, Department of Metabolism, Rome, Italy; ⁷CIGB, Centre for Genetic Engineering and Biotechnology, Habana, Cuba.

Email of corresponding author: rosella.franconi@enea.it

Plants represent a versatile production platform for high-quality biotherapeutics, tailored for different applications.

In the field of rare diseases, plants can provide cheaper and better variants of commercial molecules used for ‘enzyme replacement therapy’ (ERT), possibly with lower immunogenicity (i.e. by using glyco-engineered plants).

With respect to cancer, plants can be also a source of safe ‘activating bio-molecules’ and can provide formulations with immune-enhanced properties, able to fight the disease by stimulating cell-mediated and innate responses.

We will present our recent data about the expression in plants of the Glycogen Debranching Enzyme (GDE), whose activity is missing in Glycogen Storage Disease type III (GSDIII or Cori/Forbes disease), a rare metabolic disorder. The unique treatment for GSDIII is based on diet. ERT based on recombinant acid α -glucosidase (GAA, currently used for the treatment of Glycogen storage disease type II, GSDII) has been proposed for GSDIII.

We will also show our progress in development of plant-derived therapeutic formulations against high-risk Human Papilloma Viruses (hr-HPV) -related cancers. To improve the efficacy of our previous vaccines (based on HPV16 E7 oncoprotein antigen), E5 and E6 oncogenes-based genetic immunizations were introduced into the scheduling together with new plant-derived formulations of E7-based vaccines. The combination of vaccines against different HPV oncogenes is able to more effectively restrict tumor growth compared to the administration of the individual constructs in pre-clinical mouse models.

LIST OF PARTICIPANTS

A

Rita Abranches
Instituto de Tecnologia Quimica e Biologica,
ITQB-UNL
Oeiras, Portugal
rita@itqb.unl.pt

Linda Avesani
University of Verona
Verona, Italy
linda.avesani@univr.it

B

Antonella Baldi
Department of Health, Animal science and
Food Safety, University of Milan
Milan, Italy
antonella.baldi@unimi.it

Inge Broer
University of Rostock
Rostock , Germany
inge.broer@uni-rostock.de

Laura Bassolino
CRA-FSO
Sanremo , Italy
laura.bassolino@entecra.it

Dirk Bosch
Plant Reserch International
Wageningen, The Netherlands
dirk.bosch@wur.nl

Eugenio Benvenuto
ENEA
Rome, Italy
eugenio.benvenuto@enea.it

John Butler-Ransohoff
Bayer HealthCare AG
Wuppertal, Germany
john.butler@bayer.com

Jon Mar Bjornsson
ORF Genetics Ltd
Kopavogur, Iceland
jonmar@orf.is

Johannes Buyel
RWTH Aachen University
Aachen, Germany
johannes.buyel@rwth-aachen.de

Luisa Bortesi
RWTH Aachen University
Aachen, Germany
luisa.bortesi@molbiotech.rwth-aachen.de

C

Teresa Capell
University of Lleida
Lleida, Spain
teresa.capell@pvcf.udl.cat

Paul Christou
University of Lleida
Lleida, Spain
christou@pvcf.udl.cat

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

Udo Conrad
IPK Gatersleben
Seeland OT Gatersleben, Germany
conradu@IPK-Gatersleben.de

D

Eva L Decker
University of Freiburg
Freiburg, Germany
eva.decker@biologie.uni-freiburg.de

Christina Dickmeis
RWTH Aachen University
Aachen, Germany
christina.dickmeis@rwth-aachen.de

Sylvie De Buck
IPBO/VIB-UGent
Gent, Belgium
sylvie.debuck@vib-ugent.be

Nico Dissmeyer
Leibniz Institute of Plant Biochemistry (IPB)
Halle, Germany
nico.dissmeyer@ipb-halle.de

Ann Depicker
Gent University and VIB
Gent, Belgium
ann.depicker@psb.ugent.be

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

E

Parastoo Ehsani
Pasteur Institute of Iran
Tehran, Iran
P_ehsani@yahoo.com

F

Naomichi Fujiuchi
University of Tokyo
Tokyo, Japan
naofujiuchi@gmail.com

Rosella Franconi
ENEA
Rome, Italy
rosella.franconi@enea.it

H

John Hamill
Deakin University
Geelong, Australia
john.hamill@deakin.edu.au

Edita Holásková
Palacky University
Olomouc, Czech Republic
editaA@seznam.cz

Jean-Luc Henrioul
Root Lines Technology
Amiens, France
veronique.boitet@rootlines-tech.com

Tanja Holland
Fraunhofer IME
Aachen, Germany
tanja.holland@ime.fraunhofer.de

Goetz Hensel
IPK Gatersleben
Seeland OT Gatersleben, Germany
hensel@ipk-gatersleben.de

Jana Huckauf
University of Rostock
Rostock, Germany
jana.huckauf@uni-rostock.de

René Heppner
Martin-Luther-University Halle-Wittenberg
Halle (Saale), Germany
rene.heppner@pharmazie.uni-halle.de

Estefania Huet-Trujillo
IBMCP-CSIC
Valencia, Spain
eshuetru@upv.es

Inga Hitzeroth
Department of Molecular and Cell Biology,
University of Cape Town
Cape Town, South Africa
inga.hitzeroth@uct.ac.za

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

C.H. Hokke
Leiden University
Leiden, The Netherlands
c.h.hokke@lumc.nl

Anna Hofbauer
University of Natural Resources and Life
Sciences
Vienna, Austria
anna.hofbauer@boku.ac.at

I

Rina Iannacone
ALSIA - Research Center
Metapontum Agrobios
Metaponto, Italy
rina.iannacone@alsia.it

J

Nicolas Jacquet
UCL/Insitute of Life Sciences
Louvain-la-Neuve, Belgium
nicolas.t.jacquet@uclouvain.be

K

Oliver Kayser
TU Dortmund
Dortmund, Germany
oliver.kayser@bci.tu-dortmund.de

Victor Klimyuk
Icon Genetics GmbH
Halle (Saale), Germany
klimyuk@icongenetics.de

Miyoung Kim
St George's University of London
London, UK
mkim@sgul.ac.uk

Lilya Kopertekh
Julius Kuehn Institute
Quedlinburg, Germany
lilya.kopertekh@jki.bund.de

Kisung Ko
Chung-Ang University
Seoul, Korea
ksko@cau.ac.kr

Taeho Kwon
NBM Inc
Wanju-gun, Jeollabuk-do, Korea
thkwon@jbnu.ac.kr

L

Chiara Lico
ENEA Casaccia Research Center
Rome, Italy
chiara.lico@enea.it

M

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

Michael D McLean
PlantForm Corporation
Toronto, Ontario, Canada
mike.mclean@plantformcorp.com

Bertrand Magy
UCL/Insitute of Life Sciences
Louvain-la-Neuve, Belgium
bertrand.magy@uclouvain.be

Melnik Stanislav
University of Natural Resources and Life
Sciences
Vienna, Austria
stanislav.melnik@boku.ac.at

Axel Masloboj
University of Rostock
Rostock , Germany
axel.masloboj@uni-rostock.de

Heike Mikschofsky
Rostock , Germany
heike.mikschofsky@uni-rostock.de

Johanna Marsian
The John Innes Centre
Norwich, UK
johanna.marsian@jic.ac.uk

Maurice Moloney
CSIRO
North Ryde, Australia.
maurice.moloney@csiro.au

Stefan Martens
TransMIT Project Division for Plant
Metabolites and Chemicals
Gießen, Germany
martens@transmit.de

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

N

Catherine Navarre
Université catholique de Louvain
Louvain-la-Neuve, Belgium
catherine.navarre@uclouvain.be

O

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

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

P

Matthew Paul
St George's University of London
London, UK
mpaul@sgul.ac.uk

Fernando Ponz
Centro de Biotecnología y Genómica de
Plantas (UPM-INIA)
Pozuelo de Alarcón (Madrid), Spain
fponz@inia.es

Hoang Trong Phan
IPK Gatersleben
Seeland OT Gatersleben, Germany
hoang@IPK-Gatersleben.de

Riitta Puupponen-Pimiä
VTT Technical Research Centre of Finland
Espoo, Finland
riitta.puupponen-pimia@vtt.fi

Hadrien Peyret
The John Innes Centre
Norwich, UK
hadrien.peyret@jic.ac.uk

R

Theresa Radebner
University of Natural Resources
and Life Sciences
Vienna, Austria
theresa.radebner@boku.ac.at

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

Thomas Rademacher
Fraunhofer IME
Aachen, Germany
thomas.rademacher@ime.fraunhofer.de

Luciana Rossi
Department of Health, Animal science and
Food Safety, University of Milan
Milan, Italy
luciana.rossi@unimi.it

Ralf Reski
University of Freiburg
Freiburg, Germany
ralf.reski@biologie.uni-freiburg.de

Ed Rybicki
University of Cape Town
Cape Town, South Africa
ed.rybicki@uct.ac.za

S

Markus Sack
RWTH Aachen University,
Department of molecular Biotechnology

Stefan Schillberg
Fraunhofer IME
Aachen, Germany

Aachen, Germany
sack@molbiotech.rwth-aachen.de

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

Andreas Schiermeyer
Fraunhofer IME
Aachen, Germany
andreas.schiermeyer@ime.fraunhofer.de

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

Agata Staniek
Technische Universität Darmstadt
Darmstadt, Germany
staniek@bio.tu-darmstadt.de

stefan.schillberg@ime.fraunhofer.de

Kerstin Schmidt
BioMath GmbH
Rostock, Germany
Kerstin.schmidt@biomath.de

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

Szymon Stelter
St George's University of London
London, UK
p1205234@sgul.ac.uk

Eva Stöger
University of Natural Resources and Life
Sciences
Vienna, Austria
eva.stoeger@boku.ac.at

T

Audrey Teh
St George's University of London
London, UK
ateh@sgul.ac.uk

Jérémie Tollet
UCL/Insitute of Life Sciences
Louvain-la-Neuve, Belgium
jeremie.tollet@uclouvain.be

Alain Tissier
Leibniz Institute of Plant Biochemistry (IPB)
Halle, Germany
atissier@ipb-halle.de

U

Christoph Unger
University of Rostock
Rostock, Germany
christoph.unger@uni-rostock.de

V

Evangelia Vamvaka
University of Lleida
Lleida, Spain
evangelia.vamvaka@pvcf.udl.cat

Vikram Viridi
VIB-Gent University, Department of Plant
Systems Biology
Gent, Belgium
vikram.virdi@psb.vib-ugent.be

Marta Vázquez Vilar
Instituto de Biología Molecular y Celular de
Plantas
Valencia, Spain
marvzvi@upv.es

W

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

Lotte Westerhof
Wageningen University
Wageningen, The Netherlands
lotte.westerhof@wur.nl

Nicola Weichert
IPK Gatersleben
Seeland OT Gatersleben, Germany
weichert@IPK-Gatersleben.de

Ruud Wilbers
Wageningen University
Wageningen, The Netherlands
ruud.wilbers@wur.nl

Y

Moonsik Yang
Chonbuk National University
Jeonju, Republic of Korea
mkyang@jbnu.ac.kr

Z

Julia Zischewski
RWTH Aachen University
Aachen, Germany
julia.zischewski@rwth-aachen.de

Travel Information

Address:

Julius Kühn-Institut (JKI)
Bundesforschungsinstitut für Kulturpflanzen,
Königin-Luise-Straße 19,
14195, Berlin, Germany

Getting to JKI by car

Enter Berlin via Autobahn A 115 (AVUS), exit Hüttenweg in the direction of Dahlem, at intersection Clayallee turn left, then after about 500 m turn right into Königin-Luise-Straße. After 2 km you come to the JKI on the left.

Getting to JKI by train

At Berlin Hauptbahnhof (Berlin Central Station) proceed to top floor and take any rapid-transit train (S-Bahn) heading West to station Zoologischer Garten, change here to the underground (U-Bahn) U9 heading for Rathaus Steglitz (right up to the terminal stop), switch to Bus X83 destination Clayallee, get off at bus stop Arnimallee (= JKI Berlin-Dahlem main entrance). It takes you altogether approx. 35 minutes.

Getting to JKI by plane

via Airport Berlin-Tegel

Take Bus 109 or X9 (destination Hertzallee) to Jacob-Kaiser-Platz, underground U7 (destination Rudow) to Ferbelliner Platz, underground U3 (destination Krumme Lanke) to Dahlem Dorf, Bus X83 (destination Nahariyastraße) to bus stop Arnimallee – main entrance to the JKI is right across the street.

via Airport Berlin-Schönefeld

A 5 minute walk to the Schönefeld railway station, rapid transit S45 (destination Westend) to Schöneberg station, S1 (destination Wannsee) to Rathaus Steglitz, Bus X83 (destination Königin-Luise-Str./ Clayallee) to Arnimallee – bus stop is just at the entrance to the JKI.

Berlin Public Transport offers a service to find connections. The bus stop in front of the JKI is called Arnimallee. In case you do not mind walking 15 minutes take the underground to Dahlem-Dorf (Dahlem-Village) and walk eastwards along the Königin-Luise-Straße (Queen-Louisa-Street).

Site Map





Julius Kühn-Institut (JKI)
Bundesforschungsinstitut für Kulturpflanzen
Federal Research Centre for Cultivated Plants
www.jki.bund.de

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