

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

COST FA0804

Vienna 16-17th February 2012

Welcome to Vienna



University of Natural Resources and Life Sciences



Department of Applied Genetics and Cell Biology

Organizing Committee in Vienna:

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VIENNA

Vienna is Austria's capital and one of Europe's top city destinations. With its history over more than 5,000 years the city is a melting pot of numerous cultural influences. The centre is characterized by the 19th century architecture on Ringstrasse, with its grand buildings, monuments and parks. But also lovers of modern architecture and art will be impressed by the city's contemporary side. Vienna is clean, green and safe: it is the city of parks, bicycle paths, and the Vienna Woods, a forest area surrounding the city and acting as its "green lung". In Vienna, tradition is not only an exhibit in museums but is a pulsing part of every-day life. As the world's music capital Vienna was home for more famous composers than any other city and performances of their works still conquer international audiences.



So if you have the time....

A good starting point is **St. Stephens cathedral** (U1, Stephansplatz; in the city center). From there you can walk up "Kärntnerstraße" (shopping street) to the **Opera house** (they offer tours at least twice a day). Nearby, there's a tourist info point (Albertinaplatz / Maysedergasse, 9am to 7pm) and the **Albertina** (museum of classical modern art). If you continue along the Ring street (Ring Strasse) on the right hand side is the **Burggarten**, a nice park, with a Mozart statue and butterfly house. Next, on the left hand side, there are 2 identical buildings harbouring the **natural history** and the **art history museum**, respectively. If you continue along the Ringstrasse, the Heldenplatz comes up next across from the 2 museums (the building there is called the **Hofburg**, with the museum of ethnology, the Sissi apartment). If you walk on towards the center through the Burgtor, on the right hand side you can enter the treasury museum via the Swiss gate (right hand side). Further on towards the center, you will reach the Michaelerplatz, with roman excavations, and the Michaeler-church, and if you turn right then, there are the stables of the famous white **Lippizaner horses**, and also the national library. If you go back onto the Ringstrasse, next up, on the right hand side there's the **Volksgarten**, and then, on the left hand side, the **Parliament** (tours available). Next on the left hand side is the **city hall** with a large plaza in front, across from there on the right hand side is the **Burgtheater**. If you continue on the Ring, the main **university** is located on the left hand side. If you enjoy museums, there are a few more in the **Museumsquartier** (behind the national art/art history museum; also modern art and classical art) If you like food markets, there's the **Naschmarkt**. You can reach it if you start walking away from the city center from the opera (Operngasse). There you'll also see the **Secession**, which has this golden dome, and inside there are some frescos or Gustav Klimt to see. There are also a few nice art nouveau houses around the Naschmarkt. The **Karlskirche** is another church with a large plaza in front, it's located east of the Secession, also the Vienna city museum is nearby.

If you cross the street towards the city center, you can find the Musikverein, where they play the New Year's concert (also with tours).

Another nice park is the **Stadtpark**, there you can take a picture with the golden statue of Johann Strauss, a famous composer, and look at the Kursalon.

Typical **Viennese coffee house** are Café Sacher (Philharmonikerstraße 4, near Opera), Café Landtmann (Doktor-Karl-Lueger-Ring 4, near City hall/Burgtheater), Café Demel (Kohlmarkt 14, near Michaelertor), or Café Central (behind the Burgtheater, Strauchgasse/Herrengasse).

Sights outside the 1st district:

Schönbrunn castle (best to go there early in the day; small version of Versailles, tour of the rooms where Sissi and Franz Josef lived, with nice park, maze, zoo, which is the oldest in Europe); reach it by taking the U6 to "Längenfeldgasse", then switch to the U4, get out "Schönbrunn".

Hundertwasser house (very colourful apartment building by famous Viennese architect): Kegelgasse 34-38/Löwengasse 41-43, 1030 Wien; get there by taking the U3 to "Landstraße-Wien Mitte", exit "Landstraßer Hauptstraße". Turn left and walk down the "Landstraßer Hauptstraße" till "Seidlgasse", continue this street to the "Kegelgasse", then turn left until you reach the Hundertwasser house.

The **Belvedere** is also a nice museum, plus the building and surrounding park is nice too (there you can see impressionists, and also Viennese artists); to reach by tram 18 (leaves across from the Ibis on the Mariahilfer Gürtel) bound to Schlachthausgasse until the stop "Südbahnhof", then walk down "Prinz Eugen Strasse".

The Viennese amusement park, the Prater, with the giant ferris wheel, from which you have a great view over the city on sunny days, might also be worth a visit (reach by U1, Praterstern).

Cost Meeting Molecular Farming

16-17 February 2012, Vienna

Scientific Programme

Wednesday, 15 February 2012

17:00 **Registration at the IBIS Hotel Vienna (until 18:15)**

18:30 **Departure for dinner**
Meet us in the lobby of the hotel

Thursday, 16 February 2012

8:00 **Registration (until 9:00) and poster session set-up**

9:00 – 9:15 **Welcome and opening of the meeting**

Kirsi-Marja Oksman
Herta Steinkellner

9:15 – 10:25 **Expression of highly complex proteins (Session 1)**

Chair: Kirsi-Marja Oksman

9:15 – 9:45 **Glycosylation of therapeutically used plasma proteins**
Alfred Weber

9:45-10:05 **Recombinant IgMs expressed in mammalian cells**
Renate Kunert

10:05-10:25 **Recombinant IgMs from plants**
Andreas Loos, Clemens Gruber, Frank Hensel, Friedrich Altmann, and
Herta Steinkellner

10:25 – 10:55 **Coffee break and poster session**

Registration desk will be open

10:55 – 12:35 **Topics of general interest (Session 2)**

Chair: Herta Steinkellner

10:55-11:15 **PhD – programme BioToP – Biomolecular Technology of Proteins**
Christian Obinger

11:15-11:35 **Foundation of start up biotech-companies**
Gottfried Himmler

11:35-11:55 **Strategies for the stabilization of Fc fragments**
Gordana Wozniak-Knopp, Johannes Stadlmann, and Florian Rüker

11:55-12:15 **Proteomics: identification of glycoproteins**
Friedrich Altmann

12:15-12:35 **O-glycosylation engineering in plants**
Richard Strasser

12:35 – 14:00 **Lunch**

Registration desk will be open from 13:30-14:00

- 14:00 – 14:50** **Automatization/non invasive imaging (Session 3)**
Chair: Stefan Schillberg
- 14:00 – 14:25 **Monitoring protein concentration via automated, non-invasive image acquisition: an application for Molecular Farming**
Martina Becher, Silvia Braun, Fabio Fiorani, Nicole Raven, Stefan Schillberg, and Ulrich Schurr
- 14:25 – 14:50 **Development of online monitoring and fluorescence imaging systems for plant cell suspensions**
Wolf Klöckner, Clemens Lattermann, Tibor Anderlei, Nicole Raven, Stefan Schillberg, and Jochen Büchs
- 14:50 – 15:50** **Down stream processing, GMP production (Session 4)**
Chair: Stefan Schillberg
- 14:50 – 15:10 **DSP strategies for plant produced antibodies**
Stephan Hellwig, Jürgen Drossard
- 15:10 – 15:30 **Production, purification and characterization of spider silk proteins**
Nicola Weichert, Dominic Knoch, Valeska Hauptmann, Norman Paege, Matthias Menzel, Uwe Spohn, Mario Gils, and Udo Conrad
- 15:30 – 15:50 **BryoTechnology™: Recent developments in moss-based production of pharmaceutical proteins**
Andreas Schaaf
- 15:50 – 16:20** **Coffee break and poster session**
Registration desk will be open
- 16:20 – 17:45** **Protein accumulation and subcellular deposition (Session 5)**
Chair: Udo Conrad
- 16:20 – 16:40 **Expression and purification of recombinant proteins in tobacco BY-2 suspension cells with hydrophobin fusion technology**
Anneli Ritala, Suvi Häkkinen, Marina Petrova, and Jussi Joensuu
- 16:40 – 17:00 **Molecular farming of selected viral antigens for vaccination in *Arabidopsis* seeds**
Annelies De Paepe, Robin Piron, Els Van Lerberge, Jonah Nolf, and Ann Depicker
- 17:00 – 17:20 **Immunoglobulin A production in edible plant organs: bridging the gap between Molecular Farming and Plant Synthetic Biology**
Paloma Juárez, Alejandro Sarrión-Perdigones, Silvia Presa, Asun Fernández-del-Carmen, Antonio Granell, and Diego Orzaez
- 17:20 – 17:45 **The deposition of recombinant proteins in storage bodies**
Elsa Arcalís, Verena Ibl, Thomas Rademacher, Francesca Morandini, Linda Avesani, Mario Pezzotti, and Eva Stöger
- 18.30** **Departure for dinner**
Meet us in the lobby of the hotel

Friday, 17 February 2012

- 8:30** **Registration (until 9:00)**
- 9:00 – 10:45** **Product degradation/quality (Session 6)**
Chair: Dirk Bosch
- 9:00 – 9:25 **Degradation of recombinant proteins by plant cysteine proteinases**
Melanie Niemer, Ulrich Mehofer, Maria Verdianz, Andreas Schaller, Renier van der Hoorn, and Lukas Mach
- 9:25 – 9:45 **Novel strategies to reduce recombinant protein degradation in plant suspension lines**
Stefan Schillberg, Manoj K. Mandal, Janina Kirchhoff, Nicole Raven, and Andreas Schiermeyer
- 9:45 – 10:05 **Characterisation of the proteolytic degradation of a human IgG₁ in plant**
Raffaele Lombardi, Verena Hehle, Maria Elena Villani, Mariasole Di Carli, Matthew Paul, Julian K-C. Ma, Eugenio Benvenuto, and Marcello Donini
- 10:05 – 10:25 **Antibody production in culture cells**
Bertrand Magy, Jérémie Tollet, Catherine Navarre, and Marc Boutry
- 10:25 – 10:45 **Therapeutic proteins from mushrooms**
Elsa Berends, Karin Scholtmeijer, Han Wösten, Luis Lugones, and Dirk Bosch
- 10:45 – 11:15** **Coffee break and poster session**
Registration desk will be open
- 11:15 – 12:15** **Topics of general interest (Session7)**
Chair: Ann Depicker
- 11:15 – 11:45 **Use of SPR for quantification, characterization and quality control of recombinant antibodies and vaccine antigens produced in plant based expression systems**
Holger Spiegel and Markus Sack
- 11:45 – 12:05 **An universal expression vectors in plants: a seed delivery system**
Ofer Gover, Rita Mozes-Koch, Ilan Sela, Edna Tanne, and Haim D. Rabinowitch
- 12:05-12:25 **Presentation of the Molecular Farming Database**
Dirk Bosch
- 12:25 – 13:00** **General discussion and closing remarks**
Chair: Kirsi-Marja Oksman
- 13:00 – 14:30** **Lunch**

Abstracts

Glycosylation of therapeutically used plasma proteins

Alfred Weber

Baxter Innovations GmbH

Most therapeutically used proteins display post-translational modifications which contribute to structural features and/or the functional role of the affected protein. Such common post-translational modifications include γ -carboxylation, β -hydroxylation, amidation, sulfatation, formation of disulfide linkages, phosphorylation and glycosylation. More than a hundred different post-translational modifications have been described to date but glycosylation is probably the most common form. Most therapeutically used proteins are glycoproteins and have their carbohydrates attached via N- or O-glycosidic linkages. Carbohydrate moieties of glycoproteins have been associated with their correct folding, transport and intracellular trafficking, are important for recognition and binding to receptors and ligands, maintaining their biological activity and stability, and, last but not least, have been shown to be key regulators of their serum half-life. Although only six major monosaccharides comprise the common building blocks of these glycans (N-acetylglucosamine, fucose, mannose, galactose, N-acetylneuraminic acid and N-acetylgalactosamine), their huge number of possible combinations can create numerous glycoisoforms of a given glycoprotein. Human IgG, carrying a conserved N-glycan on its Fc part, serves as an example for this heterogeneity, which is introduced by a single glycosylation site only. In addition, this single glycan, and even single monosaccharides on the glycan, have been shown to be essential for important biological activities of IgG. Consequently, glyco-engineered variants of monoclonal antibodies are under development. On the other hand, the effects of non-human glycosylation features on monoclonal antibodies such as α -galactose or the non-human sialic acid N-glycolylneuraminic acid are under discussion. Further glycoproteins currently used or in development which will be presented are α_1 -antitrypsin, factor VIII, von Willebrand factor and butyrylcholinesterase, all of which carry more than one N-glycan.

Recombinant IgMs expressed in mammalian cells

Renate Kunert

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IgM antibodies are naturally occurring as the first defense against invading agents in the human body. They interact predominantly with phylogenetically conserved structures like nucleic acids, phospholipids and carbohydrates leading to agglutination and phagocytosis. Besides they activate the complement system highly effective and support the immune system by presentation of pathogen derived antigens on dendritic cells. Especially the specificity against the glycosylation pattern on cellular surfaces and the effective activation of the complement system render them a valuable potential in clinical application. Therefore productions processes of IgMs are under development but most of the evaluations have been done with hybridoma derived material or polyclonal antisera.

We have expressed human IgM in recombinant CHO cells and purified as well as characterized the resulting antibody fractions. In addition to pentameric and hexameric structures we also detected IgM fragments which can be assigned to IgM molecules of lower oligomerization. This observation was confirmed by different cultivation conditions for the recombinant CHO clones. Even carefully chosen storage conditions could not improve the formation of pentameric structures of some antibodies. Using a rational antibody engineering approach we were able to slightly improve the formation of pentameric structures. However, the reason for different oligomerization remains elusive. Therefore we investigated different analytical conditions to shed light on structural differences between different IgM molecules. We developed purification methods to define optimal conditions for single IgM molecules and analyzed the purified IgMs by i) circular dichroism which elucidates secondary and tertiary structures of proteins ii) microcalorimetry, which gives information on stability of the molecule and iii) spectroscopic analysis to get insights about the microconformation around specific amino acids.

Generation of different IgM glycoforms in plants

Andreas Loos¹, Clemens Gruber², Frank Hensel³, Friedrich Altmann² and Herta Steinkellner¹

¹*Institute of Applied Genetics and Cell Biology, ²Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria; ³Patrys GmbH, Würzburg, Germany*

While the importance of proper glycosylation for IgG antibodies has been unequivocally demonstrated, the impact of this important posttranslational modification is largely unknown for IgM antibodies. This is amazing since these therapeutically interesting molecules are heavily glycosylated, including sialylation, the most complex type of human glycosylation. We set out to evaluate a plant-based expression platform for the generation of different IgM glycoforms. To this end, we transiently expressed heavy chain, light chain and joining chain of Sam-6, an IgM potentially interesting for anti-tumor therapies (Pohle et al., 2004), in *Nicotiana benthamiana*. In human serum, IgMs are mainly present as pentamers consisting of 10 heavy chains, 10 light chains and 1 joining chain interconnected by disulfide bonds. The banding pattern of plant-produced Sam-6 indicates correct assembly into pentamers and binding studies revealed identical antigen-binding as mammalian cell-derived Sam-6.

The heavy chain of IgMs contains 5 potential N-glycosylation sites. Plant-produced Sam-6 was subjected to N-glycosylation analyses by mass spectrometry. Interestingly, three sites carry complex-type N-glycans, while two sites carry exclusively oligo-mannosidic structures. This mirrors the situation of human serum IgM (Arnold et al., 2005). In addition, we were able to efficiently elongate complex N-glycans with β 1,4-galactose, the required acceptor substrate for sialylation. Currently, we are in the process of generating sialylated Sam-6 in plants by co-expression of the mammalian sialylation pathway, as reported previously (Castilho et al., 2010). These studies demonstrate the capability of plants to produce and assemble even complex polymeric molecules and to equip them with human-like N-glycans. The generation of different IgM glycoforms will expand our ability to conduct structure-function investigations and thus contribute to the generation of therapeutic proteins with optimal efficacy.

PhD - programmeBioToP – Biomolecular Technology of Proteins

Christian Obinger

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Recombinant production of native and engineered proteins by biotechnological and biopharmaceutical means is a complex process that requires an inter- and multidisciplinary educational training in fields ranging from basic biochemistry and cell biology to production systems based on different organisms of varying complexity, bioinformatics and downstream processing. The doctoral programmeBioToP (Biomolecular Technology of Proteins) integrates basic and applied research in the field of modern recombinant protein science and production (from gene to product).

BioToP is a joint programme of the BOKU Departments of Applied Genetics and Cell Biology, Biotechnology, Chemistry, Food Sciences and Technology, Material Sciences and Process Engineering and Nanobiotechnology, which together constitute the VIBT (Vienna Institute of Biotechnology) of BOKU. In detail, BioToP provides comprehensive and thorough up-to-date research training in the four research and education areas of (i) analysis, design and engineering of proteins, (ii) biosynthesis, posttranslational modifications and trafficking of (recombinant) proteins, (iii) expression systems and cell factories, and (iv) bioinformatics and molecular modelling. The already existing unique cluster of basic and applied research represented by scientists in the field of biotechnology at BOKU-VIBT provides an excellent infrastructure, the critical mass and a stimulating training environment to facilitate interdisciplinary and translational research. Among others the specific curriculum includes basic courses and seminars as well as instructional courses in the four research areas as well as annual retreats and support of attend of international meetings as well as of research stays abroad.

The educational programmestarted in October 2010 and at the moment 30 qualified graduate students from all over the worldperform their theses in one of 14 research teams with excellent scientific competence and infrastructure at VIBT. The PhD-thesis projects are financed by the Austrian Science Foundation (14), BOKU (7) as well as other funding organisations. The entire duration of the FWF-program is 12 years and, BioToP, finally, should be institutionalized at BOKU – VIBT.

Strategies for the stabilization of Fc fragments

Gordana Wozniak-Knopp¹, Johannes Stadlmann² and Florian R ker¹

¹*Department of Biotechnology, Christian Doppler Laboratory for Antibody Engineering, ²Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Muthgasse 18, 1190 Vienna, Austria*

Monoclonal antibodies (mAbs) are the most successful biologically produced therapeutics today. Fcab, the antigen binding IgG1-Fc fragment has recently been established as an alternative small-size antibody format [1]. The design and introduction of additional intra-domain disulfide bonds as well as in silico-guided mutagenesis of the Fc fragment was applied with the aim to generate a more robust scaffold for further loop engineering applications. Mutations were introduced by site-directed mutagenesis and expressed in *Pichia pastoris*, purified and investigated for biophysical properties. Biophysical analysis included differential scanning calorimetry (DSC), circular dichroism spectrometry (CD) and the verification of the presence of wild-type like properties, e.g. binding to Protein A, CD16a, and FcRn. A disulfide bond in the CH3 domain which connects the N-terminus with the G-strand increased the T_m of the CH3 domain by 10°C, and another disulfide bond which connects the BC-loop with the F-sheet, increased the T_m of the CH3 domain by 5°C. Combination of both disulfide bonds increased the T_m of the CH3 domain by 15°C. The introduction of single point mutations improved the thermal stability of the Fc fragment by up to 4.5 °C. The combination of stabilizing mutations showed an additive effect on thermal stability. A shift in the T_m values of the CH2 domain by approx. 9 °C and for the CH3 domain by approx. 6 °C could be gained. The manipulations had no significant effects neither on the structure of the Fc fragment or on the binding to generic ligands.

[1] G. Wozniak-Knopp, S. Bartl, A. Bauer, M. Mostageer, M. Woissetschl ger, B. Antes, K. Ettl, M. Kainer, G. Weberhofer, S. Wiederikum, G. Himmler, G.C. Mudde, and F. R ker, Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. *Protein Eng Des Sel.* 2010 Apr;23(4):289-97

[2] G. Wozniak-Knopp, J. Stadlmann and F. R ker, Stabilisation of the Fc fragment of human IgG1 by engineered intradomain disulfide bonds. *PLoS ONE* 2011, in press

Proteomics: identification of glycoproteins

Friedrich Altmann

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1190 Vienna, Austria*

94 % of all glycoproteins prefer to be analyzed by liquid chromatography-mass spectrometry (LC-MS). The reason for this strong preference lies in the enormous analytical power of this admittedly demanding and expensive methodology. Mass spectrometric glycoprotein analysis can be performed in three ways:

(1) Entire glycoproteins: This option works well in many cases and has become industry standard for smaller and not too complex glycoproteins such as Interferons. However, even complete IgG antibodies are analyzed this way. Where ESI-MS fails, MALDI-MS may give results, albeit with drastically lower resolution.

(2) Glycopeptides: This is the standard approach that - with proper choice of the digesting protease(s) - can give site-specific glycosylation information for all glycosylation sites including potential O-glycosylation sites. Also, the degree of non-glycosylation at each site is determined and other modifications of the protein chain may become obvious, e.g. hydroxylation of proline or unexpected clipping of the N- or C-terminus. As most sugar units that comprise N- or O-glycans have different masses, the mass increment of a glycopeptide informs about the glycan composition. Roughly quantitative glycan profiles for each site are thus obtained. Isomeric structures can, however, not be resolved. A unique advantage of this method is the clear link between a particular peptide (mass) and its glycans. Thus, samples do not have to be very pure.

(3) Free oligosaccharides: These can be subjected to chromatographic methods that separate according to the structure of glycans. As an example, glycans with either a core-6-linked, a core-3-linked or a Lewis-type fucose will elute at different positions. One should not forget that free glycans no longer have an affiliation to a particular glycoprotein. Therefore, a glycoprotein analyzed this way should be very pure. A simple and fast alternative to LC-MS of free glycans is MALDI-TOF MS, which is particularly well suited for the neutral glycans found on plant glycoproteins.

O-glycosylation engineering in plants

Richard Strasser

*Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences,
Vienna*

Control of protein glycosylation is a critical step to enhance the efficacy of therapeutics and eliminate unwanted side effects resulting from non-authentic glycosylation. There are two main types of protein glycosylation - N-glycosylation and O-glycosylation - which differ in their linkage to the protein backbone. Recent developments in modification of the N-glycosylation pathway have shown that *Nicotiana benthamiana* plants are a versatile expression system for the generation of therapeutic proteins with a defined human-type N-glycosylation. O-glycosylation of serine and threonine residues is quite different from N-glycosylation as a typical consensus amino acid sequence for O-glycosylation has not been clearly identified yet and the biosynthesis is performed in a stepwise fashion involving various Golgi-located glycosyltransferases. Here, I will give an overview of different O-glycosylation engineering approaches in plants and present our strategy for the production of glycoproteins with tailored O-glycan structures in *N. benthamiana* leaves. Plants lack the machinery to produce mammalian-type O-glycosylation and recombinant human erythropoietin, which contains three N- and a single O-glycosylation site does not contain any O-linked glycans when expressed in *N. benthamiana*. Consequently, the mammalian O-glycosylation machinery has to be expressed in plants to achieve O-glycan formation on recombinant proteins. I will present our first data on engineering of tailored O-linked sugar residues on plant-produced human erythropoietin.

Monitoring protein concentration via automated, non- invasive image acquisition: an application for Molecular Farming

Martina Becher¹, Silvia Braun¹, Fabio Fiorani¹, Nicole Raven², Stefan Schillberg², Ulrich Schurr¹

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Plant phenotyping is an emerging technology addressing the need of non-invasively analysing plant structures and functions for both basic and applied research purposes. It includes laboratory or greenhouse screening approaches as well as remote sensing in the field. The Jülich Plant Phenotyping Center exploits a range of methodologies and imaging systems to estimate plant shoot and root biomass, photosynthetic performance, pigment composition, and water content.

After a brief overview on the optical methods applied to monitor in particular shoot properties of plants cultivated under controlled environmental conditions we will focus on the development of a screening system suitable for Molecular Farming approaches. Molecular Farming is the production of pharmaceutical and industrial proteins in plant cells or plants that are preferably cultivated in contained systems under standardized environmental conditions.

In our Molecular Farming project (CoMoFarm) we focus on the cultivation of intact tobacco plants which were cultivated in a hydroponic system under controlled conditions either in a greenhouse or a growth chamber. In particular, we developed a non-invasive monitoring system for intact tobacco plants based on recombinant protein fluorescence. The plants expressed a fluorescent marker protein and its production correlates to a great extent with the accumulation of a second recombinantly expressed target protein, i.e. a human IgG antibody. We successfully show that the determined marker protein fluorescence distribution at different time points during plant growth is a valid measure for the non-invasive monitoring of the target protein accumulation.

Development of online monitoring and fluorescence imaging systems for plant cell suspensions



Wolf Klöckner¹, Clemens Lattermann¹, Tibor Anderlei², Nicole Raven³,
Stefan Schillberg³, Jochen Büchs¹

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The aim of the CoMoFarm project is to develop plant based systems for the production of high value recombinant proteins in containment (www.comofarm.org). The project includes a comparison and optimisation of four different production platforms – plants grown under hydroponic conditions, hairy roots, plant cell suspensions grown in shaken bioreactors and moss grown in tubular illuminated bioreactors. Besides the evaluation of different production systems, important aspects for enhanced productivity and homogeneity, as the optimization of media and culture conditions and the applicability of novel reactor concepts are part of the project.

Optimized culture conditions for plants or plant cell suspensions require precise control- and monitoring systems to react according to altering requirements during the production cycle. The development of innovative elements for the monitoring of culture conditions is a further part of the CoMoFarm project. Cylindrical orbital shaken disposable bioreactors are surface aerated and, therefore, combine two advantages for the cultivation of plant cell suspensions: The flexibility and cost efficiency of disposable bioreactors and the lower contamination risk and hydromechanical stress of surface aerated reactors, as they do not require any invasive rotating parts.

A measuring device to investigate the oxygen transfer rate (OTR) in disposable orbitally shaken bioreactors with a nominal volume of 10, 20 and 50 litre was newly developed. Moreover, a commercial available monitoring system for the dissolved oxygen tension (DOT) was applied and tested with disposable shaken bioreactors. Ultimately, fluorescence imaging systems are used to monitor the formation of fluorescent proteins, vitamins and co-factors during the cultivation process.

DSP strategies for plant produced antibodies

Stephan Hellwig, Jürgen Drossard

Fraunhofer IME, Germany

Fraunhofer IME and the closely linked Institute of Molecular Biotechnology of the Aachen University are looking back on a track record of almost 20 years in using plants to produce monoclonal antibodies. Recent achievements include the development of a large scale (batch size 200+ kg leaf material) GMP-compliant process for the production of mAbs from transgenic, greenhouse-grown *Nicotiana tabacum* cv BY-2. The development comprised the generation and characterization of the Master Seed Bank, the cultivation and extraction process and the purification strategy of the antibody, including all regulatory affairs with respect to the manufacturing process that were necessary to obtain a permission to use the antibody in a clinical phase I trial by the MHRA.

The talk will highlight downstream processing issues in the technical runs preceding the GMP production, the GMP campaign itself and a second campaign using a different antibody with different expression properties. Technical, economic and regulatory aspects of using plants to produce biopharmaceuticals will be discussed.

Production, purification and characterization of native-sized spider silk proteins in plants

Nicola Weichert¹, Dominic Knoch¹, Valeska Hauptmann¹, Norman Paege¹, Matthias Menzel²,
Uwe Spohn², Mario Gils¹, Udo Conrad¹

¹ *IPK Gatersleben*

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During evolution spiders developed a battery of different protein-based silk materials. These silk fibers exhibit exorbitant mechanical properties in terms of toughness, hardness and elasticity. The size of spider silk proteins is assumed to be a key factor for the mechanical properties of fibers, because all spiders investigated so far produce high molecular weight spider silk proteins of at least 250 kDa to several 100 kDa. Further arguments are a higher number of motifs thus allowing more inter- and intra-chain interactions and fewer chain end defects to improve mechanical properties. The production of native-sized spider silk proteins is therefore a prerequisite for exploiting their potential for bio-based material development. The spider silk protein FLAG has been expressed in tobacco leaves flanked by Intein-C and Intein-N leading to multimerization by trans-splicing. Here we describe the optimization of plant-based production including downstream processing (purification; enrichment of large multimers; desalting) and characterization (solubility, SEM studies of microfibers).

BryoTechnology™: Recent developments in moss-based production of pharmaceutical proteins

Andreas Schaaf

Greenovation Biotech GmbH, Freiburg, Germany

Physcomitrella-based BryoTechnology is a cGMP-compliant, eukaryotic production system for demanding pharmaceutical proteins. Having all prerequisites for sustainable and economically viable API (Active Pharmaceutical Ingredients)-production in place, the system leverages the mosses unique characteristics for positioning itself in the enabling niche of toxic, glyco-designed, difficult to fold and otherwise demanding proteins.

BryoTechnology includes both, a transient production system (BryoSpeed™) as well as stable cell line development (BryoMaster™). BryoSpeed allows for quick access of feasibility amounts of high quality product. BryoMaster assures the sustainable, cGMP-compliant production of APIs by a cell-bank based and highly defined process.

Since 2009, greenovation focused on systematic process improvement, scale-up and standardisation. As a result, we could not only increase culture densities and product titers tenfold, but successfully expressed demanding proteins such as an LSD-enzyme and a mAb / toxin-fusion. For scale-up, we focused on disposable technology and were able to transfer the process into wave-bioreactors with a max. volume of 500L.

On the basis of these achievements, greenovation actually works on the consolidation of its GMP-compliant production process and the preclinical validation of several promising product candidates.

By the end of 2012, one of these candidates will be registered for clinical testing.

Expression and purification of recombinant proteins in tobacco BY-2 suspension cells with hydrophobin fusion technology

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Many foreign proteins have been expressed successfully in plant suspension cells, including antibodies, enzymes, cytokines and hormones. Tobacco has been the most popular source of suspension cells for molecular farming since these are easy to transform and handle. Tobacco BY-2 (*Nicotiana tabacum* cv. "Bright Yellow-2") suspension cells are rapidly multiplying line suitable for cultivation in bioreactors.

We have shown previously that transient overexpression of hydrophobin fusions has a capacity to induce high accumulation of target protein in plant leaves by formation of protein body-like, ER-membrane-bound organelles. Here, we report introduction of this technology to stably transformed tobacco BY-2 suspension cell lines. The presence of protein bodies in BY-2 cells was confirmed by fluorescence and light microscopy. Optimal parameters for protein expression and purification were determined and the cultivation system was scaled up from shake flasks to a 50 litre disposable plastic bag bioreactor. The transgenic lines were successfully stored via cryopreservation.

Molecular farming of selected viral antigens for vaccination in Arabidopsis seeds

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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 genes *arceline 5-I* and β -phaseolin 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 (De Jaeger et al., 2002). Also high accumulation levels of more complex scFv-Fc antibodies, corresponding to 2% of seed weight, were achieved using the same expression cassette in Arabidopsis seeds (Van Droogenbroeck et al., 2007; Loos et al., 2011).

Working towards the production of safe and effective vaccines for a number of animal pathogens, our research occupies seed specific molecular farming to generate a set of antigenic proteins and variants thereof. To this goal, selected viral protein formats from the Porcine Circovirus type 2 (PCV2) and the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) were expressed in Arabidopsis thaliana seeds. Transformants were screened and the proteins were analyzed by SDS-page analysis, Western blotting and ELISA. We have also performed purification of these antigens from the Arabidopsis seeds. The obtained results, insights and further perspectives will be discussed.

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Immunoglobulin A production in edible plant organs: bridging the gap between Molecular Farming and Plant Synthetic Biology.

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The potential of plants as production platforms for recombinant medicinal compounds is unquestionable; however, as happened earlier with other production systems, making plant platforms truly competitive will likely require complex genetic engineering.

For instance, in the case of edible plant organs as fruit, the benefits of a reduction in purification costs are counterbalanced by the risk of mixing recombinant products with regular food. A way to cope with this problem is to label the recombinant platform with distinctive features that preserve its identity. We propose the use of natural coloured plant compounds for labelling transgenic crops by means of metabolic engineering. A relatively small amount of otherwise health-promoting compounds such as anthocyanins, a natural component in many edible berries, is sufficient to label antibody-producing plants. These compounds confer an intense purple colour ensuring the traceability of transgenic fruits and their derivatives. To proof this concept, we combined in a single tomato plant four transgenes encoding the transcription factors Roseal and Delila from *Antirrhinum majus* and the heavy and light chains of a human immunoglobulin A against rotavirus. This combination resulted in transgenic purple tomatoes producing high levels of a neutralizing human antibody against the diarrhea agent rotavirus.

As highlighted in this example, the optimization of platforms and products in Molecular Farming uses increasingly complex genetic engineering approaches, involving the interplay of multiple transgenes and therefore entering the field of Synthetic Biology. However multigene engineering in plants is still a difficult “puzzle” due to the lack of shared DNA assembly methods that facilitate the building of new genetic modules/pathways from basic DNA parts in a standardized way.

To facilitate multigene engineering in plants, we have developed GoldenBraid (GB), a standardized assembly system based on type IIS restriction enzymes that allows the indefinite growth of reusable gene modules made of standardized DNA pieces. The GB system consists of a set of four destination plasmids (pDGBs) designed to incorporate multipartite assemblies made of standard DNA parts and to combine them binarily to build increasingly complex multigene constructs. We propose the use of GoldenBraid as an assembly standard for Plant Synthetic Biology. For this purpose we have GB-adapted a set of binary plasmids for *A. tumefaciens*-mediated plant transformation. Fast GB-engineering of several multigene T-DNAs, including two alternative modules made of five reusable devices each, and comprising a total of 19 basic parts has been achieved using GB. We are currently using GB-engineering to transiently express different versions of a secretory IgA as a way to optimize the production of this complex in plants.

The deposition of recombinant proteins in storage bodies

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The use of plants as bioreactors for many applications in food technology and pharmaceutical production requires the stable accumulation of a recombinant protein within the plant. To achieve this goal the subcellular destination of the recombinant protein needs to be carefully considered.

Cells of plant seeds have the natural ability to store large amounts of protein in a protected subcellular environment. The prevalent storage organelles of plant seeds are starch grains, oil bodies, protein storage vacuoles and protein bodies, which differ between species causing obvious differences in cell morphology. This is relevant for molecular farming as seeds in different crops offer several alternative subcellular destinations for the deposition of recombinant proteins that can reduce proteolytic degradation, increase the efficacy of oral delivery or facilitate recombinant protein purification.

Protein transport within storage cells may be complicated by the abundance of functionally distinct types of protein storage organelles. For example, the specialized architecture of cereal endosperm cells has been shown to influence the intracellular route of recombinant proteins. Our studies of recombinant glycoproteins in different tissues and plant species have indicated that the subcellular fate and post-translational modification of a protein is dependent on its intrinsic properties and state of assembly, but also on tissue and species-specific factors.

While native storage organelles such as protein bodies in cereal seeds offer a protective effect both in planta and after harvest, ER-derived protein storage organelles can also be induced ectopically in vegetative tissues by fusing the recombinant protein to assembler sequences, which are frequently derived from cereal prolamins or hydrophobic proteins. Fluorescent protein bodies have been obtained by fusion to a marker protein and were localized in a species- and tissue-dependent manner.

Occasionally, recombinant proteins are deposited in ER-derived bodies, although they are not hydrophobic in nature and do not contain an assembler peptide. In Arabidopsis seeds we have identified an insoluble fraction of recombinant murine interleukin-10 and localized this fraction within ER-derived protein accretions that appear very similar to Russell bodies, which occur in connection with human ER storage diseases. We speculate that plant cells may also be able to form Russell bodies as a self-protection mechanism, mirroring the situation in mammalian plasma cells where transport-incompetent molecules are stored away without blocking the normal secretory pathway.

Degradation of recombinant proteins by plant cysteine proteinases

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In recent years, plants are increasingly used as production platforms for heterologous proteins. However, plant-based expression systems frequently suffer from unwanted proteolysis. In particular, the expression of monoclonal antibodies in *Nicotiana benthamiana* can lead to the accumulation of significant amounts of degradation products. In vitro processing of antibodies with various types of proteinases suggests that their fragmentation in plants could involve cysteine proteinases, as also indicated by the stabilizing effects of co-infiltrated cysteine proteinase inhibitors in planta. For a better characterization of the *N. benthamiana* representatives of this protease subfamily, the three papain-like cysteine proteinases C14, cathepsin B and aleurain-like protease (ALP) were expressed as hexahistidine-tagged proenzymes in insect cells. After purification by nickel-chelate affinity chromatography and acid-induced autoactivation, the catalytic activity of all three recombinant enzymes was confirmed by active-site labelling with DCG-04 and assays with fluorogenic peptide substrates. However, only C14 and cathepsin B were capable of degrading antibodies to a significant extent. Similar results were obtained with an erythropoietin-Fc fusion protein as substrate. Given the far higher proteolytic activity of C14 and cathepsin B as compared to ALP, it seems that the former two enzymes are more likely to contribute to recombinant protein fragmentation in *N. benthamiana*.

Novel strategies to reduce recombinant protein degradation in plant suspension lines

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Similar to animal cells, plant suspension cells have the capacity to produce large amounts of recombinant proteins. However, the implementation of sustainable plant cell-based production processes is hampered by various factors such as the comparable big size of plant cells, making it difficult to establish high-density fermentations, the inhomogeneity in recombinant protein production, partly caused by the lack of robust cryopreservation protocols, and the significant loss of recombinant proteins in the culture medium. While selection of single highly productive cells and their establishment to monoclonal cell lines has resulted in improved homogeneity and recombinant protein yields, the obstacle of protein degradation in the culture medium has been mainly addressed by addition of protective agents to the medium or by overexpression of protective proteins. Obviously those compounds stabilize target proteins or serve as substrates for plant-derived proteases. We have identified and characterized representative enzymes from the four protease classes aspartate proteases, cysteine proteases, metalloproteases and serine proteases and have demonstrated that for example the NtMMP1 metalloprotease degrades recombinant proteins in the culture medium of tobacco BY2 suspension cells. Moreover, we have generated transgenic BY2 cells in which representatives of the four protease classes have been silenced to reduce the proteolytic activity in plant suspension cell cultures. The transgenic BY2 cells show a normal growth performance but proteolytic activity in the culture medium is significantly reduced. Importantly, overexpression of a human full-length antibody in the silenced lines led to improved stability of the antibody heavy chain and 4-fold increased antibody yields in the culture medium.

The combination of the improved features immanent in the new plant cell lines with novel selection procedures for cell line improvement as well as innovative strategies for up-scaled cultivation of plant cells will boost the competitiveness and sustainability of this protein production platform. In this context, the talk will also present recent results on the cultivation of plant suspension cultures in disposable bioreactor systems.

Characterisation of the proteolytic degradation of a human IgG1 in plant.

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Results obtained in the last 20 years demonstrated that plants are suitable hosts for the production of monoclonal antibodies (mAbs). However, proteolytic degradation of antibodies produced both in stable transgenics and using transient expression systems is still a major issue for the efficient high-yield recombinant protein accumulation. In fact, the integrity of plant-purified IgGs can be dramatically affected by unintended proteolysis, leading in some cases to a 90% yield loss of intact immunoglobulin. We previously described the efficient expression of the tumour-targeting mAb H10 in *N. benthamiana* plants by vacuum-agroinfiltration and the high purification yields (40mg/Kg FW) using a two-step purification procedure. Analysis of the final product revealed that the antibody was highly pure, free from endotoxins, plant contaminants (phenolic and alkaloid compounds) but sensitive to plant proteolytic activity.

In this study, we characterised the degradation profile of the tumour targeting H10 antibody in the attempt to identify the specific proteolytic cleavage sites of the plant produced immunoglobulin. Analysis of degradation fragments obtained from 1DE and 2DE separation of the protein A purified IgG H10, allowed the identification of antibody fragments deriving from plant proteolysis. Furthermore, N-terminal sequencing of degradation bands led to the identification of the specific cleavage sites of the immunoglobulin. Identified sequences were analyzed using the MEROPS database in order to pinpoint specific proteolytic enzymes of *N. benthamiana* responsible of the cleavage. This analysis allowed us to identify three Cystein proteases that specifically cleave the identified aminoacid sequences. All cleavage sites were localized in three inter-domain regions of the antibody (VH-CH1, CH1-CH2, CH2-CH3) that resulted exposed to the solvent, therefore, easily accessible to plant proteolytic enzymes. The identification of these cleavage sequences is a first step towards the development of new strategies to reduce antibody degradation in plants and increase the yield of intact IgG molecules.

Antibody production in culture cells depends on the isotype, the host species and the culture conditions

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Antibodies and other heterologous proteins that are expressed in plants or plant culture cells are prone to degradation by proteases. We wondered whether different antibody isotypes from different species might display different sensitivity to plant proteases.

This was first checked by incubating commercial antibodies from human (IgG1, 2, 3, 4), rat (IgG 1, 2a, 2b) or mouse (IgG1, 2a, 2b) with the culture medium recovered from a *Nicotiana tabacum* BY2 culture. Antibody degradation was observed to different extends according to the isotype and the species.

To confirm these data for antibodies synthesized by plant cells, the three most protease-resistant antibodies (human Ig2, mouse IgG2a, rat IgG2a) and the most protease-sensitive one (human IgG4) were provided with the same variable region and expressed in *N. tabacum* BY2 cells. Similar differences as those observed in vitro were obtained. For instance, the mean expression for 40 transgenic lines was 8.1 µg/ml for human IgG2 and 1.4 µg/ml for human IgG4.

To determine whether the IgG expression would be similar in another host species, *Arabidopsis Col* cells were transformed with the mouse IgG2a construct. The expression was 40% higher than that obtained with *N. tabacum*. Interestingly, at the stationary growth phase, the IgG2a was fully degraded in the *N. tabacum* culture while this was not the case in the *Arabidopsis* culture. This is in agreement with the lower protease activity observed in the *Arabidopsis* culture medium.

Finally, we compared the production of the mouse IgG2a in different culture conditions: 4-ml in a culture plate, 50-ml in an Erlenmeyer and 4-L in a bioreactor. The IgG expression in the culture medium was similar in the 4-ml and 4-L cultures but much reduced in the 50-ml culture. In the latter case, only IgG degradation products were observed.

In conclusion, the yield of antibody secreted from culture cells depends on the source species, the IgG isotype, the host species and the culture conditions.

Therapeutic proteins from mushrooms

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We have run a four-year feasibility project on development a mushroom-based production platform for human therapeutic glycoproteins. The project has yielded understanding of the mushroom's N-glycosylation machinery and the first steps towards a humanized N-glycosylation profile have been successfully achieved. We could express relevant human proteins at mg/L-levels. Some proteins were obtained as full-length products. Others were however subject to degradation by proteases. Identification of the responsible proteases and development of strategies to prevent proteolytic degradation of target proteins are ongoing. At the meeting we will present the results of our project and we would like to discuss on future plans for this platform.

Use of SPR for quantification, characterization and quality control of recombinant antibodies and vaccine antigens produced in plant based expression systems

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One of the pivotal aims of molecular farming is the production of highly valuable proteins for pharmaceutical applications in different plant expression systems. Besides the well known advantages regarding process robustness and scalability most plant expression systems come with certain disadvantages when compared to mammalian hosts like CHO, representing the most important platform for the production of therapeutic antibodies and other pharmaceutical molecules. Downstream processing of the plant material and delivery of a homogeneous, functional product that meets the requirements for pharmaceutical use or at least preliminary clinical trials can be quite a challenge and the character and the number of purification steps will certainly affect the product quality. Another well known "issue" of plant systems is the difference in the N-linked glycosylation of proteins that can affect their structure, stability, serum half-life and functionality. Since the functionality of pharmaceutical proteins is usually based on very specific interactions with receptors or ligands changes in the structure of the molecules, induced by non-native glycosylation or process conditions may result in reduced or increased affinity to the respective ligand and may influence the efficacy of the molecule. SPR allows for the characterization of molecular interactions in real-time and can be used to precisely determine total and active concentrations of pharmaceutical proteins, as well as kinetic constants of interactions, and is an excellent tool for quantification, characterization and quality control of recombinant antibodies and vaccine antigens produced in plant based expression systems, as illustrated in this presentation by different examples from molecular farming approaches.

A UNIVERSAL EXPRESSION VECTORS IN PLANTS: A SEED DELIVERY SYSTEM

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IL60 is a universal molecular platform, facilitating either expression or silencing in plants. Based on experiments with 32 plant species belonging to 12 families, including woody trees and herbaceous monocots and dicots, we are confident that this family of vectors actually delivers hereditary materials into the cells of the treated tissue(s), propagates in vivo, moves from one cell to another, and expresses the genetic information in the treated plant(s). The vector and the genetic loads persist in the plants' tissues throughout their life span without integration into the host genome and are not passed on to the offspring.

Various components of the IL60 system can work in either cis or trans thus enabling inducible silencing or expression. The system can accommodate large inserts of about 10 Kbp including an entire metabolic pathway.

Recently we have developed methods for the highly efficient delivery of IL60 vectors together with the hereditary components of interest into seeds. We have shown that the foreign DNA is actually present all over the embryo tissues, spreads, replicates and moves throughout the young seedlings and expresses for months in the entire plant. We conclude that the IL60 platform can be used for the introduction of desired traits (even of an entire metabolic system) into plants in a few weeks' time. The extent of expression compared to other known systems is under study.

Potentially, the application of this system may facilitate the production of proteins and secondary metabolite of interest in open fields, circumventing the need to bio-farm under restricted, close conditions.

The science behind the IL60 system and its potential applicative value will be discussed.

Poster Session

Optimization of the production of a human enzyme from transgenic tobacco plants grown in greenhouse.

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Recombinant alpha-mannosidase was recently expressed in stably transformed tobacco plants by *Agrobacterium*-mediated transformation (De Marchis et al., *Plant Biotechnol J.* 2011, 9:1061). The aim of this study is to develop an enzyme replacement therapy (ERT) for alpha-mannosidosis which is a rare lysosomal storage disease that leads to mental and physical deterioration. This pathology is due to progressive accumulation of undegraded oligosaccharides inside lysosomes and the cause of the disease is the deficiency of the alpha-mannosidase enzyme, which normally cleaves alpha-linked mannose residues from [glycoproteins](#) during their ordered degradation. Purified alpha-mannosidase from tobacco leaves of in vitro grown plants was biochemically similar to the enzyme obtained from human tissue and it can be internalised and processed by alpha-mannosidosis fibroblast cells. These results encouraged us to consider plants as a promising expression system for the production of recombinant alpha-mannosidase and to further study its expression in tobacco. We are currently growing transgenic tobacco plants in the greenhouse to investigate the accumulation of the heterologous protein in tissues (leaves and seed) at different developmental stages by Western blot. The enzyme will be then purified from the most promising tissues, biochemically evaluated and hopefully tested in knock-out alpha-mannosidosis mice.

Development and production of VHH and VHH-Fc antibodies as functional tools for plant research

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Antibodies and antibody derived fragments are excellent tools for the detection and purification of proteins. However, only a few antibodies targeted against plant proteins are currently available on the market. Plant researchers therefore are forced to fuse their protein of interest to one of the many different protein tags (GFP, His, ...). This step is not only time consuming, the protein tag might also interfere with the function of the protein. We are currently developing an approach that will meet this demand for high quality antibodies against *Arabidopsis thaliana* specific proteins.

Due to the large size and complex nature of conventional antibodies (150kDa), researchers progressed towards the development of smaller antibody fragments that fully retain their antigen-binding ability: Fab (50kDa) and scFv (25kDa). However, these antibody derivatives still require the assembly of two variable domains to bind their respective antigens. Camelids on the other hand produce heavy-chain antibodies (HCAbs) which are devoid of light chains [1]. Their N-terminal domain, called a Nanobody® or VHH, represents the smallest antigen-binding domain (15kDa).

So far, we have identified VHHs against two major seed storage proteins of *Arabidopsis thaliana*: 2S albumin and 12S globulin. Together, these proteins make up approximately 80% of the seed proteome. The VHHs were produced in *E. coli* and are being analyzed for their use in ELISA, co-immunoprecipitation and immunolocalisation. However, because fusion to an Fc-fragment will further increase the stability and sensitivity of the antigen-binding molecule, we are also producing the VHHs as dimeric VHH-Fc antibodies [2]. Here we report the VHH-Fc antibodies accumulating to levels of 0,1 to 15% of total soluble protein in *Arabidopsis* seeds. As these VHH-Fc antibodies copurify with their targets, we will shift the production of anti-seed protein antibodies towards the transient system of *Nicotiana benthamiana* leaves [3].

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Recombinant protein expression in plastids: adjuvants, viral capsomeres and envelope domains

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The expression system based on chloroplast transformation is a novel method to produce foreign proteins in a cost-effective manner, as protein yields in plastids can reach up to 70% of total soluble proteins (TSP). Furthermore, concerning the biosafety, tobacco is a self-pollinating crop and transgenes that are introduced into the maternally inherited chloroplast genome cannot spread through pollen (Clarke and Daniell 2011, Lössl and Waheed 2011).

In our current projects, three proteins are expressed in tobacco chloroplasts: A pentamer forming L1 from Human papillomavirus (HPV), the pentameric heat labile enterotoxin subunit B from *E. coli* (LTB) as an adjuvant and the Dengue virus envelope protein domain (ED III).

HPV causes cervical cancer in women worldwide, which is currently prevented by expensive vaccines based on virus-like particles (VLPs). An alternative to VLPs consists in pentameric L1 capsomeres which are highly immunogenic (Schädlich et al. 2009). Moreover, the immunogenicity of a given antigen can be improved by coupling of an adjuvant (Stevenson et al. 2004). Having demonstrated successful expression of capsomeres in tobacco plastids (Waheed et al. 2011a), we now proved the expression of a modified HPV-16 L1 antigen coupled with LTB as an adjuvant (Waheed et al. 2011b).

Dengue fever is a virus infection, transmitted by the mosquito *A. aegypti*, which threatens millions of people the tropics. Successful vaccination against all four virus serotypes must be achieved at once, in order to avoid severe side effects due to antibody dependent enhancement (ADE). In our approach we work with a modified fusion protein consisting of the EDIII of all four serotypes which has already proven immunogenicity in mice (Etemad et al. 2008). Expression of this complex tetravalent protein in chloroplasts is particularly challenging, as their original coding sequences are homologous up to 67%. In the plastid expression system this homology could cause aberrant recombination events which might result in deletion of the protein. Therefore we started with insertion of a monovalent and the tetravalent EDIII gene using a constitutive expression cassette and an ethanol-inducible expression system in parallel (Lössl et al. 2005). First transformants carrying the monovalent form have regenerated and they contain the whole expression cassette correctly within the plastid genome.

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Tobacco hairy roots as production hosts for recombinant proteins

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antibody, hairy root, recombinant, secretion, therapeutic, tobacco

Our aim is to develop plant-based systems that can be used to produce large amounts of pharmaceutical and industrial high-quality recombinant proteins in contained systems. In the frame of EU-funded CoMoFarm project (www.comofarm.org) we are establishing ways to standardize the growth and behaviour of whole plants and plant tissue- and cell cultures to achieve a consistent yield and product quality. In the concept of plant molecular farming, diagnostic and therapeutic antibodies are important targets. In this project, three different proteins are being expressed and produced by different production hosts. The monoclonal antibody M12 binds to vitronectin that has been thought to be involved in hemostasis and tumor malignancy¹. Two other proteins are secreted and membrane-integrated versions of the influenza hemagglutinin antigens. In this setup, the impact of localization of the protein is being investigated. Ultimately, the goal of this project is to generate results and knowhow to help to reduce costs involved in the production of industrial and pharmaceutical proteins.

When it comes to industrial scale production of valuable proteins, plant hairy roots offer an attractive choice by yielding generally more stable production patterns than those of undifferentiated cells. Transgenic *Nicotiana tabacum* cv Petite Havana SR1 plants expressing M12 antibody were infected with a wild type *Agrobacterium rhizogenes* strain LBA9402/12 for hairy root induction. Secretion of M12 antibody to culture medium was triggered by various chemical compounds including phytohormones, signalling and stabilizing agents and macronutrients. The secretion capacity of hairy roots after applying these agents was studied by using statistical experimental design (Modde, Umetrics). Another recombinant protein studied in this project, a membrane-integrated version of hemagglutinin antigen, was successfully expressed in *N. tabacum* SR1 hairy roots. The functional testing of these hairy roots are currently under process.

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Poster Session

Expression of chimeric non-mammalian galactosyltransferases in plants

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Two isoforms of the human β 1,4-galactosyltransferase 1 (GalT) exists, a long form being 13 aa longer at the N-terminus compared to the short form. Expression of both isomers in tobacco revealed clear differences in galactosylated N-glycans of leaf glycoproteins. The majority of galactosylated N-glycans obtained by the short form were of the so-called hybrid type N-glycans whereas the long form showed primarily complex type N-glycans. Non-mammalian GalTs lack the N-terminal extension and in that respect resemble the truncated version of human GalT. In this study, we showed that N-terminal extension of chicken and zebrafish β 1,4-GalT homologues with a 13 aa sequence, that differentiates, acts to increase the levels of complex type galactosylated N-glycans in tobacco leaf glycoproteins compared to expression of the wild-type versions of each gene. Furthermore, it is demonstrated that replacement of the CT and TMD of both GalTs by the corresponding region of rat α 2,6-sialyltransferase has a similar, but stronger effect.

Biotechnological experimental models for the development and selection of plant molecules with potential applications in human health

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The development process that leads to the obtainment and validation of new molecules to be used as drug is quite long and expensive. It happens frequently that, after an intense screening activity, a compound that was proved to be effective in early stage experimental trials shows toxic effects and has to be discarded. The high costs of development could be sensibly reduced if intermediate experimental protocols, providing information about toxicity of the new products before experimental practices on animals or humans, become available. The project that our group is carrying on has the specific target to set up protocols and models to identify potential toxic effects of new compounds in an early experimental phase. We are using, as starting material, molecules extracted from plants (proteins and peptides belonging to the HSP family, antioxidants, low molecular weight molecules such as glycoalkaloids, terpenoids, phenolic compounds) or produced in recombinant organisms (plants, yeasts, bacteria). Arabidopsis HSP70 proteins, purified from recombinant organisms, were already demonstrated to have activity on maturation and differentiation of Human Dendritic Cells (DC) (Iannacone et al, manuscript in prep). The purified HSP70 proteins (native and recombinant) will be also used as starting material for toxicology analyses. We will test the effect of these organic compounds and peptides on proteins which catalyze transport through cell membranes. These proteins indeed, represent the first molecular contact for xenobiotic compounds. Thus, several toxic effects could be mediated by interaction of the compounds with these proteins. In case of interaction of the compounds with membrane transport system, the absorption of nutrients, such as amino acids, sugars or cofactors, could be impaired. These alterations will appear as derangement of intestinal and renal functions. The experimental protocols for studying interaction of the compounds with membrane transporters consist in the *in vitro* experimental procedure of proteoliposome system. This system are assembled with artificial phospholipid membranes, mimicking the cell membranes, in which purified transport systems are inserted using appropriate methodologies which have been pointed out by the biochemistry and molecular biotechnology laboratory of University of Calabria. The prediction of alteration of the membrane absorption at the intestinal and renal level will be very helpful in the selection of compounds which do not exert such effects for further experimentation on animals and then on humans. The ability of the suggested models to identify toxic effects of potential pharmaceutical active molecules in the early phase of research will be discussed.

Oral delivery of plant-derived HIV-1 p24 antigen in low doses shows a superior priming effect in mice compared to high doses

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Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immune deficiency syndrome (AIDS), is a severe sexually transmitted infection (STI) that is implicated in one of the world's most severe human epidemics. In 2009, approximately 33 million individuals globally were estimated to be living with HIV-1, and to date, at least 25 million people have succumbed to AIDS-related diseases. Resource-poor settings, especially the sub-Saharan region, are struggling the most with the HIV-1 epidemic. Since the beginning of the HIV-1 epidemic, a period of almost 30 years, substantial efforts have been directed at developing a protective vaccine against HIV-1 infection, with unsuccessful results. Because of the capability of HIV-1 to transfer genetic information to the genome of the infected host and its extensive capability to mutate, the development of a vaccine has been a much more complex issue than initially thought.

Several vaccine strategies have been explored in animal systems and in human trials in the quest for an HIV-1 vaccine. A somewhat neglected approach for the administration of vaccines is to target the large number of immune competent cells present in the gut-associated lymphoid tissue (GALT) in the gastrointestinal tract. A subunit vaccine system capable of exposing the vaccine antigen to the GALT is believed to be the basis of a potential vaccination strategy, especially when working with STIs. Furthermore, for HIV-1 in particular, the GALT vaccination site is promising because early HIV-1 infection is associated with a rapid depletion of CD4⁺ immune cells in the GALT.

The use of edible transgenic plants as a production system for the p24 antigen followed by oral delivery has among others been reported by our group to successfully produce systemic immune responses in mice [1]. Briefly, transgenic *Arabidopsis* expressing low levels of p24 antigen was established and generated in an initially limited pilot feeding experiment in mice, and detectable levels of anti-p24-IgG antibodies were found in serum. In addition, a priming effect was also shown in a subsequent feeding experiment using fresh p24 transgenic plants. This was followed by the development of improved transgenic *Arabidopsis* containing increased levels of the p24 antigen and in transgenic *Daucus carota* (carrot), which also expressed the p24 antigen [2] ().

In the present study, we used these two HIV-1 p24 transgenic plant systems (*Arabidopsis* and carrot), in oral immunization experiments. Both transgenic plant systems showed a priming effect in mice and induced humoral immune responses, which could be detected as anti-p24-specific-IgG in sera after an intramuscular p24 protein boost. Initial dose-dependent antigen analyses using transgenic *Arabidopsis* indicated that low p24 antigen doses were superior to high p24 antigen doses.

[1] Lindh et al., 2008 Feeding of mice with *Arabidopsis thaliana* expressing the HIV-1 subtype C p24 antigen gives rise to systemic immune response. *APMIS* 116:985-994

[2] Lindh et al., 2009 Production of the p24 capsid protein from HIV-1 subtype C in *Arabidopsis thaliana* and *Daucus carota* using an endoplasmic reticulum-directing SEKDEL sequence in protein expression constructs. *Prot. Expr. Pur.* 66:46-51

THERAPEUTIC VACCINES FROM PLANTS

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Genetically engineered plants can efficiently deal with the production of safe and high-quality protein-based therapeutics. Plant-produced antibodies and enzymes have already reached Phase III and FDA programs and are approaching the market. Although plant molecular farming research in the area of therapeutic (i.e. anti-cancer) vaccines could contribute to improving the efficacy of current cancer immunotherapeutic regimens, this field is still far behind.

Human Papilloma Virus (HPV) cancers have no specific/effective pharmacological treatment and related surgical/chemo/radio-therapy interventions exert a considerable impact on health services without resolving disease recurrence. The available preventive recombinant HPV are blocking new infections and protect against high-grade cervical intraepithelial neoplasia. However, they have some limitations as they are primarily type-specific and expensive and provide no healing for already infected/tumour-affected large number of subjects. Therefore, HPV tumours represent a good target both for developing biosimilar preventive vaccines and to develop an urgently needed therapeutic vaccine.

Plant production of candidate HPV prophylactic and therapeutic vaccines is proven, with evidence of efficacy in animals. In particular, we expressed non-oncogenic E7 protein variants at high levels (0.5 mg/g fresh leaf tissue after purification) using a second-generation viral vector for transient plant expression (collaboration with Fraunhofer CMB, USA). With the same plant virus-based technology, but using a rhizogenic strain of *Agrobacterium*, we were able to produce clonal roots expressing high levels of the E7 protein in containment. That could more easily reach public acceptance about the use of plants for pharmaceutical production and fulfil GMP guidelines. In this direction, we are developing strategies to express E7 protein by genetic engineering of the chloroplast of the green microalga *C. reinhardtii* (collaboration with G. Giuliano, ENEA, Italy).

In studies carried out on large numbers of animals (50 per treatment), after two doses of purified plant-derived E7 tumor antigen (tAg, 20 µg), very aggressive E7-expressing experimental tumours growth block was achieved by inducing a huge tAg-specific T-cell response also in absence of adjuvant. Being the pre-clinical evaluation pivotal to foresee the efficacy of a vaccine also in humans, we have developed a new mouse model that represents better the natural history and response to therapies of some HPV tumors in humans and onto which we are testing our formulations.

There is growing evidence that weakly immunogenic tumour antigens, when given in the appropriate/adjuvanting context in a vaccine, stimulate immune rejection and tumour clearance. Interestingly, plant-produced antigens and plant-derived proteins seem to be very promising in this sense (Franconi R, Spanò L, Venuti A, Massa S. PCT/IT2010/00324 'Vaccines based on genetic chimera of viral and/or tumoral antigens and plant proteins'). We are, therefore, now exploring the value of plant-expressed fusions of the E7 tAg with the inactive mutant saporin protein (SAPKQ) from *Saponaria officinalis*. This protein has features (plant-origin, high immunogenicity, inflammation and apoptosis induction) that might represent unique advantages in tumor therapy and that has been already proven to improve the efficacy of genetic E7-based therapeutic vaccines.

In the long term, these products might contribute to the sustainability of treatments, technological innovation/modernization of health research and to improving the efficacy of cancer immunotherapeutic regimens.

EXPRESSION OF N-TRUNCATED GAD65mut FORMS IN A PLANT-BASED PLATFORM

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Type 1 diabetes (T1D) is an autoimmune disease characterized by the T-cell mediated destruction of insulin-secreting pancreatic β -cells, causing the need of life-long insulin therapy. The 65 kDa isoform of glutamic acid decarboxylase (GAD65) present in the pancreatic cells is one of the major autoantigen involved in disease development. In the last years antigen-specific immunotherapy (ASI) based on the delivery of GAD65 has emerged as an appealing approach for treating T1D; recent phase II clinical trials have shown that human administration of two injections of 20 μ g of alum-formulated GAD65 lead to a significant preservation of residual insulin secretion without serious adverse effects. Large-scale phase III confirmatory studies are underway in Europe and in the USA. The major disadvantage of this approach is the high-cost associated with the current molecule production system based on Baculovirus/insect cells (500,000 €/g). In the perspective of the use of GAD65 for autoimmune treatment a cost-effective recombinant system for the production of the immunoreactive protein would be highly desirable.

It is well documented that GAD65 undergoes some post-translational modifications in the N-terminal domain that result in a firmly membrane-anchored protein, which is highly hydrophobic. In vitro, GAD65 requires detergent to be solubilised. However, because detergents are extremely cytotoxic, a detergent-free preparation is mandatory for vaccination; moreover the presence of detergents can complicate the purification process. The production of a soluble form of the protein would simplify the downstream processing of the molecule and, eventually, the final pharmaceutical formulation.

We have previously shown that GAD65 and a mutated catalytically-inactive form of the protein (GAD65mut) can be expressed in transgenic tobacco plants. GAD65mut accumulates 10-fold higher than GAD65 and retains the immunogenic properties.

In order to develop a system for the high-efficient production and purification of GAD65, we engineered GAD65mut to various extents to obtain soluble forms of the molecule. In the present work we describe and discuss the solubility and accumulation levels of three N-truncated forms of GAD65mut in comparison with full-length GAD65mut and GAD65 in a plant-based platform. This system, based on transient expression in *N. benthamiana*, was chosen for its high-throughput and fast expression of molecules.

The importance of the 3' UTR in controlling the translation efficiency of Cowpea mosaic virus (CPMV) RNA-2-based constructs

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A transient expression system based on a deleted version of Cowpea Mosaic Virus (CPMV) RNA-2, termed CPMV-HT has been successfully used for the plant-based transient expression of a wide range of scientifically and economically important protein products [1, 2]. The CPMV-HT system relies on the use of the untranslated regions (UTRs) of the CPMV RNA-2 to achieve enhanced expression of a protein of interest after agroinoculation. Specific mutations in the 5'-UTR have been shown to modulate the translational efficiency of constructs [1, 2]. The ability to modulate translation means that it is possible to express multiple proteins in differing amounts within the same plant cell. This ability has been exploited to produce virus-like particles (VLPs) of Bluetongue virus (BTV), an important veterinary pathogen, which have been shown to raise an appropriate immune response in sheep.

Although mutations in the 5'-UTR have been shown to modulate translation efficiency in the CPMV-HT system, the influence of the 184 nucleotide (nt) 3'-UTR on expression levels has not been determined. To investigate this, mutations were introduced into 3'-UTR of a CPMV-HT construct containing GFP and the effect on GFP expression levels assessed after agro-infiltration of *Nicotiana benthamiana* leaves. Deletion of the entire 3'UTR led to a 3-fold decrease in GFP expression; re-insertion of the sequence in the correct orientation restored expression while re-insertion in the opposite orientation did not. Deletion of the 5' 65nt of the 3' UTR actually led to a slight enhancement of GFP expression compared to when the entire 3' UTR was present. Mutagenesis of a region of the 3' UTR previously shown to form a Y-shaped secondary structure important for RNA-2 replication [3] showed that this structure is also involved in controlling translational efficiency. These effects have been shown to be due to changes in mRNA stability rather than a direct effect on translatability.

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Process development for production of a human antibody in BY 2 suspension cells.

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The production of high value recombinant proteins, such as vaccines or antibodies, is a well established process with mammalian cell as well as in microbes and yeast. For plant cell suspension culture this production stage was established a few years ago giving the advantage of low cost chemically defined media, no by production of endotoxins and a functional glycosylated assembly of multi subunit proteins. [1] Several plant derived recombinant proteins for diagnostics are already on the market and some pharmaceutical proteins, especially vaccines, are in pre clinical and clinical trials. [2, 3]

This work shows the production of a full length antibody using a transformed tobacco suspension cell line. The cell line is designed to secrete the antibody into the apoplast. Two different media compositions were used during the cultivation in the disposable bioreactor Appliflex® (Applicon, Schiedam, NL). The 5L working volume experiments resulted in product concentrations in the supernatant of over 150 mg L⁻¹ (>600 µg/gFW) with the optimised process strategy.

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Meeting Dinner on the 16th

We are having our COST meeting dinner at the wine tavern 10er Marie (Ottakringer Straße 224, 1160 Vienna)



http://www.fuhrgassl-huber.at/piv_e/archive.php?c=m_main&w=&t=m_front.html

Directions

We will travel all together but in case you get lost....

From Wien Westbahnhof take the U3 (orange line) to Ottakring (final stop). Once you exit follow Heigerleingasse and turn left into Ottakringerstrasse



Last subway runs at 00:22! After that: take Nightbus N46 (bound to Kärntner Ring/Oper, runs at 4min and 34min past the hour as of 1a.m.) to Thaliastrasse, then switch to the N64 (bound to Alterlaa) until Westbahnhof; will take about 30min)

TRAVELLING INFORMATION

Going to the Vienna International Airport

By Taxi:

Fares: from 31€ (4 passengers)

Full address of Hotel:

Hotel Ibis Wien Mariahilf, Mariahilfer Gürtel 22-24, 1060 Wien

Airport Driver

Reservierung (24 h Service)  +43-1-7007-36422 

Fax +43-1-7007-36393

Stadtbüro  +43-1-22-8-22 

Airport Jet - Set Service  +43-1-7007-33140 

Reservierung (24h Service)  +43-1-7007-33141 

ATS Airport Transfer Service  +43-1-7007-35910 

Reservierung (24 h Service)  +43-1-7007-35905 

Fax +43-1-7007-35920

 +43-1-7007-33280  ,

C & K Airportservice -33281

Fax +43-1-7007-33289

Stadtbüro  +43-1-44-444 

Flughafentaxi.at  +43-1-7007-33690 

Fax +43-1-7007-33377

Stadtbüro  +43-1-330-15-05 

By Bus:

Vienna Airport Lines, Bus number 1187:

Fares:

One-way ticket: 7€

Tickets are purchased at the bus driver

Time table:

Wien West – Vienna Airport

	Daily													
Fahrtnummer	1187 101	1187 103	1187 105	1187 107	1187 109	1187 111	1187 113	1187 115	1187 117	1187 119	1187 121	1187 123	1187 125	
Fußnoten														
<u>Wien Westbahnhof (Europaplatz)</u> ① ② ab	5.00	5.30	6.00	6.30	7.00	7.30	8.00	8.30	9.00	9.30	10.00	10.30	11.00	
<u>Wien Dörfelstraße (Meidling Bahnhof)</u> ① ②	5.15	5.45	6.15	6.45	7.15	7.45	8.15	8.45	9.15	9.45	10.15	10.45	11.15	
<u>Flughafen Wien Ankunftsebene</u> ← an	5.45	6.15	6.45	7.15	7.45	8.15	8.45	9.15	9.45	10.15	10.45	11.15	11.45	

	Daily													
Fahrtnummer	1187 127	1187 129	1187 131	1187 133	1187 135	1187 137	1187 139	1187 141	1187 143	1187 145	1187 147	1187 149	1187 151	
Fußnoten														
<u>Wien Westbahnhof (Europaplatz)</u> ① ② ab	11.30	12.00	12.30	13.00	13.30	14.00	14.30	15.00	15.30	16.00	16.30	17.00	17.30	
<u>Wien Dörfelstraße (Meidling Bahnhof)</u> ① ②	11.45	12.15	12.45	13.15	13.45	14.15	14.45	15.15	15.45	16.15	16.45	17.15	17.45	
<u>Flughafen Wien Ankunftsebene</u> ← an	12.15	12.45	13.15	13.45	14.15	14.45	15.15	15.45	16.15	16.45	17.15	17.45	18.15	

	Daily											
Fahrtnummer	1187 153	1187 155	1187 157	1187 159	1187 161	1187 163	1187 165	1187 167	1187 169	1187 171	1187 173	
Fußnoten												
<u>Wien Westbahnhof (Europaplatz)</u> ① ② ab	18.00	18.30	19.00	19.30	20.00	20.30	21.00	21.30	22.00	22.30	23.00	
<u>Wien Dörfelstraße (Meidling Bahnhof)</u> ① ②	18.15	18.45	19.15	19.45	20.15	20.45	21.15	21.45	22.15	22.45	23.15	
<u>Flughafen Wien Ankunftsebene</u> ← an	18.45	19.15	19.45	20.15	20.45	21.15	21.45	22.15	22.45	23.15	23.45	

By S-Bahn (S7):

Tickets can be bought at ticket vending machines of the ÖBB (Wien Westbahnhof to Flughafen Wien).

Fares:

One-way ticket: 3.60€



Time table:

Trains usually leave on platform 3 from Wien-Mitte, Landstrasse.

Wien Mitte - Airport				
Leaves	Train	Arrives	Duration	
6:16	S 7 𠄎	6:42	0:26	daily
6:46	S 7 𠄎	7:12	0:26	
7:16	S 7 𠄎	7:42	0:26	
7:46	S 7 𠄎	8:12	0:26	
8:16	S 7 𠄎	8:42	0:26	
8:46	S 7 𠄎	9:12	0:26	
9:16	S 7 𠄎	9:42	0:26	
9:46	S 7 𠄎	10:12	0:26	
10:16	S 7 𠄎	10:42	0:26	
10:46	S 7 𠄎	11:12	0:26	
11:16	S 7 𠄎	11:42	0:26	
11:46	S 7 𠄎	12:12	0:26	
12:16	S 7 𠄎	12:42	0:26	
12:46	S 7 𠄎	13:12	0:26	
13:16	S 7 𠄎	13:42	0:26	
13:46	S 7 𠄎	14:12	0:26	
14:16	S 7 𠄎	14:42	0:26	
14:46	S 7 𠄎	15:12	0:26	
15:16	S 7 𠄎	15:42	0:26	
15:46	S 7 𠄎	16:12	0:26	
16:16	S 7 𠄎	16:42	0:26	
16:46	S 7 𠄎	17:12	0:26	
17:16	S 7 𠄎	17:42	0:26	
17:46	S 7 𠄎	18:12	0:26	
18:16	S 7 𠄎	18:42	0:26	
18:46	S 7 𠄎	19:12	0:26	
19:16	S 7 𠄎	19:42	0:26	
19:46	S 7 𠄎	20:12	0:26	
20:16	S 7 𠄎	20:42	0:26	
20:46	S 7 𠄎	21:12	0:26	
21:16	S 7 𠄎	21:42	0:26	
21:46	S 7 𠄎	22:12	0:26	
22:16	S 7 𠄎	22:42	0:26	
22:46	S 7 𠄎	23:12	0:26	

