Annual Meeting

COST Action FA0804

Molecular Farming: Plant as a Production Platform for High Value Proteins

Programme and Abstracts

Hotel Aequa, Vico Equense (NA), Italy
October 6-8, 2010

Local organizers:
Nunzia Scotti, CNR-IGV, Portici (NA), Italy
Teodoro Cardi, CRA-ORT, Pontecagnano (SA), Italy

Action FA0804: Molecular farming: plants as a production platform for high value proteins

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United Kingdom (MC Member)	Dr Lesley TORRANCE
United Kingdom (MC Substitute Member)	Dr Sean CHAPMAN

PROGRAM

Wednesday 6.10.2010

18:00 -	Registration and hanging up the posters
20:00 – 22:00	Welcome dinner

Thursday 7.10.2010

09:00	Kirsi-Marja Oksman: Current status of the Action
09:15	Working groups 2 and 3 Stefan Schillberg and Dirk Bosch: Introduction and presentation of the goals of the WG2 and 3
09:45	Yoseph Shaaltiel (Protalix, Israel): Molecular farming approach for production of recombinant glucocerebrosidase in carrot cells
10:15	Maurice Moloney (Rothamsted Research, UK) Oilseed-based biopharmaceutical production: from clone to clinic
10:45	Discussion: What are the best platforms and product candidates for molecular farming?
11:30	Coffee break
12:00	Working group 1 Paul Christou: Introduction and presentation of the goals of the WG1
12:15	WG1 focus groups Paul Christou and Bart van Droogenbroeck: goals, action plan, broadening participation and discussion
12:30	FG1 Regulatory framework (leader Joachim Schiemann) ▪ Joachim Schiemann: The regulatory frame for molecular farming (10 min.) ▪ Inge Broer (University of Kiel, Germany): Approaches to reduce the regulatory burden for experimental field conditions and placing on the market (20 +10 min.)
13:15	Lunch
14:15	FG1 Regulatory framework (continue) ▪ Maurice Moloney (Rothamsted Research, UK): How to meet GMP requirements for PMP production under open field conditions (20 + 10 min.)

- General discussion (including an open letter drafted by Joachim Schiemann)

15:00	FG2 Public perception/stakeholder interactions (leader Bart van Droogenbroeck)
15:45	General discussion, action points, deliverables and outputs, assignments of FG1 and FG2
16:30	FG3 Developing country aspects (leaders: Julian Ma and Paul Christou)
17:00	Sylvia Burssens (IPBO, Belgium): Industrial Biotechnology Applications for Developing Countries
17:20	General discussion, action points, deliverables and outputs, assignments of FG3 and the whole WG1
17:50 – 19.30	Poster presentation with aperitif
20:00 –	Pizza dinner in a local Pizzeria (optional)

Friday 8.10.2010

09:00	WG2 and WG3 (continue) Franco M. Buonaguro (Cancer Institute, Naples, Italy): Development of a vaccine for HIV
09:30	Einar Mäntylä (OrfGenetics, Iceland): Molecular farming approach for cosmetic products
10:00	Discussion on production platforms, products, down-stream processing, future activities (action points, deliverables)
11.00	Coffee break
11:30	Dirk Bosch: Status of molecular farming database, demonstration
12:00	Karin Metzlaff (Executive Director, EPSO, Brussels): How can we further increase impact and visibility of plant science in Europe? (30 min talk + 30 min discussion)
13:00	Lunch
14:30 - 18:00	Management committee meeting (for only MC members)

Abstract 1

EXPRESSION OF BIOLOGICALLY ACTIVE PROTEINS IN AN INDUSTRIAL SCALE PLANT CELL CULTURING DEVICE

YOSEPH SHAALTIEL

Protalix biotherapeutics, 20100 Carmiel, Israel

For many years industrial attempts were made to adjust plant cell cultures for commercial application. Numerous designs were made but failed to materialize into an industrial process. The main problems with the industrial size reactors were low biological efficiency in terms of cell mass production and low economical efficiency.

Protalix has developed a proprietary plant cell culturing device with both high biological efficiency and high economical efficiency. It is easily scaled up, and is very low cost in capital investment and maintenance. The system has proven its efficiency with a large number of different plant species and was found to produce high plant cell mass in all cases.

The plant cell culturing device can be used for secondary metabolite production as well as recombinant protein expression.

Protalix is using the system for the production of pharmaceutical complex proteins. Several different protein classes were expressed in our cell culture system and all were found to be biologically active.

Our lead product Glucocerebrosidase is produced presently in large scale, processed to high purity of above 99% purity. This product is developed under FDA IND guidelines. According to FDA requirements this product went through extensive toxicological studies in rodents and two primate species of primates. The protein was found to be safe and as a result a clinical path was approved and phase I and Phase III pivotal study were terminated extension, switchover study and compassionate studies are ongoing treating over 100 patients.

Our system is since then used to develop other protein products to broaden our pipeline some will be discussed.

Abstract 2

APPROACHES TO REDUCE THE REGULATORY BURDEN FOR EXPERIMENTAL FIELD RELEASES AND PLACING ON THE MARKET

INGE BROER[1] AND KERSTIN SCHMIDT[2]

[1]University of Rostock, Justus-von-Liebig-Weg 8, D-18059 Rostock
[2]BioOK GmbH, Thünenplatz 1, D- 18190 Groß Lüsewitz

Risk assessment on PMP, development of methods, decision support system

BioOK is an interdisciplinary network of researchers and small sized companies that develop an effective and competitive risk assessment procedure for transgenic plants aiming on the examination and post-market monitoring of genetically modified organisms (GMO) and derived food and feed as a "One-Stop-Agency". The partners of BioOK are located in the same region, investigate the same plant material and share the combined expertise present in the system.

Abstract 3

INDUSTRIAL BIOTECHNOLOGY APPLICATIONS FOR DEVELOPING COUNTRIES

SYLVIA BURSSENS[1], IVAN INGELBRECHT[1], DULCE DE OLIVEIRA[1], G. TZOTZOS[2], AND MARC VAN MONTAGU[1]

[1]Institute of Plant Biotechnology for Developing Countries, Department of Plant Biotechnology and Genetics, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent
[2]The United Nations Industrial Development Organization, Biotechnology Unit, Wagramerstrasse 5, P.O. BOX 400, A-1400 Vienna

Industrial Biotechnology, Green Biopharma, Network

Developing a sustainable bio-based economy that uses eco-efficient bioprocesses and renewable bioresources in new value chains is one of the global key challenges in the 21st century. To achieve this, a new set of industries must emerge whose main products and services use knowledge to decrease costs and create opportunities for growth in an environmentally and socially responsible manner. These technology intensive industries rely on a constant infusion of new knowledge, of which the principle source is fundamental research. Designing a sustainable future will therefore demand close cooperation between industry, academia, and policymakers.

Industrial Biotechnology has been recognized as one of the main drivers of a sustainable knowledge based bio-economy. The International Industrial Biotechnology Network (IIBN) is dedicated to promote the use of novel biotechnologies as a means of adding economic value to hitherto underutilized or unexplored biological resources in developing countries. The focus of the IIBN is on non-food applications derived from so called 'orphan' or underutilized plants and organisms that can help the development of green, sustainable economies in developing and emerging regions. The goal is to include less resourced farmers as feedstock providers for the new industry that is arising from the knowledge based bio-economy.

Green biopharma, including the production of pharmaceutical compounds, vaccines, and antibodies in plants, is considered as one of the priority themes within IIBN. Possibilities for cooperation within the framework of IIBN will be discussed.

Abstract 4

DEVELOPMENT OF A VACCINE FOR HIV

LUIGI BUONAGURO[1], NUNZIA SCOTTI[2], MARIA LINA TORNESELLO[1], TEODORO CARDI[2], FRANCO MARIA BUONAGURO[1†]

[1]Istituto Nazionale Tumori "Fond. G. Pascale" – IRCCS, Via Mariano Semmola 142, 80131 Napoli, Italy
[2]CNR-IGV, Institute of Plant Genetics, Via Università 133, 80055 Portici (NA), Italy

HIV, Vaccine, VLPs, plant-based pharmaceuticals, neutralizing immunity

Development of a preventive HIV Vaccine is worldwide pursued in order to achieve a valid biomedical tool to contain and possibly eradicate HIV infection. Several strategies have been elaborated spanning from monomeric recombinant gp120 to multi-component structured viral particles. Our group has been involved in developing candidate HIV vaccines, in baculovirus systems as well as in tobacco plants, with the aim of inducing broad-spectrum neutralizing antibodies. To achieve such goal either env or gag proteins have been produced as monomers or enveloped VLPs, with or without env spikes, purified and administered via mucosal and parenteral routes, alone or in adjuvant-combined formulations in animal models. Furthermore prime-boost strategies, able to induce both humoral as well as cellular immune response, with combination of several immunogens produced in different expression systems (including priming DNAs, etc.), have been used.
Although immunological studies with plant-derived HIV proteins are still limited, available results show an immunogenicity higher than, or comparable to, other systems. In particular Env-based vaccines expressed in *N. benthamiana* have shown the ability to induce IgG with neutralizing activity. Moreover, the recent discovery that the human influenza virus-H1N1 hemagglutinin (HA) is able to induce assembly of particles at the plant plasma membrane, with subsequent accumulation of several VLPs at budding sites, has prompted us to verify whether HIV-Env glycoproteins are able to be presented alone or fused to the HA trans-membrane region onto VLPs. This approach would allow the production of plant-derived Env-based enveloped VLPs, whose immunogenicity could be tested in parallel with already available chimeric non-enveloped VLPs obtained by the fusion of specific Env epitopes to self-assembling HBsAg. Finally, plants can contribute to the containment of HIV infection with products able to back up our immune system (such as adjuvants, for active immunotherapy, and monoclonal antibodies, for passive immunotherapy), as well as reduce/minimize viral exposure (i.e. microbicides, for their local antiviral activities).

Abstract P1

PLANT HEAT SHOCK PROTEIN 70 AS CARRIER FOR IMMUNIZATION AGAINST A PLANT-EXPRESSED REPORTER ANTIGEN

BURIANI G., MANCINI C., LICO C., BENVENUTO E., BASCHIERI S.

ENEA, UT-BIORAD, Laboratory of plant biotechnology, C.R. Casaccia, Via Anguillarese 301, 00123, Rome, Italy.

Plant biofactories - HSP70 - Subunit vaccines - Recombinant antigen delivery

Mammalian Heat Shock Proteins (HSP), have potent immune-stimulatory properties due to the natural capability to associate with polypeptides and bind receptors on antigen presenting cells. The present study was aimed to explore whether plant HSP, and in particular HSP70, share similar properties. We wanted in particular to evaluate if HSP70 extracted in association to naturally bound polypeptides from plant tissues expressing a recombinant "reporter" antigen, carry antigen-derived polypeptides and can be used to activate antigen-specific immune responses. This application of HSP70 has been very poorly investigated so far. The analysis started by structurally modeling the plant protein and defining the conditions that ensure maximal expression levels and optimal recovery from plant tissues. Afterwards, HSP70 was purified from Nicotiana benthamiana leaves transiently expressing a heterologous "reporter" protein. The purification was carried out taking care to avoid the release from HSP70 of the polypeptides chaperoned within plant cells. The evaluation of antibody titers in mice sera subsequent to the subcutaneous delivery of the purified HSP70 demonstrated that it is highly effective in priming humoral immune responses specific to the plant expressed "reporter" protein. Overall results indicated that plant-derived HSP70 shares structural and functional properties with the mammalian homologue. This study paves the way to further investigations targeted at determining the properties of HSP70 extracted from plants expressing foreign recombinant antigens as a readily available immunological carrier for the efficient delivery of polypeptides derived from these antigens.

Abstract P2

PEA DERIVED VACCINES AGAINST RABBIT HAEMORRHAGIC DISEASE VIRUS (RHDV)

HEIKE MIKSCHOFSKY[1], HORST SCHIRRMEIER[2], INGE BROER[1]

[1]University of Rostock, Agrobiotechnology, Justus-von Liebig-Weg 8. 18059 Rostock
[2]Friedrich-Loeffler-Institute, Südufer 10, 17493 Greifswald-Insel Riems

Plant derived vaccines, CTB, VP60, seed specific expression

Vaccines against rabbit haemorrhagic disease virus (RHDV) are commercially produced in experimentally infected rabbits. A genetically engineered and manufactured version of the major structural protein of RHDV (VP60) is considered to be an alternative approach for vaccine production. Pea has the potential to become an excellent recombinant production system. Here we demonstrate constitutive and seed specific approaches to express high amounts of VP60 for the protection of rabbits against RHDV.

Abstract P3

EXPRESSION AND ANALYSIS OF RECOMBINANT HUMAN GLUTAMIC ACID DECARBOXYLASE (GAD) AUTOANTIGENS IN TRANSGENIC MAIZE TO PREVENT INSULIN-DEPENDENT DIABETES MELLITUS

BRUNA MIRALPEIX[1], LINDA AVESANI[2], TERESA CAPELL[1], MARIO PEZZOTTI[2], PAUL CHRISTOU[1,3], KOREEN RAMESSAR[1]

[1]Departament de Produccio Vegetal I Ciencia Forestal (PVCF), Universitat de Lleida, Av. Alcalde Rovira Roure, 177, Lleida, E-25198, Spain

[2] Dipartimento Scientifico e Tecnologico, Università degli studi di Verona, Strada le Grazie 15, 37134, Verona, Italy

[3] Institucio Catalana de Recerca i estudis avancats (ICREA)

Diabetes mellitus, glutamic acid decarboxylase, autoantigen, maize

Diabetes mellitus is a chronic disease occurring when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Type 1 diabetes (T1DM) is the most frequent chronic childhood disease. Due to the life-long need for insulin and the clinical relevance of its chronic complications, diabetes has a strong social impact. The autoimmune destruction of pancreatic islet cells causing T1DM is targeted against several islet autoantigens, including the smaller isoform of glutamic acid decarboxylase (GAD65). Animal studies have shown that parenteral administration of GAD65 can prevent (or delay) the onset of the disease. However, poor GAD protein solubility in bacteria and inadequate production levels from eukaryotic cells have so far precluded the use of this approach for large-scale GAD65 production for oral tolerance studies. Plants are one of the most promising alternative production systems because of cost benefits and safety. Maize is particularly attractive because it has been developed as a commercial platform for recombinant protein production and its success as a production system for pharmaceutical proteins is widely documented. We expressed diabetes mellitus autoantigens, GAD65 and the chimeric molecule GAD67/65 in maize. We present our most recent results aiming towards the high level expression of GAD65 in maize

Abstract P4

HIGH-YIELD PRODUCTION OF THE VACCINIA VIRUS A27L AND A33R IMMUNOGENIC PROTEINS IN TRANSGENIC AND TRANSPLASTOMIC TOBACCO PLANTS

M. MANUELA RIGANO[1], CARMELA MANNA[2], ANNA GIULINI[3], EMANUELA PEDRAZZINI[3], MARIA CAPOBIANCHI[4], CONCETTA CASTILLETTI[4], ANTONINO DI CARO[4], GIUSEPPE IPPOLITO[4], PAOLA BEGGIO[5], CARLO DE GIULI MORGHEN[5], ALESSANDRO VITALE[3], TEODORO CARDI[2,6]

1Dept. Soil, Plant, Environmental and Animal Production Sciences, Univ. of Naples, Portici, Italy;
[2]CNR-IGV, Inst. Plant Genetics, Portici, Italy
[3]CNR-IBBA, Ist. Biologia e Biotecnologia Agraria, Milan, Italy
[4]National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy
[5]Dept. Medical Pharmacology, Lab. of Molecular Virology, Univ. of Milan, Italy
[6]present address: CRA-ORT, Res. Ctr. for Vegetable Crops, Pontecagnano, Italy

Smallpox, transplastomic plant, tobacco, vaccine.

Recently, orthopoxviruses (OPV) have received increasing attention, because of the fear of bioterrorism and the occurrence of zoonotic OPV outbreaks, highlighting the need for the development of new-generation vaccines against smallpox and related viruses. In this respect, the production of subunit vaccines in transgenic plants is an attractive approach, being them safe and less expensive to produce and distribute. In this study, we produced the A27L and A33R immunogenic proteins of vaccinia virus in the non-food, non-feed crop tobacco (cv. Petit Havana) by Agrobacterium-mediated transformation of the nuclear genome and biolistic transformation of the plastome. The protein A27L was expressed in the stroma of transplastomic plants in soluble form and accumulated to about 18 % of total soluble protein. Such high A27L expression levels altered the organization of chloroplasts and caused a mutant phenotype. Nevertheless, the level of protein accumulation did not decline during leaf development in mature plants, suggesting that the protein is stable in transgenic tobacco chloroplasts. Chloroplast-synthesized A27L protein was similar to the native protein found in the virus particle and in infected cells, based on size, oligomerization state and reactivity against specific antibodies. Moreover, the plant-made A27L was recognized by serum from a patient recently infected by a zoonotic orthopoxvirus. Additionally, since optimal protection to the challenge is provided by a multicomponent smallpox vaccine, we performed Agrobacterium-mediated co-transformation experiments with the A33R and A27L genes. Preliminary results showed high frequency of co-transformation and co-expression of the two vaccinia virus immunogenic proteins in transgenic tobacco plants. Taken together, these results demonstrate that plants are an attractive and versatile production platform for the expression of OPV subunit vaccines.

Abstract P5

PLANT-BASED ANTI-HIV-1 STRATEGY: EXPRESSION OF THE PR55^{GAG} POLYPROTEIN IN TRANSGENIC TOBACCO CHLOROPLASTS

NUNZIA SCOTTI[1], LORENZA SANNINO[1], SILVIA SILLETTI[1], LUIGI BUONAGURO[2], FRANCO MARIA BUONAGURO[2], TEODORO CARDI[1,3]

[1]CNR-IGV, Institute of Plant Genetics, Via Università 133, 80055 Portici (NA), Italy
[2]Istituto Nazionale Tumori "Fond. G. Pascale" – IRCCS, Via Mariano Semmola 142, 80131 Napoli, Italy
[3]present address: CRA-ORT, Research Centre for Vegetable Crops, Via dei Cavallegeri 25, 84098 Pontecagnano (SA), Italy

plant-based vaccine, tobacco, plastid transformation, HIV-1

The Pr55^{gag} polyprotein, the main structural protein of HIV-1, is an interesting candidate vaccine able to elicit both humoral and cellular immune responses. The Pr55^{gag} polyprotein precursor is processed by viral protease into four distinct domains, that participate in the production of HIV-1 mature particles and capable of assembling into Virus-Like Particles (VLPs).
In our laboratory, the expression of the Pr55^{gag} polyprotein was pursued using different plant-based strategies. Best results were obtained by sequence integration in the plastid genome, an alternative approach to nuclear transformation that offers several advantages: precise integration of transgenes by homologous recombination; high production level of recombinant proteins due to the large number of transgene copies in homoplasmic transformants; reduction of transgene flow through pollen, being the plastid genome maternally inherited in most crop plants. Highest protein yield (7-10 % of total soluble proteins, corresponding to 338-400 mg/kg of fresh tissue) was obtained with an optimized vector (pNS40), where the *gag* coding region was expressed by the strong rrn promoter fused with the 5' translation control region (TCR) that includes the 5'-UTR, the 42 N-terminal nucleotides, and the 3'-UTR of the plastid rbcL gene. These plants showed a pigment-deficient phenotype in both heterotrophic shoot cultures *in vitro* and in autotrophic plants grown in soil, a growth retardation, and a reduction of the total protein content (Scotti et al. 2009. Planta 229: 1109-1122) most probably due to strong down-regulation of RbcL protein. Hence, we have investigated the possible consequences of the over-expression of the HIV-1 Pr55^{gag} protein on chloroplast development and regulation of plastid gene expression by electron microscopy and microarray analyses, respectively
Plastid ultrastructure in leaf mesophyll cells of the HIV-1 Pr55^{gag} transplastomic plants revealed an irregular shape and the absence of stacked thylakoids membranes (similar to proplastid) compared to that of control plants (wild-type and transplastomic plants transformed with an empty vector). Since no significant differences were found at transcriptional level between HIV and transplastomic control plants by microarray analysis, further analyses based on chloroplast proteome and precise localization (stroma or membrane) of the Pr55^{gag} polyprotein in transgenic chloroplasts will be carried out.
Preliminary immunological results with plant-derived Pr55^{gag} protein showed an immunogenicity higher than, or comparable to, other systems.

Abstract P6

PLANT-PRODUCED OUTER-MEMBRANE PROTEINS FROM BRUCELLA ABORTUS AND THEIR IMMUNOGENICITY IN MICE

CHRISTINE SEITHER[1], JULIA BORKOWSKI[1], KARINA A. PASQUEVICH[2,3], JULIANA CASSATARO[2,3], HERIBERT WARZECHA[1]

[1]Technische Universität Darmstadt, Department of Biology, 65287 Darmstadt, Germany
[2]Laboratorio de Inmunogenética, Hospital de Clínicas "José de San Martín", Facultad de Medicina, Universidad de Buenos Aires (UBA). Buenos Aires. Argentina
[3] Instituto de Estudios de la Inmunidad Humoral (IDEHU-CONICET), Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina

Brucella abortus, outer membrane proteins, immunogenicity, adjuvant

Brucellosis caused by infection with *Brucella abortus* threatens health and productivity of a large number of livestock animals as it provokes abortion and infertility, therefore causing important economic losses. Since the outer membrane lipoproteins Omp16 and Omp19 are promising vaccine candidates we established a transient expression system for both proteins in *Nicotiana benthamiana*. Both proteins could be obtained at levels of 2% and 1,3% TSP for Omp16 and Omp19, respectively. Although both proteins were produced in an non-lipidated form, either systemic or oral immunization with purified or crude protein elicits a Th1-specific response in mice. These abilities indicate that the proteins exhibit self-adjuvanting properties which makes them ideal vaccine candidates for adjuvant-free oral delivery.

Abstract P7

HIV, *CHLAMYDIA TRACHOMATIS*, AND *HELICOBACTER PYLORI* VACCINE ANTIGEN AND PROTEINACEOUS ADJUVANT PRODUCTION IN ARABIDOPSIS AND CARROT

ÅKE STRID[1], INGRID LINDH[1], IRINA KALBINA[1], LARS ENGSTRAND[2], NILS LYCKE[3], ELLEN MARKS[3], STEVE APPLEQUIST[4], SÖREN ANDERSSON[2]

[1]Örebro University, School of Science & Technology, Örebro Life Science Center, 70182 Örebro
[2]Swedish Institute for Infectious Disease Control (SMI), 17182 Solna
[3]Department of Medicine - Huddinge, Karolinska Institute, 14186 Stockholm
[4]Department of Microbiology & Immunology, Göteborg University, 40530 Göteborg, Sweden

Adjuvant, Chlamydia, Helicobacter, HIV, Subunit vaccine antigen

In our project, we use plants for synthesis of vaccine antigens and adjuvant proteins. Among targeted diseases are HIV, *Chlamydia trachomatis* (CT) and *Helicobacter pylori* (HP) infections. Novel genetic constructs have been designed for production of optimized antigen proteins and several transformation techniques and intracellular protein production sites are being examined for yield optimization. Both *Arabidopsis thaliana* (HIV, CT, HP) and carrot (HIV, CT) have been successfully used as production hosts and plant-produced antigens against HIV (primarily the p24 protein), CT (own designed chimera of the MOMP protein) and HP (different versions of the TonB protein). For HIV and CT these antigens have been used in immunization trials in laboratory mice, giving rise to systemic immune responses. For this purpose both consumption of plant tissue and distribution of purified antigens have been used. For CT, the administration of the recombinant protein has also been shown to protect against the disease in mice. Moreover, to further increase the effect of vaccine antigens, we also use plants to produce protein-based adjuvants for inclusion in vaccine formulations. These adjuvants are based either on the bacterial flagellin protein or are cholera toxin-derived chimeric proteins.

Abstract P8

OVEREXPRESSION AND PURIFICATION OF THE ELASTIN-LIKE POLYPEPTIDE FUSION INFLUENZA H5N1 ANTIGENS IN *NICOTIANA TABACUM*

HOANG TRONG PHAN[1], BINH TRAN LE[2], DOREEN FLOSS[3], JUERGEN SCHELLER[3], UDO CONRAD[1]

[1]Leibniz Institute of Plant Genetics and Crop Plant Research, Correnssstrass 3, D-06466 Gatersleben, Germany

[2]Institute of Biotechnology, 18 Hoang Quoc Viet road, Cau Giay, Ha Noi, Viet Nam

[3]Institute of Biochemistry, Christian-Albrechts-University, Kiel, Germany

H5N1 avian influenza A virus, hemagglutinin, neuraminidase, matrix protein (M1), inverse transition cycling (ITC), elastin-like polypeptides (ELPs)

The H5N1 avian influenza A virus, commonly called "bird flu", is a highly contagious and deadly pathogen in poultry. Furthermore, H5N1 influenza viruses transmitted from poultry to humans in Asia cause high mortality (289/489) and pose a pandemic threat [1]. Vaccination is the most effective approach to reduce illness and death from pandemic influenza. Influenza vaccines (inactivated and live attenuated influenza vaccines) in general use today are derived from viruses grown in hens' eggs. In reality, the H5N1 virus was highly pathogenic for poultry and also lethal for hen's eggs [2]. Therefore, the HA content of the H5N1 candidate vaccine viruses grown in embryonated chicken eggs was low [3].

Plant expression systems are attractive because they offer a number of potential advantages: inexpensiveness; safety; ability of a rapid, economical scale-up. The most disadvantages of these expression systems are low concentration of recombinant proteins and the lack of efficient purification methods for recovering these proteins have hindered the development of plant biotechnology applications. Elastin-like polypeptides (ELPs) are artificial biopolymers consisting of repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG), where Xaa is any amino acid except Pro [4]. ELP fusion proteins can be easily purified by inverse transition cycling (ITC). In this study, the ELP was fused to avian influenza H5N1 globular, ectodomain hemagglutinin; neuraminidase and matrix protein 1 and expressed in stably transgenic tobacco plants. Fusions with the ELP tag significantly enhance the accumulation of recombinant influenza antigens in tobacco leaves and seeds. A simple and scalable membrane-based technology was optimized to recover the ELP fusion influenza antigens from leaf extracts *via* ITC. This method is very fast, highly efficient, especially to avoid degradation of recombinant influenza antigens caused by plant proteases during ITC.

References

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Abstract P9

CHLOROPLAST-BASED EXPRESSION OF A MODIFIED HUMAN PAPILLOMA VIRUS (HPV16) L1 PROTEIN FORMING CAPSOMERES

WAHEED M.T.[1], LÖSSL E.[1], HASSAN W.[1], THÖNES N.[2], GOTTSCHAMEL J.[1], KAUL H.[1], LÖSSL A.[1]

University of Natural Resources and Applied Life Sciences (BOKU), Gregor-Mendel-Strasse 33, 1180 Vienna, Austria
Deutsches Krebsforschungszentrum, F035, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Capsomeres, HPV16, L1 Plastids, sedimentation analysis, Male sterility,

Objective: For approximately 70% of invasive cervical carcinoma, one of the most common cancers in women, Human papillomavirus (HPV) types 16 and 18, have been identified as being responsible. However, since a complete VLP-based vaccination costs around 500-800 Euro they are hardly available to people in developing countries, where most incidences of cervical cancer arise. Out of this situation these countries need a cost saving solution.
In the last five years a high immunogenicity of capsomeres has been proven. Due to their advantages they allow to provide a cost effective substitute to conventional HPV vaccines based on VLPs.
Methodology: Nicotiana tabacum chloroplasts were transformed with the modified HPV-16 L1 (L1_2xCysM) gene for the viral coat protein. Leaves were transformed using particle gun (PDS1000He, Bio-Rad, CA, USA). After bombardment, leaves were regenerated to whole plants on spectinomycin containing medium. Analysis of novel plant protein was examined by cesium chloride (CsCl) density gradient centrifugation and sucrose sedimentation.
Results: The present study demonstrates the possibility to express a mutated HPV-16 L1 (L1_2xCysM) gene in plant chloroplasts. In these organelles the recombinant L1 protein accumulates in form of assembled capsomeres up to ca. 1,5 % of total soluble protein.
The correct form assembly of capsomeres was determined by cesium chloride density gradient centrifugation and sucrose sedimentation analysis. Antigen capture ELISA confirmed the formation of capsomeres by using a conformation specific monoclonal antibody, recognizing conformational epitopes. Transplastomic tobacco plants exhibited normal growth and morphology. However, all transplastomic lines obtained were male sterile in all the following generations up till now.
Conclusion: In summary our results give rise to the hope for a production of capsomere-based vaccine a low cost in plant chloroplasts (Waheed et al. 2010).

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Waheed MT, Thönes N, Müller M, Hassan SW, Lössl E, Kaul HP, Lössl A (2010) Transplastomic expression of a modified human papillomavirus L1 protein leading to the assembly of capsomeres in tobacco: A step towards cost effective second generation vaccines. DOI: 10.1007/s11248-010-9415-4 Transgenic Research 2010

Abstract P10

PLANT SEEDS AS BIOREACTORS FOR THE PRODUCTION OF HIGH-VALUE PROTEINS

ANNELIES DE PAEPE[1], SYLVIE DE BUCK[1], THOMAS DE MEYER[1], KIRSTEN DE WILDE[1], ROBIN PIRON[1], VIKRAM VIRDI[1], GEERT DE JAEGER[1], BART VAN DROOGENBROECK[2], ROLINDE DEMEYER[2] , MARC DE LOOSE[2], ANN DEPICKER[1]

[1]Department of Plant Systems Biology, Ghent University, Flanders Interuniversity Institute for Biotechnology (VIB), 9052 Gent, Belgium
[2]Institute for Agricultural and Fisheries research (ILVO), Unit technology and Food (T&V), 9820 Merelbeke, Belgium

seeds, Arabidopsis, antibodies, vaccines, greenhouse production

Besides the conventional production platforms such as animal and insect cell cultures, yeast and bacteria, the use of transgenic plants is a promising alternative production system for high value recombinant proteins. Plants offer general advantages in terms of production scale and economy, product safety, and ease of storage and distribution. Recently, plants have been used as recombinant biofactories to express a number of proteins including pharmaceuticals and potential vaccines. Levels of expression are critical and vary greatly depending on the protein expressed, the plant species used for expression, and the expression construct design. Seeds have the useful advantage of accumulating proteins in a relatively small volume and stable environment. Using the regulatory sequences of the seed storage protein gene *arceline 5-I* and the *β-phaseolin* promoter of *Phaseolus vulgaris*, a single-chain variable fragment (scFv) accumulated to exceptionally high levels as high as 36,5% of total soluble protein (TSP) in *Arabidopsis* seeds which is equivalent to approximately 7% of seed weight. Also, when using this seed-specific expression vector for the expression of a more complex format, scFv-Fc antibodies accumulated to very high levels (2% of seed weight) and showed proper processing and functionality. Currently, the production of other antibody formats and subunit vaccines is evaluated.
Given the high expression levels in *Arabidopsis* seeds, the use of *Arabidopsis* seeds in greenhouses (with a yield of about 1.8 ton seed/hectare/year) might become an attractive alternative for recombinant protein production since it is a flexible, contained, non-food, rapid and low cost host for the commercial production of heterologous proteins, such as pharmaceuticals or vaccines.

Abstract P11

PRODUCTION OF RECOMBINANT PROTEINS IN TRANSGENIC BARLEY GRAINS

STEPHANIE GOEDEKE, GÖTZ HENSEL, CATRIN KAYDAMOV, SYLVIA BROEDERS, ISOLDE SAALBACH, JOCHEN KUMLEHN

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Plant Reproductive Biology, Correnssstr. 3, 06466 Gatersleben, Germany

barley, expression, molecular farming, recombinant antibody

Molecular farming is considered a promising strategy to produce valuable proteins not only in human and veterinary medicine, but also in agriculture. Compared with other bioproduction systems such as bacteria, yeasts, mammalian or plant cell cultures, transgenic crop plants may provide an inexpensive and convenient platform for the large scale production of recombinant proteins. The particular advantage of barley as a production system is on the one hand that the endosperm lays the foundations to depose a high protein amount and on the other hand that the essential large-scale production processes which include cultivation, harvest, transport and storage are well established. Moreover, outcrossing can be neglected, since barley is a stringent self-pollinator. In this study, we aim to utilise transgenic barley grains to stably express recombinant antibodies in mature endosperm. To achieve strong endosperm-specific expression, the *α-GLIADIN* promoter of wheat (*Triticum aestivum*) as well as a signal peptide sequence for the targeting of the gene product via the endoplasmic reticulum to subcellular protein storage compartments is used. To further enhance expression and accumulation of recombinant proteins in barley grains, diverse regulatory elements such as heterologous 5' and 3' untranslated regions, including the 5'UTR of the seed-specific *LEGUMIN B4* promotor of broad bean (*Vicia faba*) and the 3`UTR of the *ARC5-I* gene of common bean (*Phaseolus vulgaris*) have been tested. Furthermore, a combination of the *α-GLIADIN* promoter with an enhancer region of the *CaMV 35S* promoter was used to enhance the accumulation of GFP, which was utilised as a model recombinant protein in this study. Based on this expression system, transgenic barley lines have been generated which carry a gene encoding the recombinant antibody BA11 (kindly provided by Novoplant GmbH). This single-chain antibody effectively prevents piglets from being infected by enterotoxigenic *E. coli* strains. Enterotoxins are the major cause of diarrhoea and death in neonatal pigs right after being weaned. Milled grains can be directly fed to piglets for disease prevention. Expression analyses revealed an effectual scFvBA11 yield as high as 1 g/kg mature grain.

Abstract P12

TOBACCO HAIRY ROOT CULTURES AS PRODUCTION HOST FOR THE HUMAN ANTIBODY M12

SUVI T. HÄKKINEN[1], NICOLE RAVEN[2], TONI PAASIKALLIO[1], JUHA-PEKKA PITKÄNEN[1], KIRSI-MARJA OKSMAN-CALDENTEY[1], STEFAN SCHILLBERG[2], ANNELI RITALA[1]

[1]VTT Technical Research Centre of Finland, P.O. Box 1000, 02044 VTT, Finland
[2]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Forckenstrasse 6, 52074 Aachen, Germany

antibody, hairy root, recombinant, therapeutic, tobacco

In addition to mainstream production systems, such as those based on microbes and mammalian cells, plants offer a well established alternative for the production of high-value recombinant proteins. Compared to whole plants, the use of cell culture systems offers advantages to produce metabolites or recombinant proteins in a contained and a controlled environment, independent of climatic factors and under conditions in which the different production parameters can be optimized. *Agrobacterium rhizogenes* infection induced hairy root cultures can be exploited as production vehicles having commonly more stable production pattern than those of undifferentiated cells. Furthermore, the efficient genetic engineering accompanied with the up-scaled production possibilities make hairy roots an attractive option for industrial scale production. Diagnostic and therapeutic antibodies are an important target in plant molecular farming. For example, the human M12 antibody is supposed to bind to the membrane-bound mucin molecule MUC-1 present on epithelial cancer cells, such as breast, pancreas, colorectal and lung cancers[1].

The antibody M12 was constructed by fusion of the constant domain of human IgG, to the variable domains of the scFv M12, which were earlier selected from a naïve human phage display library directly on a MUC-1-expressing breast carcinoma cell line[1]. Hairy roots were initiated by infecting transgenic *Nicotiana tabacum* cv Petite Havana SR1 plants expressing M12 antibody with a wild type *Agrobacterium rhizogenes* strain. Hairy roots cultivation was performed either in shake flasks or in a Medicel® cultivation unit[2], comprising of 30 individual bioreactors. Cultivation parameters were optimized by using statistical experimental design.

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Abstract P13

PLANT PRODUCTION OF THE TUMOUR-TARGETING HUMAN IgG H10: CONSIDERATIONS ON EXPRESSION STRATEGIES AND PURIFICATION PROCESS

RAFFAELE LOMBARDI, MARIA ELENA VILLANI, MARIASOLE DI CARLI, CARLA MARUSIC, EUGENIO BENVENUTO, MARCELLO DONINI

ENEA, UT-BIORAD, Laboratory of plant biotechnology, C.R. Casaccia, Via Anguillarese 301, 00123, Rome, Italy.

plantibody; agroinfiltration; proteolysis; antibody purification, endotoxin

The tumour-targeting antibody mAb H10 can be transiently expressed and purified at high levels in *Nicotiana benthamiana* by using a vacuum-agroinfiltration system boosted by the use of the artichoke mottled crinckle virus (AMCV) P19 virus silencing suppressor protein. In this work we analysed the different steps of protein extraction from agroinfiltrated leaves to optimise the purification process of the secretory mAb H10 providing new insights in the field of large-scale plant production. Two different extraction procedures (mechanical shearing/homogenisation and recovery of intercellular fluids -IFs-) were evaluated and compared in terms of purified antibody yields, antibody degradation and total phenolic compounds content. Mechanical grinding from fresh leaf tissues gave the highest purification yield (75 mg / Kg Fresh Weight -75% intact tetrameric IgG-) and total phenolics concentration in the range of 420 mg / g FW. The second extraction procedure, based on the recovery of IFs, gave purification yields of 15-20 mg / Kg FW (corresponding to 27% of total soluble protein) in which about 40% of purified protein is constituted by fully assembled IgG with a total phenolic compounds content reduced by one order of magnitude (21 mg / g FW). Despite a higher antibody degradation, purification from intercellular fluids demonstrated to be very promising since extraction procedures resulted extremely fast and amenable to scaling-up. Overall data highlight that different extraction procedures can dramatically affect the proteolytic degradation and quality of antibody purified from agroinfiltrated *N. benthamiana* leaves. Based on these results, we optimised a pilot-scale purification protocol using a two-step purification procedure from batches of fresh agroinfiltrated leaves (250 g) allowing purification of milligram quantities (average yield 40 mg / Kg FW) of fully assembled and functional IgG with a 99.4% purity, free of phenolic and alkaloid compounds with low endotoxin levels (< 1 EU / ml).

Abstract P14

NEXT GENERATION COMBINATION MICROBICIDES TO COMBAT HIV

M. SABALZA[1], K. RAMESSAR[1,2], C. VAN DOLLEWEERD[3], T. CAPELL[1], B. O'KEEFE[2], J. MA[3], P. CHRISTOU[1,4]

[1]Departament de Produccio Vegetal I Ciencia Forestal (PVCF), Universitat de Lleida, Av. Alcalde Rovira Roure, 177, Lleida, E-25198, Spain
[2] Molecular Targets Laboratory, National Cancer Institute at Frederick (NCI), Frederick, MD 21702, USA
Molecular Targets Development Program, National Cancer Institute at Frederick (NCI), Frederick, MD 21702, USA
[3] Centre For Infection, Division of Cellular and Molecular Medicine (CMM), St. George's University of London Room 2.148, London SW17 0RE, United Kingdom
[4] Institucio Catalana de Recerca I estudis avancats (ICREA)

Anti-HIV monoclonal antibodies, griffithsin, CV-N, vombination microbicides, molecular pharming

Protein microbicides against HIV are promising alternatives to the current generation of small molecule drugs. An effective microbicide will need to consist of a cocktail of anti-HIV molecules to prevent the rapid evolution of HIV resistance and to provide sufficient cross-clade protection. It is unlikely that such drugs will be broadly available because of their high production and distribution costs. Their production in plants (molecular pharming), could provide a reliable low-cost platform for the manufacture of protein microbicide candidates. Amongst various plant production systems, cereal seeds offer high stability of proteins when dry and can be easily stored for long periods of time. We present an alternative production platform for some of the most promising microbicide candidates that are being evaluated to date: griffithsin, cyanovirin-N and human neutralizing antibodies (2G12, 4E10, 2F5 and b12). We aim to produce three different combination microbicides by co-expressing these proteins/antibodies in transgenic corn seeds. A first combination includes the 4 human HIV neutralizing monoclonal antibodies which will be produced in one plant, whereas a second combination consists of the anti-HIV proteins griffithsin and cyanovirin-N, together with the b12 monoclonal antibody. A third combination comprises monoclonal antibodies 2G12, 4E10 and 2F5. These particular combinations were selected for three reasons. The first one is to study the effectiveness of each combination against HIV and in the process exploit possible synergistic effects. The second reason pertains to regulatory issues: to date, regulations for antibodies produced in mammalian cells exists, however no regulations are in place for therapeutic proteins produced against HIV. The third reason is to obtain transgenic corn plants expressing all 4 human HIV neutralizing antibodies, griffithsin and cyanovirin-N within the same plant by cross-pollinating corn plants expressing the last two combinations. We had previously shown high-level, endosperm-specific production of 2G12 in corn seed, demonstrating equal (if not superior) HIV-neutralization when compared to 2G12 produced in mammalian cells. We will evaluate corn seeds as a production system for combinations of these recombinant pharmaceutical proteins. Further analysis will confirm HIV-binding efficacy and consequently the ability to block virus entry into host cells. Here we put forward our experimental strategy and describe early experiments in the creation of such transgenic plants for further development.

Abstract P15

INDUCTION AND PURIFICATION OF HSP70, A POTENTIAL PLANT DERIVED ADJUVANT, FROM ALFALFA AND RECOMBINANT ORGANISMS

IANNACONE R., JALIASHVILI T., ARMENTANO N., MELFI D., SEMERARO L., SUMMERER S., TADDONIO G., RAHIMI H., RUGHETTI A., NUTI M. CELLINI, F.

[1]Metapontum Agrobios S.S. Jonica 106 Km 448,2 -75012 Metaponto (MT)
[2]Dip.Medicina Sperimentale, Sapienza Università di Roma Viale Regina Elena, 324 00161 Roma - Italy

Alfalfa, HSP70, Chaperones, Adjuvants, Heat stress

Heat Shock Proteins (HSPs) represent a class of proteins highly conserved in all organisms; they are activated by environmental stresses and act mainly as molecular chaperones.
Human origin HSP70 has been demonstrated to act as general immune-stimulants of both innate and acquired immunity via binding several surface receptors of different immune cells that results in dendritic cell maturation and NKs activation.
Due to the high similarity between human and plant HSP70, it can be inferred that plant HSP70 can have the same role than human HSP70. It was demonstrated previously that there is activation of mammalian immune system mediated by plant HSP70 (Kumaraguru *et al*. 2003).
Plant bio-mass can be used as cheap source of HSPs upon induction of HSPs in field condition using physical methods or by up-regulation of content of HSPs by the means of genetic engineering.
In our work we used both methods to increase HSP70 content in plant tissues. Firstly we set up a method that allows the accumulation of HSP70 in green tissues upon treatment with a hot stream in field condition for industrial applications (Patent N° WO 00/070932). The feasibility of accumulation and extraction of plant HSP70 from green tissues using a pilot plan was accomplished and the purified HSP70 was characterized by biochemical methods. Secondly genes codifying HSP70 were isolated from *Arabidopsis* and transferred in yeast and plants (tobacco, alfalfa, *Arabidopsis*) using constitutive and inducible promoters.
HSP70 protein purified from in-field induced alfalfa or from recombinant organisms was used in assays with Human Dendritic cells (DCs). Preliminary results show the influence of both native and recombinant HSP70 on the maturation and differentiation of DCs.
Data regarding the purification and characterization of plant HSP70 from yeast and tissues and its effects on human DC will be discussed.

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Abstract P16

CLONING AND EXPRESSION ANALYSIS OF *SPPA* GENE ENCODING BACTERIOCIN SAKACIN P INTO *NICOTIANA BENTHAMIANA*

VILMA NARBUTAITE[1], GRAZINA JUODEIKIENE[1], LORETA BASINSKIENE[1], GEORGE P. LOMONOSSOFF[2]

[1]Department of Food Technology, Faculty of Chemical Technology, Kaunas University of Technology, Kaunas, Lithuania
[2] Department of Biological Chemistry, John Innes Centre, Norwich NR4 7UH, United Kingdom

expression, GATEWAY, sakacin P, sppA, N.benthamiana

Sakacin P is a 61 amino acid residue long class IIa bacteriocin encoded by the structural gene *sppA*. An important research goal is to develop the efficient methods for heterologous expression of bacteriocins produced by lactic acid bacteria. Heterologous expression of bacteriocins allows creating strains that would have improved protective properties differing in the ability to produce certain bacteriocin, therefore facilitating the scientific evaluation of the bacteriocin effect.

The main aim of this study was to apply versatile expression vectors (CPMV-*HT* vector, pEAQ-*HT)* for the direct cloning of genes encoding bacteriocin sakacin P from lactic acid bacteria (LAB) into the binary plasmid. Cloning experiments were focused on the GATEWAY cloning system, when the gene of interest was first cloned into an Entry vector (pENTR) and then transferred to a Destination vector (pDEST) by site-specific recombination: the insert of a correct pENTR-sppA entry clone possessing both start and stop codons was used in GATEWAY recombination (LR) reactions with pEAQ-HT-DEST1 and pEAQ-HT-DEST3 destination vectors, resulting in pEAQ-GW-sppA and pEAQ-GW-His-sppA, respectively. To examine the gene transfer to plants and the performance of the pEAQ-based vectors for transient expression, pEAQ-GW-sppA and pEAQ-GW-His-sppA constructs were transformed into *Agrobacterium tumefaciens* strain LBA 4404 by electroporation and bacterial suspensions were used to agro-infiltrate *Nicotiana benthamiana* leaves. The protein expression was determined by the analysis of soluble protein extracts by SDS-PAGE, followed by the western blot and immunodetection (SNAP-id) analysis.

SDS-PAGE of protein extracts revealed no expressed levels of sakacin P (4.4 kDa), as detected by Coomassie Blue staining. Moreover, the unfused and His-tagged sakacin P variants were not detectable in Western blots and immunodetection using the His-Tag monoclonal and an anti-mouse horseradish peroxidase conjugate antibodies. The non-expression of the protein into *Nicotiana* was confirmed with the applied rapid purification of soluble target protein under native conditions using His•Bind Resin chromatography.

Abstract P17

PLANT-BASED EXPRESSION, PURIFICATION AND BIOMECHANICAL CHARACTERIZATION OF SPIDER SILK PROTEIN DERIVATES

VALESKA HAUPTMANN[1], FRAUKE JUNGHANS[2], BENOIT LEPICHIER[3], MATTHIAS MENZEL[2], JACQUES GUEGUEN[3], UWE SPOHN[2], UDO CONRAD[1]

[1]Leibniz Institute of Plant Genetics and Crop Plant Research, Corrensstraße 3, 06466 Gatersleben, Germany
[2] Fraunhofer Institute for Mechanics of Materials, Walter-Hülse-Strasse 1, 06120 Halle (Saale), Germany
[3] Institut National de la Recherche Agronomique, Rue de La Géraudière-BP 71627, 44316 Nantes Cedex 03, France

Spider silk, synthetic genes, recombinant expression, protein purification, atomic force microscopy

Spider silks possess impressive mechanical properties, e.g. extremely high tensile strength and elasticity[1]. Such high value proteins are therefore interesting biomaterials for industrial and medical purposes. Conceivable application could be the reinforcement of materials or coating of implants, which also could enhance the biocompatibility[2]. Spiders synthesize six different types of silk fibres and one glue-like silk and combine the different proteins, consisting of short blocks of amino acid repeats, into one material[3]. So far it is unknown how these different amino acid sequence elements contribute to the extraordinary mechanical properties.
Plants are attractive expression systems of recombinant proteins as they are inexpensive and versatile systems, amenable to rapid and economical scale-up. The goal of the presented project is the investigation how the size and the primary structure of plant produced spider silk derivates influences the resulting mechanical properties.
Therefore various newly synthesized spider silk like proteins have been fused with Elastin-like peptides (ELPs), which enables the purification by ITC (*inverse transition cycling*) and they may also contribute to elasticity and solubility of these proteins. ELPs are artificial structural proteins of highly repetitive character[4]. Furthermore they can enhance the expression level in plant cells[5]. During a STSM (COST) further purification was performed by size exclusion chromatography of the prepurified proteins. In addition characterization of material properties of spider silk by atomic force microscopy is presented.

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Abstract P18

PLANT VIRAL PARTICLES AS NANO-SCAFFOLDS FOR CONTROLLED POSITIONING OF ENZYMES ON SOLID SUPPORTS

JANE BESONG[1], NOELLE CARETTE[2], DANIELA CARDINALE[2], JOCELYNE WALTERS[2], THIERRY MICHON[2], KRISTIINA MÄKINEN[1]

[1]Department of Food and Environmental Sciences, University of Helsinki, Finland.
[2] INRA Centre of Bordeaux, France.

Virus, capsid protein (CP), virus-like particle (VLP), enzyme, enzyme nano-carrier (ENC)

The VIRUSCAF project which was launched in November 2009 belongs to the research field of solid film supported bio-catalysis with potential applications for the technology of micro-reactors and biosensors. It intends to study the possibilities to control the distribution of active enzymatic systems on solid supports. The project's outcomes will serve the technology for enzymatically assisted catalysis in chemical synthesis. The control of enzymes structure and function develops their catalytic potential for innovative and effective synthetic routes limiting the number of intermediate steps, toxic effluents and energy cost. In this respect, the cautious use of biosystems should sustain renewable and environmentally safe processes. The systems concerned are either constituted of a single enzyme, or of a couple of enzymes catalysing a cascade of two consecutive reactions. The later illustrating an example of a simple bio mimetic system inspired from the metabolic chain reactions in living cells. The close spatial location of the two enzymes increases the reaction efficiency, the product from the first enzyme catalysed reaction, being the substrate of the second (diffusion limitation or channelling). Virus particles are precisely defined nanometer-sized objects formed by the self-association of capsid proteins monomers (CP for Capsid Protein, Coat Protein or capsomers). In order to finely tune the distribution of enzymes on surfaces, it has been planned to use these particles as enzyme supports. Recently the Bordeaux team demonstrated that using the natural resources of a plant, it is possible to decorate the surface of filamentous phytovirus particles with an active lipase. Non infectious virus-like particles (VLPs) have previously been reconstructed in bacterial and plants. This same approach can be used to produce VLPs *in vitro* from CPs which have been modified to display specific anchoring sites for the enzymes of interest. The Helsinki team has constructed a PVA-based vector for heterologous protein expression which will be used to express these modified enzymes *in planta*. Nanolytographic patterning will be used to finely tailor the positional assembly of the virus particles on solid supports. The enzymes purified from plant leaves will be linked to the particles through the complementary peptide motives previously introduced. The resulting enzyme nano-carrier (ENC) surfaces will be characterized for morphology, enzymes distribution and catalytic efficiency

Abstract P19

IN PLANTA PROTEIN SIALYLATION THROUGH OVER-EXPRESSION OF THE RESPECTIVE MAMMALIAN PATHWAY

ALEXANDRA CASTILHO[1,2], RICHARD STRASSER[1,2], JOHANNES STADLMANN[3], JOSEPHINE GRASS[3], JAKUB JEZ[2], PIA GATTINGER[2], RENATE KUNERT[4], HERIBERT QUENDLER[5,6], MARTIN PABST[3], RENAUD LEONARD[3], FRIEDRICH ALTMANN[3], HERTA STEINKELLNER[2]

[1] These authors contributed equally to this work
[2]Department of Applied Genetics and Cell Biology
[3]Department of Chemistry
[4]Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria,
[5]Polymun Scientific GmbH, Muthgasse 18, 1190 Vienna, Austria
[6]present address: Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037, USA

Recombinant therapeutic proteins, glycosylation, sialylation, monoclonal antibodies

Many therapeutic proteins are glycosylated and require terminal sialylation to attain full biological activity. Current manufacturing methods based on mammalian cell culture allow only limited control of this important posttranslational modification which may lead to the generation of products with low efficacy. Here we report *in vivo* protein sialylation in plants. This was achieved by the introduction of an entire mammalian biosynthetic pathway in *Nicotiana benthamiana,* comprising the coordinated expression of the genes for (i) biosynthesis, (ii) activation, (iii) transport and (iv) transfer of Neu5Ac to terminal galactose. We show the transient overexpression and functional integrity of six mammalian proteins that act at various stages of the biosynthetic pathway and demonstrate their correct subcellular localisation. Coexpression of these genes with a therapeutic glycoprotein, a human monoclonal antibody (mAb), resulted in quantitative sialylation of the Fc domain. Sialylation was at great uniformity when glycosylation mutants that lack plant-specific N-glycan residues were used as expression hosts. Finally we demonstrate efficient neutralization activity of the sialylated mAb, indicating full functional integrity of the reporter protein. We report for the first time the incorporation of the entire biosynthetic pathway for protein sialylation in a multicellular organism naturally lacking sialylated glycoconjugates. Beside the biotechnological impact of the achievement this work may serve as a general model for the manipulation of complex traits into plants.

Abstract P20

NEED FOR SPEED - TOOLS FOR PROTEIN EXPRESSION AND PURIFICATION IN PLANTS

JUSSI JOENSUU[1], ANDREW CONLEY[1,2], MICHAEL LIENEMANN[1], MARKUS LINDER[1], RIMA MENASSA[2]

[1]VTT Technical Research Centre of Finland, FIN-02044 Espoo, Finland
[2]Agriculture and Agri-Food Canada, N5V4T3, London, Ontario, Canada

protein purification,high-throughput cloning, protein body induction, hydrophobin, elastin-like polypeptide

Insufficient accumulation levels and the lack of efficient purification methods often hinder the feasibility of recombinant protein production in transgenic plants. Transient expression in *Nicotiana benthamiana* leaves via agroinfiltration has established itself as a benchmark tool to study protein over-expression in plants. This technique has a capacity of testing high number of expression constructs to provide mg-amounts of protein of interest in a matter of few days. It also provides valuable information on the behaviour of recombinant proteins in stable transgenic plants. In order to reach good expression levels (i.e. > 5 % total soluble protein), there is a need to empirically evaluate the optimal expression parameters for each candidate protein. Therefore, high-throughput methods for construct cloning and subsequent protein purification are needed. We have studied fusion protein technologies to facilitate protein over-expression and purification in plants. Synthetic elastin-like polypeptides and fungal hydrophobins were fused to several different target proteins and were found to enhance the protein accumulation by intracellular protein body formation. Subsequently, these fusion partners also allowed efficient non-chromatographic purification of the target proteins from the complex leaf extracts. The implications of these results for the production of recombinant proteins in plants will be discussed. Latest advances for high-throughput expression vector cloning will be also summarized.

Abstract P21

AGGREGATION AS A BOTTLENECK FOR IL-10 PRODUCTION IN *NICOTIANA BENTHAMIANA*

LOTTE WESTERHOF, RUUD WILBERS, ARJEN SCHOTS

Wageningen University, Laboratory of Molecular Recognition and Antibody Technology, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

3D domain swapping, aggregation, biopharmaceuticals, cytokine, interleukin-10

Production of the anti-inflammatory cytokine interleukin 10 (IL-10), an often-suggested promising biopharmaceutical, has not met market demands. The human IL-10 (hIL-10) gene encodes a 160 amino acid (aa) protein preceded by an 18aa signal peptide for secretion. The mature protein consists of six alpha helices (A-F) with two internal disulfide bridges. To confer complete biological activity two mature proteins form a non-covalent homodimer of 37kDa by intertwining their helices A-D with the other's helices E and F based on strong hydrophobic patch interaction. Human and mouse IL-10 (mIL-10) exhibit 73% homology and share a potential glycosylation site that is not glycosylated. But, mIL-10 has yet another site which is glycosylated. Studying human and mouse IL-10 production in *Nicotiana benthamiana*, we discovered that human IL-10 aggregates *in planta* into multimers due to its intrinsic 3D-domain swapping characteristic, while mouse IL-10 aggregation is almost completely absent. Removal of the extra glycosylation site of mIL-10 showed that aggregation is in part prevented by its glycosylation. However, introduction of the second glycosylation site of mIL-10 in hIL-10 would most likely render it nonfunctional, since mIL-10 is unable to trigger a response from human cells while hIL-10 can trigger responses from mouse cells. Instead a flexible linker was introduced in between the alpha helices D and E of hIL-10 to allow folding of helices E and F of one hIL-10 protein into its own A-D region, creating the possibility of a stable monomer (hIL-10^{mono}). This indeed prevented aggregation and 10-fold more IL-10 could be extracted from plant material. But, as expected hIL-10^{mono} was shown not to have biological activity as determined by a cell-based assay. Successful strategies to restore biological activity in order to obtain an IL-10 variant that can be used for medical applications will be discussed.

Abstract P22

STABILITY AND DEGRADATION OF ANTHOCYANINS IN FRUIT AND ORNAMENTALS

MICHAL OREN-SHAMIR

Volcani Center, Department of Ornamental Horticulture, 50250 Bet-Dagan, Israel

Anthocyanins are the largest group of plant pigments that accumulate in the vacuoles and are responsible for colons ranging from red to violet and blue. The biosynthesis pathway of anthocyanins and its regulation have been well characterized. In contrast, little is known about the fait of anthocyanin pigments after they have accumulated in the vacuoles. There is evidence showing that anthocyanins are degraded in plants due to changes in environmental conditions such as increased temperature, or due to developmental changes in leaves, fruit and flowers. The degradation can be due either to active enzymatic degradation or turnover of the pigments following chemical changes occurring in the plant vacuoles. The goals of our study are to find ttreatments that stabilize the anthocyanins in the vacuoles, causing increased accumulation of the pigments in plants, and to study the enzymatic processes involved in active *in planta* anthocyanin degradation.We have characterized active *in planta* anthocyanin degradation in *Brunfelsia* flowers, in which the change of color from purple to white is dependant on the induction of genes and synthesis of novel proteins. In our earlier studies we have also shown that treatment of ornamentals with magnesium salts caused increased accumulation of anthocyanins. In an attempt to understand this phenomenon, we studied the effect of magnesium treatment on the grape cell suspension of *V. vinifera* cv. Gamay Red. Magnesium caused a four fold increase in the anthocyanin concentration of the cells, with no induction of synthesis. When anthocyanin synthesis was inhibited, a steady decrease in anthocyanin accumulation was detected. Addition of magnesium to the inhibited cells prevented the decrease in anthocyanins, suggesting that magnesium increases the stability of the pigments and prevents the natural turnover occurring in the cells. Further proof of this hypothesis is the fact that were most affected by the magnesium treatment were those with a low molecular weight and lower stability, suggesting that the main role of the metals is stabilizing the pigments and increase their half life time. This study suggests that turnover of anthocyanins occurs in fruit cells, and preventing this process may enable increased accumulation of the pigments even under conditions in which synthesis rate is low.

LIST OF PARTICIPANTS

1. AVESANI Linda
   University of Verona, Dept. of Biotechnology
   37134 Verona, Italy
   linda.avesani@univr.it

2. BARONE Amalia
   University of Naples "Federico II"
   Dept. Soil, Plant, Environmental and Animal Production Sciences
   80055 Portici, Italy
   ambarone@unina.it

3. BASCHIERI Selene
   ENEA, BIOTEC-GEN Casaccia Research Center
   00123 S. Maria di Galeria (RM), Italy
   selene.baschieri@enea.it

4. BASINSKIENE Loreta
   Kaunas University of Technology, Dept. of Food Technology
   50254 Kaunas, Lithuania
   loreta.basinskiene@ktu.lt

5. BELLUCCI Michele
   CNR-IGV, Institute of Plant Genetics
   06128 Perugia, Italy
   michele.bellucci@igv.cnr.it

6. BORTESI Luisa
   University of Verona, Dept. of Biotechnology
   37134 Verona, Italy
   luisa.bortesi@univr.it

7. BOSCH Dirk
   Plant Research International, Wageningen UR
   6708 PB Wageningen, The Netherlands
   dirk.bosch@wur.nl

8. BROER Inge
   University of Rostock, Institute for Land Use,
   Dept. of Agrobiotechnology
   18059 Rostock, Germany
   inge.broer@uni-rostock.de

9. BUONAGURO Franco M.
   National Cancer Institute 'Fond. G. Pascale',
   Dept. of Experimental Oncology,
   Molecular Biology & Viral Oncology Unit – AIDS Reference Center,
   80131 Naples, Italy
   irccsvir@unina.it

10. BUONAGURO Luigi
National Cancer Institute 'Fond. G. Pascale',
Dept. of Experimental Oncology,
Molecular Biology & Viral Oncology Unit – AIDS Reference Center,
80131 Naples, Italy
irccsvir@unina.it

11. BURSSENS Sylvia
Ghent University, Institute Plant Biotechnology for Developing Countries,
Dept of Plant Biotechnology and Genetics
B-9000 Gent, Belgium
Sylvia.Burssens@UGent.be

12. CAPELL Teresa
University of Lleida, Dept. of Crop and Forest Sciences
E25198 Lleida, Spain
teresa.capell@pvcf.udl.es

13. CARDI Teodoro
CRA - ORT
Agriculture Research Council, Research Centre for Vegetable Crops
84098 Pontecagnano (SA), Italy
teodoro.cardi@entecra.it

14. CHRISTOU Paul
University of Lleida, Dept. of Crop and Forest Sciences
E25198 Lleida, Spain
christou@pvcf.udl.es

15. CONRAD Udo
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),
Dept. of Molecular Genetics
06466 Gatersleben, Germany
conradu@ipk-gatersleben.de

16. DE PAEPE Annelies
University of Gent, VIB Dept. of Plant Systems Biology
9052 Gent, Belgium
anpae@psb.vib-ugent.be

17. DEPICKER Ann
University of Gent, VIB Dept. of Plant Systems Biology
9052 Gent, Belgium
anpic@psb.vib-ugent.be

18. DONINI Marcello
ENEA, BIOTEC-GEN Casaccia Research Center
00123 S. Maria di Galeria (RM), Italy
marcello.donini@enea.it

19. FAYE Loic
University of Rouen, ANGANY Genetics
76821 Mont Saint Aignan, France
loic.faye@anganygenetics.com

20. GRILLO Stefania
CNR-IGV, Institute of Plant Genetics
80055 Portici (NA), Italy
grillo@unina.it

21. GUEDES-PINTO Henrique
Centre of Genomics and Biotechnology,
Institute for Biotechnology and Bioengineering
5001-801 Vila Real, Portugal
h.gp@hotmail.com

22. HAKKINEN Suvi
VTT Technical Research Centre of Finland
02044 VTT, Finland
Suvi.Hakkinen@vtt.fi

23. HAUPTMANN Valeska
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),
Dept. of Molecular Genetics
06466 Gatersleben, Germany
Hauptmann@IPK-gatersleben.de

24. HOANG Phan Trong
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),
Dept. of Molecular Genetics
06466 Gatersleben, Germany
hoang@IPK-gatersleben.de

25. IANNACCONE Rina
Metapontum Agrobios
75010 Metaponto (MT), Italy
riannacone@agrobios.it

26. JÄRVEKÜLG Lilian
Tallin University of Technology, Dept. of Gene Technology
12618 Tallin, Estonia
lilian.jarvekulg@ttu.ee

27. JOENSUU Jussi
VTT Technical Research Centre of Finland
02044 Espoo, Finland
jussi.joensuu@vtt.fi

28. JUODEIKIENE Grazina
Kaunas University of Technology, Dept.of Food Technology
50254 Kaunas, Lithuania
grazina.juodeikiene@ktu.lt

29. KUMLEHN Jochem
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),
Dept. of Plant Reproductive Biology
06466 Gatersleben, Germany
kumlehn@ipk-gatersleben.de

30. LIAIMER Anton
AMB, BFE, University of Tromsø
9037 Tromsø, Norway
anton.liaimer@uit.no

31. LOMONOSSOFF George
John Innes Centre, Dept. Biological Chemistry
Norwich NR4 7UH, UK
george.lomonossoff@bbsrc.ac.uk

32. LÖSSL Andreas
University of Natural Resources and Applied Life Sciences,
Dept. of Applied Plant Sciences and Plant Biotechnology
1180 Vienna, Austria
andreas.lossl@boku.ac.at

33. MA Julian
St. George's University of London,
Molecular Immunology Unit
London SW17 0RE, UK
jma@sgul.ac.uk

34. MÄKINEN Kristiina
University of Helsinki, Dept. of Food and Environmental Sciences
00014 University of Helsinki, Finland
kristiina.makinen@helsinki.fi

35. MÄNTYLÄ Einar
Orf Genetics
203 Kópavogur, Iceland
einar@orf.is

36. METZLAFF Karin
European Plant Science Organisation, EPSO
1000 Brussels, Belgium
Karin.Metzlaff@epsomail.org

37. MIKSCHOFSKY Heike
University of Rostock, Institute for Land Use,
Dept. of Agrobiotechnology
18059 Rostock, Germany
heike.mikschofsky@uni-rostock.de

38. MIRALPEIX ANGLADA Bruna
University of Lleida, Dept. of Crop and Forest Sciences
E25198 Lleida, Spain
bruna.miralpeix@pvcf.udl.cat

39. MOLONEY Maurice
Rothamsted Research
AL5 2JQ Harpenden, UK
maurice.moloney@bbsrc.ac.uk

40. MORISSET Dany
National Institute of Biology,
Dept. of Biotechnology and Systems Biology,
1000 Ljubljana, Slovenia
Dany.Morisset@nib.si

41. NEUHAUS Jean-Marc
Institute of Biology, University of Neuchatel
2009 Neuchatel, Switzerland
jean-marc.neuhaus@unine.ch

42. OKSMAN - CALDENTEY Kirsi-Marja
VTT Technical Research Centre of Finland
02044 Espoo, Finland
kirsi-marja.oksman@vtt.fi

43. OREN-SHAMIR Michal
Volcani Center
50250 Bet Dagan, Israel
vhshamir@agri.gov.il

44. ORZAEZ Diego
Institute of Plant Molecular and Cellular Biology,
CSIC-University Polytechnic of Valencia
46022 Valencia, Spain
dorzaez@ibmcp.upv.es

45. PAALME Viiu
Tallin University of Technology, Institute of Gene Technology
19086 Tallin. Estonia
viiu.paalme@gmail.com

46. POGGENDORF Iris
Zurich University of Applied Sciences
School of Life Sciences and Facility Management – Biotechnology
CH-8820 Wädenswil, Switzerland
iris.poggendorf@zhaw.ch

47. RIGANO Manuela
University of Naples "Federico II"
Dept. Soil, Plant, Environmental and Animal Production Sciences
80055 Portici, Italy
manuelarigano@hotmail.com

48. RITALA Anneli
VTT Technical Research Centre of Finland
02044 Espoo, Finland
anneli.ritala@vtt.fi

49. SABALZA GALLUES Maite
University of Lleida, Dept. of Crop and Forest Sciences
E25198 Lleida, Spain
msabalza@pvcf.udl.cat

50. SCHIEMANN Joachim
Julius Kuehn Institute, Federal Research Centre for Cultivated Plants,
Institute for Biosafety of Genetically Modified Plants
06484 Quedlinburg, Germany
joachim.schiemann@jki.bund.de

51. SCHILLBERG Stefan
Fraunhofer Institute for Molecular Biology and Applied Ecology,
Dept. of Plant Biotechnology
52074 Aachen, Germany
stefan.schillberg@ime.fraunhofer.de

52. SCHOTS Arjen
Wageningen University, PSG Nematology
6700 ES Wageningen, The Netherlands
Arjen.Schots@wur.nl

53. SCOTTI Nunzia
CNR-IGV, Institute of Plant Genetics
80055 Portici (NA), Italy
nscotti@unina.it

54. SHAALTIEL Yoseph
PROTALIX Biotheraputics
20100 Carmiel, Israel
shaaltiel@protalix.com

55. SILLETTI Silvia
CNR-IGV, Institute of Plant Genetics
80055 Portici (NA), Italy
silvia.silletti@libero.it

56. SIRKO Agnieszka
Polish Academy of Sciences,
Institute of Biochemistry and Biophysics
02-106 Warsaw, Poland
asirko@ibb.waw.pl

57. STEINKELLNER Herta
University of Natural Resources and Life Sciences,
Dept. for Applied Genetics and Cell Biology
1190 Vienna, Austria
herta.steinkellner@boku.ac.at

58. STÖGER Eva
BOKU University Vienna
1190 Vienna, Austria
eva.stoger@boku.ac.at

59. STRID Ake
Örebro University, Örebro Life Science Center
70182 Örebro, Sweden
ake.strid@oru.se

60. VAN DROOGENBROECK Bart
Institute of Agricultural and Fisheries Research, Unit Technology and Food
9820 Merelbeke, Belgium
Bart.VanDroogenbroeck@ilvo.vlaanderen.be

61. VANEK Tomas
Joint Laboratory of Institute of Experimental Botany
and Research Centre of Crop Production
165 02 Prague 6, Czech Republic
vanek@ueb.cas.cz

62. WARZECHA Heribert
Technische Universität Darmstad, Dept. for Biology
64287 Darmstadt, Germany
warzecha@bio.tu-darmstadt.de

63. WESTERHOF Lotte
Wageningen University
6708 PB Wageningen, The Netherlands
lotte.westerhof@wur.nl

64. WILBERS Ruud
Wageningen University
6708 PB Wageningen, The Netherlands
ruud.wilbers@wur.nl

Notes