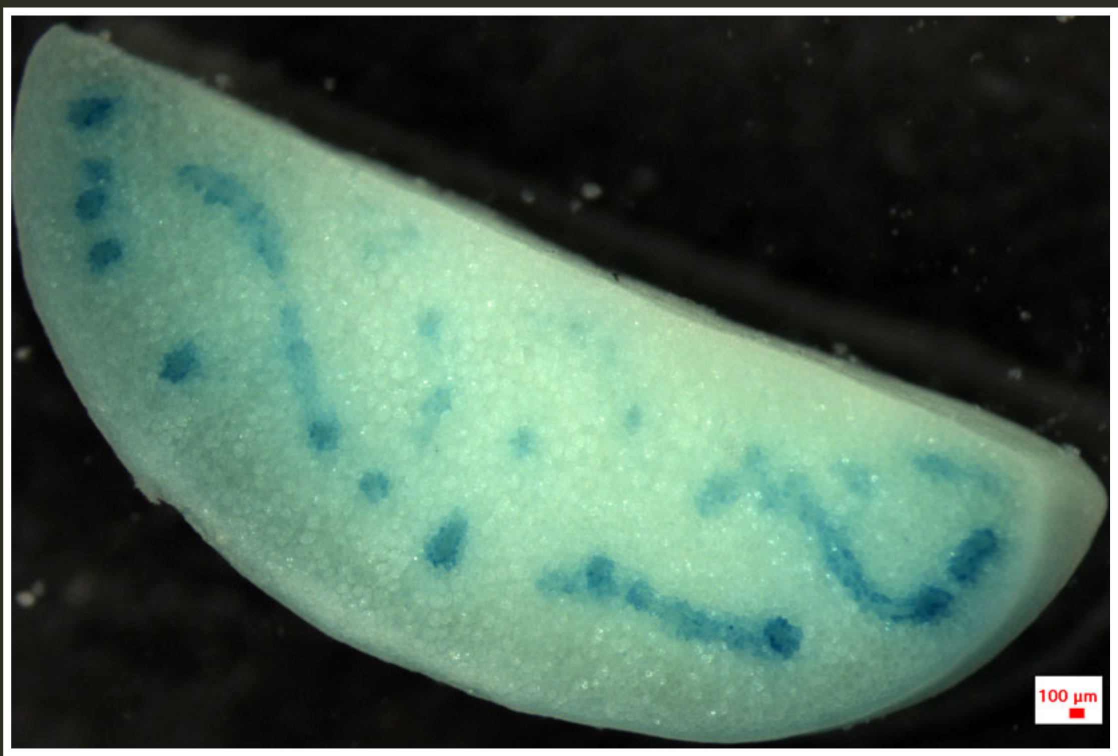
# Plant Biotech Denmark
# Annual meeting 2011
# March 3-4

Faculty of Life Sciences
University of Copenhagen

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Cover photo: Iron distribution in a cross section of a cotyledon from the *Phaseolus vulgaris* (common bean) genotype Bat A.
The iron is stained blue using the Perls' Prussian Blue method.
Work by Cristina Cvitanich, Department of Molecular Biology, Aarhus University

# Programme

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| 9.00 - 9.30 | *Registration and coffee* | |
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| 16.10 - 16.30 | **Elemental fingerprinting of plant tissue using laser ablation**, by Søren Husted, Professor, Department of Agriculture and Ecology, LIFE, University of Copenhagen | 18 |
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**FRIDAY- March 4, 2011**

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***Key Note Talk***

**A new story of an old protein: Sensing and transport are independent functions of the same protein CHL1**

*Yi-Fang Tsay*

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Ions are not only essential nutrients for higher plants, but also act as signal molecules. Little is known about how plants sense changes of nutrient concentrations in soil. Nitrate transporter gene *CHL1* was isolated twenty years ago using a nitrate uptake mutant. In addition to exhibiting nitrate uptake defect, *chl1* mutant is also defective in several nitrate responses. These defects suggest that CHL1 participates in nitrate signaling, but because of its transport activity, it is difficult to conclude whether CHL1 is directly or indirectly involved. Using a decoupled mutant with a mutation of P492L between putative $10^{th}$ and $11^{th}$ transmembrane domains, we showed that uptake activity is not required for the sensing function of CHL1, demonstrating that CHL1 is a nitrate sensor (transceptor)

Soil nitrate concentration can vary by four orders of magnitude. CHL1 is a dual-affinity nitrate transporter involved in both high- and low-affinity nitrate uptake. CHL1 phosphorylated at threonine residue 101 is a high-affinity nitrate transporter and dephosphorylated CHL1 is a low-affinity nitrate transporter. As a sensor, using dual-affinity binding, CHL1 s can detect a wide range of nitrate concentration changes. CHL1 functions as a sensor for a nitrate-induced transcriptional response. In response to low concentrations of nitrate, CIPK23 will phosphorylate CHL1 at T101, and T101-phosphorylated CHL1 will induce a low-level transcriptional response. When exposed to high-concentrations of nitrate, T101 phosphorylation is prohibited and dephosphorylated CHL1 leads to high level transcriptional response. Therefore, using dual-affinity binding and phosphorylation switching, CHL1 can sense a wide range of nitrate concentrations in the soil and thus trigger different levels of nitrate response.

**Keywords:** *nitrate, Arabidopsis, sensor, transporte*

## A membrane-bound signalosome controls plant growth

*Anja T. Fuglsang[1]*, Jörgen Persson[1], Tracey A. Cuin[2], Waltraud X. Schulze[3], Kristina H. Thuesen[1], Teis E. Sondergaard[1], Cecilie K. Ytting[1], Yoshikatsu Matsubayashi[4], Sergey Shabala[2], Michael G. Palmgren[1]*

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In eukaryotes, current models of receptor kinase mediated signal transduction reveal complex pathways resulting in altered gene transcription. Here we report direct receptor kinase mediated post-translational regulation of a final target without preceding signal amplification, which provides a novel concept for receptor kinase signaling. At the plant surface, the peptide receptor kinase (PIRK) and the major plasma membrane proton pump ($H^+$-ATPase; AHA2) form an integrated signalosome. When secreted growth-promoting peptide PSY1 binds to the extracellular receptor domain of PIRK, PIRK phosphorylates the cytoplasmic autoinhibitory domain of AHA2, which results in increased proton extrusion, a prerequisite for cell elongation.

## Cereal bioengineering: Amylopectin-free and hyper-phosphorylated barley starch

*Massimiliano Carciofi[1], Shahnoor S. Shaik[2], Susanne L. Jensen[2], Jan T. Svensson[2], Andreas Blennow[2], Preben B. Holm[1], Kim H. Hebelstrup[1]*

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*[2] VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, 1870 Frederiksberg, Denmark*

Barley lines producing grains with either amylopectin-free or hyper-phosphorylated starches were made by transgenic methods. Cereals producing these kind of starches have not been reported before.

Amylopectin-free barley was generated by simultaneously silencing the three genes encoding the starch branching enzymes SBEIIa, SBEIIb and SBEI by a chimeric hairpin. The construct was inherited as a single locus with a distinct 1:3 segregation in consecutive generations. The transgenic grains were shrunken and the yield was around 80% of that found in wildtype. The starch granules were irregularly, elongated and globose shaped. Transgenic grains also had a higher beta-glucan content.

In order to increase barley starch phosphorylation, endosperm specific overexpression of glucan water dikinase from potato (StGWD) was conducted. The content of phosphate esters in starch from consecutive generations ($T_0$ and $T_1$) of transgenic grains was tenfold higher than from vector control and wild type grains. Amylose content was not affected in hyper-phosphorylated grains. Hyper-phosphorylated starch granules had several pores on the surfaces, similar to pores seen on enzymatically semi-degraded granules. This provides support for the presence of a general mechanism in starch degradation in the plant kingdom that phosphorylation carried out by ectopic expression of StGWD tags barley starch granules for degradation executed by endogenous enzymes.

Our work demonstrates two new strategies for *in planta* starch bioengineering of cereals. Bioengineering may be used to obtain novel and technologically interesting cereal starches and to elucidate the complex pathways of starch biosynthesis and the roles of individual starch biosynthetic enzymes.

**References:**

Blennow, A., Engelsen, S. B. (2010). Helix-breaking news: fighting crystalline starch energy deposits in the cell. *Trends in Plant Science* Vol. 15, 4:236-240

Hebelstrup, K. H., Christiansen, M. W., Carciofi, M., Tauris, B., Brinch-Pedersen, H., Bach Holm, P. (2010). UCE: A uracil excision (USER™)-based toolbox for transformation of cereals. *Plant Methods* 6:15

***Key Note Talk***

**Strategies for durable resistance from an understanding of RXLR effectors from the potato late blight pathogen *Phytophthora infestans***

*Paul Birch[1,2], Miles Armstrong[1,2], Petra Boevink[1], Susan Breen[1,2], Tatyana Bukharova[1], Eleanor Gilroy[1], Ingo Hein[1], Hazel McLellan[1,2], Leighton Pritchard[1], Rosalind Taylor[1], Steve Whisson[1]*

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Plants are under constant threat from microbial pathogens. Two layers of inducible plant defense provide obstacles to infection. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) follows the perception of conserved microbial molecules at the surface of plant cells. Plant pathogens secrete effector proteins that suppress PTI. Prokaryotic pathogens secrete and ‘inject’ effector proteins inside living host cells, via a type III secretion system (T3SS). T3SS effectors possess a range of enzymatic functions and have been shown to directly interact with host proteins to suppress key defence signaling, regulatory and mechanistic processes, thus creating a susceptible environment for infection. Effectors may be recognized by plant disease resistance (R) proteins, resulting in effector triggered immunity (ETI) that often involves the hypersensitive response (HR), a form of programmed cell death (PCD), coinciding with restriction of the invading pathogen.

Despite huge advances in our understanding of bacterial pathogenesis, very little is understood about how filamentous eukaryotic pathogens (fungi and oomycetes) cause disease, and yet these represent the major threats to global crop production.

We and others have shown that the oomycete pathogen *Phytophthora infestans*, cause of late blight on potato and tomato, secretes potentially hundreds of effectors containing a conserved amino acid motif, RXLR (where R is arginine, X is any amino acid and L is leucine), which is required for the translocation of these proteins inside living plant cells (Whisson et al., 2007; Birch et al., 2008). More recently, we have demonstrated the mode-of-action of one such effector, AVR3a, upon delivery into host cells (Bos et al., 2010). AVR3a interacts with and stabilizes the host ubiquitin E3 ligase, CMPG1, preventing its normal, degradative role in mediating PCD in response to a range of pathogen elicitors which are detected at the host plasma membrane.

We are studying the expression, sequence diversity, localisations (within the host cell) and host protein targets of key RXLR effectors from *P. infestans*. Moreover, we are investigating how and where, within the plant cell, these effectors are recognized by host resistance proteins. This talk will describe our current understanding of the roles RXLR effectors play in virulence, and will indicate that their recognition by R proteins follows the Guard Hypothesis, and is mediated by interactions through host proteins that are virulence targets of effectors.

Birch PRJ, *et al* (2008) *Curr Opin Plant Biol* 11:373-379.

Bos JI, *et al* (2010). *Proc Natl Acad Sci U S A.* 107:9909-9914.

Whisson SC, *et al* (2007) *Nature* 450:115-118.

## Autoimmunity in Arabidopsis acd11 is mediated by Epigenetic Regulation of an Immune Receptor

*Stephan Thorgrimsen[1].,Kristoffer Palma[1]., Frederikke Gro Malinovsky [1¤a], Berthe Katrine Fiil[1], H. Bjørn Nielsen[2], Peter Brodersen[1], Daniel Hofius[1], Morten Petersen[1], John Mundy [1*]*

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Certain pathogens deliver effectors into plant cells to modify host protein targets and thereby suppress immunity. These target modifications can be detected by intracellular immune receptors, or Resistance (R) proteins, that trigger strong immune responses including localized host cell death. The accelerated cell death 11 (acd11) ‘‘lesion mimic’’ mutant of Arabidopsis thaliana exhibits autoimmune phenotypes such as constitutive defense responses and cell death without pathogen perception. ACD11 encodes a putative sphingosine transfer protein, but its precise role during these processes is unknown. In a screen for lazarus (laz) mutants that suppress acd11 death we identified two genes, LAZ2 and LAZ5. LAZ2 encodes the histone lysine methyltransferase SDG8, previously shown to epigenetically regulate flowering time via modification of histone 3 (H3). LAZ5 encodes an RPS4-like R-protein, defined by several dominant negative alleles. Microarray and chromatin immunoprecipitation analyses showed that LAZ2/SDG8 is required for LAZ5 expression and H3 lysine 36 trimethylation at LAZ5 chromatin to maintain a transcriptionally active state. We hypothesize that LAZ5 triggers cell death in the absence of ACD11, and that cell death in other lesion mimic mutants may also be caused by inappropriate activation of R genes. Moreover, SDG8 is required for basal and R protein-mediated pathogen resistance in Arabidopsis, revealing the importance of chromatin remodeling as a key process in plant innate immunity.

**References:**

PLoS Pathogens 6 (10). Pii: e1001137

### The *Lotus japonicus spontaneous nodule formation 4* (*snf4*) mutant has a mutation in a DNA polymerase ξ gene

*Sandal, Niels[1], Tirichine, Leïla[1], Andersen, Stig Uggerhøj [1], Nielsen, Kåre Lehmann [2], Bek, Anita [1], Blaise, Mickael [1], Sato, Shusei [3], Tabata, Satoshi [3] and Stougaard, Jens [1]*

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Three monogenic plant loci called *spontaneous nodule formation snf1*, *snf2* and *snf4* were found in a screen for plant mutants that were able to develop spontaneous nodules in the absence of rhizobia. The spontaneous nodules developing on these mutants were similar to wild type nodules in respect to nodule structure, upregulation of early nodulin genes and repression of nodule formation in the presence of nitrogen in the growth medium.
Map based cloning of *snf1* lead to the identification of a calcium calmodulin dependent kinase (*CCaMK*) gene with a gain of function mutation resulting in the *snf* phenotype (1). Similarly it was found that the *snf2* encodes a gain of function (*Lhk1*) cytokinin receptor (2). So far the *snf4* locus has not been cloned and the mutation causing the spontaneous nodulation phenotype has remained undescribed. Recently, a new whole genome sequencing method using Illumina/Solexa sequencing has been developed in Arabidopsis to identify genes with single base mutations (3). We have now applied this method in *L. japonicus* using the *snf4* locus as a test case. Our traditional marker based mapping delimited the *snf4* locus on chromosome 3 within a region of less than 150 kb and whole genome sequencing of nuclear DNA extracted from *snf4* mutant plants identified a mutation in a DNA polymerase **ξ** catalytic subunitgene located in the *snf4* region. This mutation leads to a change of an amino acid conserved through evolution. This amino acid is placed close to the nucleotide binding amino acid in the DNA repair part of polymerase **ξ** and modeling indicates that this could lead to a reduced effiency in proofreading.

**References:**

1. Tirichine et al. 2006a Nature 441, 1153-1156.
2. Tirichine et al. 2006b Science 315, 104-107.
3. Schneeberger, K. et al. 2009 Nature Methods 6, 550-1.

***Key Note Talk***

**Prospects for using genomic selection in plant breeding**

*Leif Skøt*

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Plant breeding can be defined as the improvement of heritable traits by selection and recombination. Target traits usually include crop yield (grain or biomass), abiotic stress tolerance, disease resistance, nutrient use efficiency and crop quality. Most traits of interest are quantitative, i.e. governed by many genes and environmental effects. The proportion of the phenotypic variation explained by each of these quantitative trait loci (QTL) is usually small, which limits the benefits of traditional marker assisted selection (MAS). However, genomic selection (GS) is a different type of MAS in which all marker, locus or haplotype effects are estimated simultaneously in order to predict a genomic estimated breeding value (GEBV). The aim of GS is 1) to develop a predictive mapping (function) between genetic markers, environment, and phenotype, and 2) to use this predictive function to best select the next generation for breeding. GS is generally considered to be a regression problem as the phenotypes are typically quantitative, rather than qualitative. It is technically challenging because:

- There are typically many markers – therefore over-fitting needs to be avoided. The price of DNA sequencing is dropping at a remarkable speed, resulting in ever cheaper marker data, making this problem more acute.
- The genotype markers are not independent, but are related positionally on chromosomes.
- The organisms are not independent, i.e. there is a kinship structure.
- The basic architecture of the traits is still poorly understood, and varies between traits: are there only a few alleles involved with strong effects, or many alleles with weak effects? This distinction is important because different learning methods have "biases" that make *a priori* assumptions about the structure of the data.

GS has shown its value in animal breeding, particularly in dairy cattle, but has still to make the same impact in plant breeding. This is likely to change. I will discuss some of the challenges and opportunities in GS, with particular reference to implementation in the existing perennial ryegrass and other forage crop breeding programmes at IBERS.

**Identification and molecular characterization of the vernalization response in timothy (*Phleum pratense*)**

*Alice Fiil[1], Louise Bach Jensen[1], Siri Fjellheim[2], Thomas Lübberstedt[3] and Jeppe Reitan Andersen[1]*

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Timothy (*Phleum pratense* L.) is the major forage species within the genus *Phleum* and is widely grown in cool-season regions of the world, including the Nordic countries. Most temperate perennial grasses have a dual induction requirement for flowering. Primary induction is achieved by a prolonged period of low temperature, i.e., vernalization, and/or short days, while secondary induction requires transition to long days and increased temperatures. While induction of flowering in timothy requires secondary induction, a vernalization response has not previously been reported for this species. We studied the vernalization response in 38 genotypes of diverse geographic origin and found that vernalization accelerated heading in all genotypes. In addition, considerable variation in the vernalization response was observed between genotypes and a requirement for vernalization to induce flowering was indicated in genotypes of Northern origin. While heading time in general was found to be correlated to geographic origin of genotypes, a strong vernalization response was identified in genotypes of contrasting geographic origin. Candidate genes for the vernalization response genes *VRN1*, *VRN2* and *VRN3* (also named *FT*) were identified and transcript analysis during primary and secondary induction showed that *VRN1* transcription was induced by vernalization. However, when not vernalized, genotypes with a strong vernalization response showed a prolonged repression of *VRN1* transcription throughout secondary induction. In contrast, in genotypes with a mild vernalization response *VRN1* transcription was induced by transfer to secondary induction. We conclude that significant genetic variation for the vernalization response is present within timothy and suggest that differential regulation of *VRN1* transcription discriminates genotypes with contrasting vernalization responses.

## Investigating xylanase activities and inhibitors in barley grains

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The mature barley grain *(Hordeum vulgare)* is intensively used as feed and contains a battery of hydrolytic enzymes that are synthesized during seed development and deposited in an inactive form in the grain. These enzyme activities are of biological significance as they are involved in initiating seed germination and are considered important for maximizing nutrients in food and feed. Some of the already known enzymes include β-amylases, phytases, proteases, β-glucanases and xylanases. Although barley proteome has been substantially studied, the current knowledge about these enzyme activities at molecular level is scarce, despite their potential for improvement of product quality.
The present project sets out to investigate xylanase (microbial vs. plant) and its endogenous inhibition activities in different barley cultivars. Activity assays coupled with proteomics analysis (1D SDS-PAGE, 2D gel electrophoresis, MALDI-TOF mass spectrometry) were conducted on different barley cultivars. Preliminary results suggest that there is a considerable intercultivar variation both in the level of microbial and endogenous xylanase activities. Inhibition assays are in progress and will provide detailed information about the level and ratio of xylanase and its inhibitors.

*This work is supported by the Danish Directorate for Food, Fisheries and Agri Business (DFFE), Technical University of Denmark (DTU), and the Danish Center for Advanced Food Studies.*

## High speed 4 D bioimaging

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The basis of bioimaging is to capture biological processes as they happen in the living cell. From a technical point of view this approach is limited by the resolution and recording speed that can be achieved with current microscopes. Recent developments in microscopy resulted not only in improved resolution but also in much faster acquisition. Confocal microscopes equipped with a resonant scanner can acquire 25 frames per second, a 10 fold increase over standard confocals. Spinning disc confocal microscopes can even reach acquisition speeds of 50 frames per second.

In order to obtain a 3D image of an object with confocal microscopy, a stack with images from multiple focus planes, called optical sections, has to be recorded (see Fig. 1). The high recording speeds make it now possible to acquire image stacks at very short intervals. This means that cellular processes can be followed with full spatial and temporal resolution (3D plus time = 4D).

As an application example, data will be presented from photoactivation experiments in intact plant tissue. We were able to activate fluorescence inside one cell and then measure its spread to neighboring cells. With the three dimensional data we could calculate accurate quantitative values for cell coupling at different interfaces, which was not possible before because of the sensitivity of intercellular connections to preparation.

Future potential and current limitations to 4D bioimaging will be discussed.

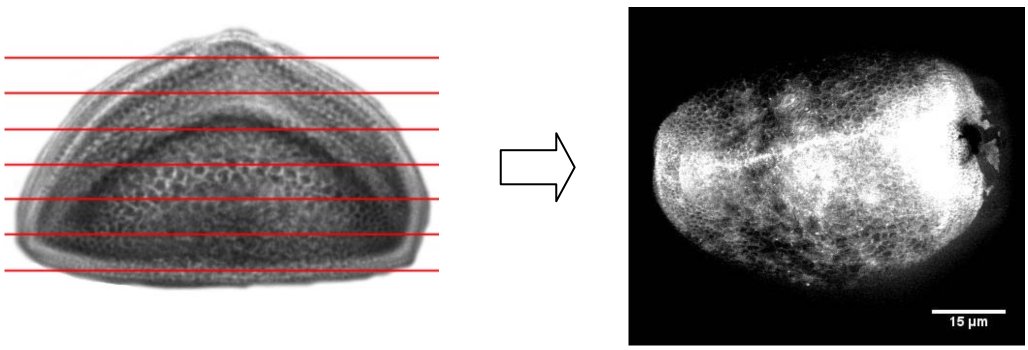


Fig. 1: 3D imaging with confocal microscopes; A Bright field image of a pollen grain to illustrate optical sectioning through an object; red lines symbolize the images taken at different focal planes; B Maximum projection of a pollen grain derived from an image stack with 227 optical sections

**A roadmap for zinc transport into the developing barley grain. Gene expression profiling using laser microdissection**

*Søren Borg and Birgitte Tauris*

*Department of Genetics and Biotechnology, Research centre Flakkebjerg, Faculty of Agricultural Sciences, Aarhus University*

Nutrients destined for the developing cereal grain encounter several restricting barriers on their path towards their final storage sites in the grain. In order to identify transporters and chelating agents that may be involved in transport and deposition of zinc in the barley grain, expression profiles have been generated of four different tissue types: the transfer cells, the aleurone layer, the endosperm, and the embryo. Cells from these tissues were isolated with the 'laser capture microdissection' technology and the extracted RNA was subjected to three rounds of T7-based amplification. The amplified RNA was subsequently hybridized to Affymetrix 22K Barley GeneChips. Due to the short average length of the amplified transcripts and the positioning of numerous probe sets at locations more than 400 base pairs (bp) from the poly(A)-tail, a normalization approach was used where the probe positions were taken into account. On the basis of the expression levels of a number of metal homeostasis genes, a working model is proposed for the translocation of zinc from the phloem to the storage sites in the developing grain.

## Single cell analysis using laser microdissection

*Kirsten Jørgensen, Radhakrishna Shetty, Hans L. Jørgensen, Birgit Jensen, Karin Olsen and Natascha K. Hansen*

*Department of Plant Biology and Biotechnology, LIFE, University of Copenhagen*

Laser microdissection microscopy enables analysis of the transcript, protein and metabolite profile of specific isolated plant cells. This may provide a more detailed analysis of the function of individual cell types by avoiding masking of the profiles with constituents present in neighboring cells or the entire tissue.

Miniature roses are often infected by *Podosphaera pannosa.* This powdery mildew infection results in economic losses because of the appearance of greyish-white mycelium on the plants. Infected epidermal cells fluorescence under UV light and this response has been correlated with a hypersensitive response (HR) in roses (Conti et al. 1985, Dewitte et al. 2007). Transcript levels of callose synthase, *GSL5,* and the HR specific ,*hsr203J,* are often up-regulated following analysis of infected leaves. A manyfold further up-regulation of these transcript levels was observed in the specific fluorescent epidermal cells isolated by laser microdissection.

The laser component of the Palm laser microdissection system may also be used to cut out special tissue parts without the catapulting abilities. Cassava contains cyanogenic glucosides that are mainly being produced in the young developing leaves and then transported to the tubers. To identify specific cell layers that would accumulate the major portion of the cyanogenic glucosides deposited in the tubers, the laser was used to isolate consecutive cell layers from mildly fixated tuber tissue without catapulting the cells. The cell layers were separated under stereomicroscope and then analysed by LC-MS.

The assignment of synthesis and deposition of bioactive natural products to specific cell types and the regulation of the synthesis, turn-over and degradation to environmental challenges maybe studied in much greater detail using laser micro dissection microscopy.

Conti GG, Bassi M, Maffi D and Bonecchi R (1985) Host-parasite relationship in a susceptible and a resistant rose cultivar inoculated with *Sphaerotheca pannosa*.I. Fungal growth, mechanical barriers and hypersensitive reaction. Phytopathologische Zeitschrift 113: 71-80

Dewitte A, Leus L, Van Huylenbroeck J, Van Bockstaele E and Höfte M (2007) Characterization of reactions to powdery mildew (*Podosphaera pannosa*) in resistant and susceptible rose genotypes. Journal of Phytopathology 155: 264-272

## Elemental fingerprinting of plant tissue using laser ablation

*Søren Husted*

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Elemental bioimaging is an essential analytical tool in order to improve our understanding of loading, assimilation and distribution of mineral elements in plant tissue. Recently, we have characterized the tissue compartmentation and molecular speciation of Fe and Zn in the cereal grain. We have shown that Fe and Zn are bound to different ligands in the grain and that their tissue compartmentation is very different. In order to further characterize the distribution of Fe and Zn in plant tissue, we have adopted a new analytical technique based on laser ablation (LA) hyphenated with ICP-MS, which enables us to perform bioimaging of most essential plant nutrients with a hitherto unmatched sensitivity. In this presentation the technique will be described and a number of exciting applications will be shown.

**Study of barley seed development by spatially resolved mass spectrometrical analysis**

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The presentation will introduce the developments and the current state of mass spectrometry imaging (MSI) techniques. The current achievements of MSI will be shown by selected examples from the literature and from our own work on barley seed development, and the challenges for the further development of the technique will be discussed. Finally we will mention parallel proteome analysis of barley seed development based on laser caption micro-dissection of tissue followed by LC-MS/MS.

The introduction of mass spectrometry into bioanalytical techniques has been a prerequisite for the development of proteomics and metabolomics as untargeted approaches. This development has also fundamentally broadened the application of targeted biochemical assays to analyse specific proteins, to monitor products from enzymatic reactions, or to profile selected classes of compounds. In the majority of applications, sample extraction is performed on whole organs, resulting in a mixed profile summing up the compounds from all the underlying differentiated cells and tissues. As a consequence, spatial information is lost, and compounds associated with low abundant tissues or cell types within the organ might be overlooked. To achieve spatial resolution for transcript profiling on a large scale for plant tissues, approaches have been introduced based on cell sorting of protoplasts labelled with a tissue specific fluorescent reporter (1), or by isolation of specific mRNA populations with the help of tagged ribosomes (2). To obtain related information on the spatial distribution of proteins and metabolites, approaches based on mass spectrometry have been introduced. MALDI-TOF based imaging has become very popular now, and pioneering work to introduce this technique has been performed in the lab of Richard Caprioli (3) in the context of clinical applications. Tissue sections are covered with a suitable matrix, and from the whole area or selected positions, MS spectra are recorded. MALDI MSI has now been used to reveal the molecular signatures associated with different types of tumours or to study the distribution and metabolism of drugs or drug candidates as a means to understand the cellular responses upon treatment.

Despite the current achievements, MS based imaging still holds many challenges to improve applicability. Major factors are sensitivity and spatial resolution. Other aspects include the identification of unknown compounds from the limited amounts available at the surface of tissue sections, and the development of new matrices. Moreover, improvements in post-acquisition analysis will significantly contribute to MSI.

In plant biology, imaging mass spectrometry has only recently been introduced, and we have summarized the current state for proteomic studies (4). We have initiated work to monitor the spatial distribution of metabolites and proteins in the process of barley seed development. We will present selected examples from this work and integration of the results into earlier 3-D models of barley caryopses.

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**Crop improvement and biofortification - – An international perspective**

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Food security and healthy nutrition are not longer problems of developing countries, but in our highly industrialized world have become global issues. Although the recent financial crisis and economic downturn have seemingly removed some of the global concerns about food shortages, hunger and human health, the challenge of achieving food security and producing nutritional crops will undoubtedly remain one of the great societal challenges that now lie before us. According to the World Health Organization, for example, approximately two billion people suffer from iron deficiency. Women and children are particularly affected in developing countries, where rice is the major staple food. Peeled rice, also called polished rice, does not have enough iron to satisfy the daily requirement, even if consumed in large quantities.  Similarly, cassava is a major staple food for 600 million people, mostly in tropical countries, but the crop suffers from many diseases and the root is of poor nutritional quality.

While there is a general recognition that we need better crops to improve human health and nutrition, the use of gene technology in crop improvement and biofortification is controversially discussed, particularly in Europe—even if conventional breeding is difficult and time-consuming to achieve this goal. Governments, corporations and the public need to understand, however, that there is an urgency for new and long-term investments in crop research, breeding and biotechnology in order to avert the impending food crisis and reduce malnutrition. Scientists are challenged to produce novel crop varieties that can fight of diseases efficiently, cope with rapidly changing environmental conditions, and provide a healthy and balanced nutrition.  Using examples from research in laboratories around the world and from our own efforts to engineer cassava and rice for improved nutritional qualities, I will illustrate and discuss new advances in crop improvement.  These examples show that plant biotechnology can make important contributions to food security and deliver increased nutritional qualities and health improvement to broad segments of the human population.

**About the author**
Prof. Wilhelm Gruissem has been full Professor of Plant Biotechnology in the Institute of Plant Sciences at the ETH Zürich (Swiss Federal Institute of Technology) since 2000. He was elected President of the European Plant Science Organization (EPSO) in 2006. After obtaining his Ph.D. in 1979 he was appointed as professor of plant biology at the University of California at Berkeley in 1983. He was Chair of the Department of Plant and Microbial Biology at UC Berkeley from 1993 to 1998, and from 1998 to 2000 he was Director of a collaborative research program between the Department and the Novartis Agricultural Discovery Institute in San Diego. Since 2001 he is Co-Director of the Functional Genomics Center Zurich. In addition to his research on systems approaches to understand pathways and molecules involved in plant growth control, he directs a biotechnology program on trait improvement in cassava, rice and wheat. He is elected fellow of the American Association for the Advancement of Sciences and Editor of Plant Molecular Biology and on the editorial boards of several journals. He published the acclaimed book 'Biochemistry and Molecular Biology of Plants'. He has received several prestigious awards, including a price from the Eiselen Foundation in Germany for his trait improvement work in cassava. In 2007 he was elected lifetime foreign member of the American Society of Plant Biologists.

## Bioengineering and simple plant systems

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Synthetic Biology is an emerging field that employs engineering principles for constructing genetic systems. The approach is based on the use of well characterised and reusable components, and numerical models for the design of biological circuits – in a way that has become routine in other fields of engineering. Synthetic Biology is providing a conceptual and practical framework for the systematic engineering of gene expression and behaviour in microbes, facilitating the design of novel regulatory networks, including synthetic oscillators, switches, logic gates, intercellular signaling systems and metabolic pathways. Synthetic Biology approaches also show great potential for the engineering of multicellular systems. (1) The greatest diversity of cell types and biochemical specialisation is found in multicellular systems, (2) the molecular basis of cell fate determination is increasingly well understood, and (3) it is feasible to consider creating new tissues or organs with specialized biosynthetic or storage functions by remodelling the distribution of existing cell types. Of all multicellular systems, plants are the obvious first target for this type of approach. Plants possess indeterminate and modular body plans, have a wide spectrum of biosynthetic activities, can be genetically manipulated, and are widely used in crop systems for production of biomass, food, polymers, drugs and fuels.

Current GM crops generally possess new traits conferred by single genes, and expression results in the production of a new metabolic or regulatory activity within the context of normal development. However, cultivated plant varieties often have enlarged flowers, fruit organs or seed, and are morphologically very different from their wild-type ancestors. Recent genetic studies have provided detail of the molecular processes underlying plant development. The next generation of transgenic crops will contain small gene networks that confer self-organizing properties, with the ability to reshape patterns of plant metabolism and growth, and the prospect of producing neomorphic structures suited to bio production.

Morphogenesis is a cellular process, driven by interplay between gene expression and a growing network of cell interactions. We have developed a battery of microscopic and genetic tools to allow clear visualization of individual cells inside living plant tissues and have the means to manipulate them. These techniques are well suited to study of simple experimental systems based on a freshwater alga, *Coleochaete orbicularis*, and a lower plant *Marchantia polymorpha*. Many of the molecular genetic tools that have been developed in Arabidopsis can be transferred to Coleochaete and Marchantia. These types of simple systems will become increasingly important for plant morphogenetic studies.

### That creative and intelligent fat

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The traditional view of lipids as dull structure-builder molecules has changed considerably over the last couple of decades, and the importance of lipids for cell function is becoming more recognized[1]. Lipids are as important for life as proteins and genes. Lipids lead to intriguing structures with very unusual and subtle materials properties that have been optimized by evolutionary principles over billions of years. These properties are consequences of the fundamental physical principles of self-organization that rule when many molecules act in concert. A key player in this concert is water that forces lipid molecules to self-assemble and organize into subtle structures. In particular, lipids in water can spontaneously form lipid bilayers and membranes. Lipid membranes are extended thin layers that are only two molecules thick. These layers constitute the backbone of all biological membranes which are a ubiquitous structural element in all living cells. The unique structural, dynamical, and functional properties of lipid bilayers constitute the basis for their use in synthetic biology.
An important membrane function is purely topological to compartmentalize living matter into cells and subcellular structures. Furthermore, membranes present themselves to macromolecules such as enzymes as highly structured interfaces at which important biochemical processes are carried out and catalyzed. The structure and molecular organization of the lipid-bilayer component of membranes hold the key to understanding the functioning of membranes[2]. Due to the fact that lipids form membranes by self-assembly processes that do not involve strong chemical forces, membranes are pieces of soft matter. Softness is a materials feature that lipid membranes share with other forms of condensed matter like polymers and liquid crystals. During evolution, Nature has evolved biological membrane structures as an optimal form of micro-encapsulation technology that, on the one hand, imparts the necessary durability to the particular soft condensed matter that membranes are made of and, on the other hand, sustains the lively dynamics that are needed to support and control the mechanisms of the many essential cellular functions associated with membranes[3]. Many lipid species are now also known to play a very active role, serving as so-called second messengers that pass on signals and information in the cell. Lipids also play the roles of enzymes, receptors, drugs, as well as regulators of, e.g., neural activity. Lipids are known to modulate the expression of genes, a phenomenon now being clearly recognized for poly-unsaturated lipids in the context of human brain evolution and the rise of diet-related human diseases.
Based on the unique properties of lipid and lipid membranes, the talk will give an overview of the use of lipid membrane biophysics in synthetic biology with a focus on liposome technology, principles for nano-scale molecular organization of membranes, enzymatic remodeling of membranes, and the *de-novo* design of cell-like functional and active membrane capsules.

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**Synthetic Biology by Massive Combinatorial Genetic Chemistry – Application to Today's Biotechnology Challenges**

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Industrial biotechnology has rapidly evolved in the last decades and has led to many processes that are both competitive and economical. Today, purified enzymes or whole cell biocatalysts are successfully applied in the chemical, pharmaceutical, food & feed, pulp &paper industries as well as the energy sector.

Despite these achievements, the design of multistep biosynthetic pathways to build strains capable of one-step biotransformations from cheap starting materials to the desired commercially interesting end products remains a challenge with the conventional tools of the trade such as metagenomics, directed evolution and metabolic engineering. Here, Evolva's Genetic Chemistry platform plays an enabling role, combining modern synthetic biology tools with nature's principles of recombination and selection.

Evolva's Genetic Chemistry approach taps into nature's rich chemical diversity to produce commercially important natural products in the baker's yeast Saccharomyces cerevisiae. Thousands of biosynthetic genes from multiple species (e.g. microbes, fungi, and plants) are mixed in a combinatorial fashion in expressible Yeast Artificial Chromosomes (or eYACs). The random assortment of expressed genes in principle allows for the creation of novel or reconstitution of known biosynthetic pathways which can deliver valuable products. The resulting yeast cell factories can be screened to identify the high producers of the desired metabolite, which can then be analyzed to identify the biosynthetic genes responsible for the high production to obtain a producer strain that can be further optimized using Systems Biology and directed evolution tools.

We have assembled a significant number of functional metabolic pathways (e.g. tetraterpenoids, flavonoids and diterpenoids) and selected the best combinations of genes originating from multiple biological species across all kingdoms. For example, a seven step flavonoid pathway was reconstituted in yeast using genes from plants, fungi and other yeasts, which were randomly combined on eYACs and transformed into yeast to create a variety of flavonoid producing yeast cell factories. Randomly picked clones were analyzed, and approximately half of them showed production of the flavanone naringenin, and a third of them produced the flavonol kaempferol in various amounts. This reflected the assembly of 5–7 step multispecies pathways converting the yeast metabolites phenylalanine and/or tyrosine into flavonoids, normally only produced by plants.

With Evolva's Genetic Chemistry platform literally millions of different combinations of biosynthetic genes with numerous applications in the Industrial Biotechnology sector are possible. Besides delivering novel multistep biosynthetic pathways for highly efficient production of valuable metabolites in microorganisms, the technology may also be used to isolate genes for missing steps in biosynthetic pathways, to create metabolic pathways not seen in nature and to optimize the carbon flow into the desired pathways.

**Light-driven biosynthesis of bioactive compounds**

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View poster: Construction of a synthetic light-driven enzymatic supra-metabolon by Lassen LMM, Nielsen AZ, Jensen K, Møller BL, Jensen PE. Abstract page 66.

## Bioactive components in Algae

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Algae are already produced and utilized worldwide, and macroalgae accounts for 40% of marine aquaculture production[1]. Algae have been used as a nutraceutical and for medical purposes for millenniums in Asia[2], but in the Western world we want scientific proofs of the bioactive components of the algae. Recent reviews on bioactive components of both micro and macroalgae sum up the scientific proofs of e.g. anti-bacterial, antioxidant, anti-obesity, anti-cancer and anti-virus activities[3,4,5]. These activities have been shown in extracts and in the use of the entire algae, but also by the specific component, whether it is the pigment, polysaccharide, protein or fatty acids that accounts for the activity.

High production of fatty acids in microalgae and proteins in macroalgae make these suitable as feed or feed supplement for agriculture or fish. The high content of polysaccharides in the macroalgae mainly consist of dietary fibres; good for the dietary tract, however with very few calories.

Algae are attracting more attention in Denmark, because of their potential source of e.g. nutraceuticals, bioenergy or all inclusive in biorefineries. Biorefineries will utilize the low volume high value-added components for e.g. nutraceutical purposes and the high volume but low value components (waste) will be used for energy purposes. This concept is the basis of the research of a recent Danish Indian collaboration and also in agreement with the recommendations from the Seaweed Network of Denmark. The algae are too valuable because of the neutraceutical characteristics, but also too expensive to grow for bioenergy purposes alone.

The Seaweed Network has recently pointed out recommendations within business and research of macroalge of e.g. production including breeding, enzyme development to utilize the biomass, and clear guidelines of legislation in regard to non or “novel food” according to EU and daily intake recommendations. A steering committee will now initiate the making of a white paper with recommendations concerning these fields, aimed at guiding decision makers within research grants, business, local authorities and national politicians in Denmark.

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## Biodiesel fuels derived from microalgae

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Production of triacylglycerols in agricultural plants like canola, soybean, palm tree or other oil producing plants for biodiesel cannot be scaled up without seriously compromising global food supply. Economical production of lipids in microalgae requires an efficient and cost-effective cultivation of microalgae species that produces high amounts of lipids.

We have chosen to study a limited number of microalgae species for oil production. These species are being mutagenized and variants with proper phenotypes are being selected. Targets for improvements are: Increased growth rate, increased cell size, elevated lipid content, improved salt tolerance (for seawater algae), and enhanced lipid extraction yield. Analyses including fatty acid composition of neutral and polar lipids by liquid- and gas-chromatography coupled with mass spectrum analyses as well as fluorescence spectroscopy and flow-cytometry employing specific dyes. Screening of existing culture collections as well as algae collected from natural habitats will also be performed in order to identify species that may accumulate even higher amounts of lipids. The characteristics of the ultimate microalgae for large-scale lipid production are: Easy to cultivate in inexpensive media; fast growth and high biomass production; resistance to biological contamination; enhanced and consistent lipid production; easy to harvest; simple lipid recovery

The well-characterized green algae, *Chlamydomonas reinhardii* has been chosen as a model. Mutations are induced by genetic engineering and by conventional methods. The experiments serve as “proof of concept” for various genetic modifications and selection methods. If successful, similar protocols will be used for improvement of other microalgae species without employing *in vitro* DNA-techniques.

**The past meets the future: Evolutionary importance of the charophycean green algae and the prospects for developing them as model systems for plant biology.**

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The Charophycean Green Algae or CGA represent the group of extant green algae that are most closely related and ancestral to modern land plants. Their simple morphology and associated developmental strategies, notable biochemical similarities to land plants and ease of experimental manipulation make them potentially valuable model organisms for basic and applied plant research. Several CGA taxa have recently piqued considerable interest in research communities including the desmid, *Penium margaritaceum.* This alga represents a convenient and powerful unicellular model system for studying biochemistry, secretory pathways and development of plant extracellular matrices. *Penium* grows rapidly and produces only a primary cell wall consisting primarily of pectin, cellulose and arabinogalactan proteins. The homogalacturonan pectin domain is manifested in a series of tightly compressed fibrils organized in a “lattice” attached to the outer surface of the wall. Post-secretory modulation is focused at the cell’s central isthmus and proceeds in a distinct bipolar fashion. These events can be precisely monitored using live cell-labeling protocols with commercially available monoclonal antibodies along with various types of light and electron microscopy based technologies. Secretory events can also be elucidated using microplate-based experimentation with various inhibitors, enzymes and exogenous polysaccharides. Additionally, the internal secretory machinery of *Penium* consists of well over 100 Golgi bodies per cell that initiate spectacular vesicle transport and secretion mechanisms. These features make *Penium* an exceptional organism not only for basic biological studies but also for applied endeavors in plant polysaccharide processing and production. Other CGA also provide exceptional systems for studies of developmental patterning (*Coleochaete*), rapid gene induction (*Spirogyra*), bioadhesion (placoderm desmids), and heavy metal remediation (desmids). Molecular studies of the CGA are presently underway and once sufficient data banks become available, CGA-based studies will rapidly expand.

# Poster abstracts

## Products

# Productivity



# Nutrition

# Diseases

## Breeding

# Systems Biology

### Overview of GT-family-31 of the Carbohydrate Active EnZYmes Database

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Arabinogalactan-proteins (AGPs) are highly glycosylated macromolecules usually comprising 90-98% carbohydrate and 2-10% protein. They belong to a large family of plant cell wall (CW) glycoproteins, the hydroxyproline-rich glycoproteins (HRGPs), that are ubiquitous throughout the plant kingdom. AGP backbones are typically rich in hydroxyproline (Hyp), which are usually substituted by type II ArabinoGalactan (AG) chains. AGP protein backbones are extensively modified in the endoplasmic reticulum/Golgi apparatus prior to secretion to the cell surface. This includes *O*-glycosylation by GTs (*i.e.* the addition of type II AG chains), and the addition of a glycosylphosphatidylinositol (GPI) anchor, that enables the attachment of AGPs to the plasma membrane. In addition to the important roles in plant growth and development, AGPs have numerous applications as functional food ingredients and are increasingly recognized as having health benefits. However, the biosynthetic machinery involved in the synthesis of the glycan component of AGPs is poorly understood.
We will present an overview of the glycosyltransferase members of CAZy GT-family-31 using our recent research as a working paradigm (Egelund et al., 2011; Ellis et al., 2010; Qu et al., 2008).

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**Identification of long distance glucosinolate transporters in Arabidopsis provides insight in secondary metabolite redistribution**

*Tonni Grube Andersen[1], Nour-Eldin H H[1], Burow M[1] Fuller V[1], Jørgensen M E[1] , Olsen C CE[2], Hedrich R[3] , Geiger D[3] and Halkier B A[1]*

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Glucosinolates, also known as mustard oil glucosides, are a large group of plant secondary metabolites found mainly in the agriculturally important *Brassicaceae* family. Glucosinolates have been shown to be subject to long distance transport in the vasculature, and several observations indicate a source-sink transport network for glucosinolate redistribution.

We have identified two symporters capable of facilitating glucosinolate transport in the model plant *Arabidopsis thaliana* belonging to the NRT/PTR family. Biochemical characterization revealed that both facilitate a specific high-affinity proton-driven of glucosinolates. These transporters were hence named Glucosinolate TRansporter 1 and 2 (GTR1 and GTR2) respectively.

Upon bolting, Arabidopsis plants accumulate glucosinolates to high levels in seeds, whereas senescent leaves become depleted. In a double knockout mutant of GTR1 and GTR2, no glucosinolates were detected in seeds, but were concomitantly retained in leaves. In combination, our data shows that GTR1 and GTR2 are essential for long distance transport of glucosinolates in Arabidopsis.

These remarkable findings provide us with powerful knowledge for studying aspects of dynamical redistribution along the glucosinolate transport pathway as well as elucidating other secondary metabolite transport pathways.

In a broader perspective, identification of glucosinolate transporters provides new tools for exploitation of tissue-specific manipulation of glucosinolates in other related crops such as economically important rapeseed and cabbage species.

### Dissection of a secondary metabolite transport pathway

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An emerging area of glucosinolate research today, is to gain knowledge on transport processes of these secondary metabolites. We have recently identified AtGTR1 and AtGTR2 as glucosinolate transporters critical for long distance transport from leaves to seeds. A double knockout mutant in these transporters shows only trace amount of glucosinolates in known sinks. This remarkable feature is correlated with comparable increases in potential source tissues without affecting plant viability.

In wildtype and double mutant, we have analyzed the total glucosinolate content at various developmental stages of the whole plant including roots, rosette leaves, as well as inflorescences dissected into stem, flowers, cauline leaves, silique walls and seeds. The obtained data have enabled us to experimentally generate a comprehensive developmental glucosinolate transport map and to identify new sinks and sources.

Our data indicate that Arabidopsis carefully manages its content of glucosinolates and that glucosinolates are mobilized and redistributed during development to the tissue with highest fitness value. Identification of the transporters provides a powerful tool for dissecting the glucosinolate transport pathway, and serves as a model system for transport of secondary metabolites.

## Starch bioengineering in *Brachypodium distachyon*

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*Brachypodium distachyon* was recently introduced as a model plant for temperate cereals (Opanowicz *et al*., 2008). We aim to establish Brachypodium as a model for cereal starch metabolism. Grain starch from two lines: Bd21 and Bd21-3 are being characterized. Microscopic, chemical and structural data including amylopectin chain length distribution, phosphate content and amylose content provided further evidence for the close relationship to temperate cereals even though starch content and starch granule size were considerably lower than that for barley (*Hordeum vulgare*). Bioinformatics analyses identified starch biosynthesis genes including seven soluble starch synthases (SS), three granule bound starch syntheses (GBSS), four starch branching enzymes (SBE), two glucan- and one phosphoglucan- water dikinases (GWD, PWD). Phylogenetic analysis based on the SS genes provided evidence for a close relation to barley and wheat. Putative carbohydrate-binding modules (CBMs) of the families CBM20, CBM45, SBM48 and BM53 were identified and the positions of these were conserved. Based on these preliminary data we suggest that *Brachypodium distachyon* can provide a valuable model for starch bioengineering in temperate cereals.

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## Unravelling the Biosynthetic Pathway of Arabinogalactan Proteins

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Arabinogalactan proteins (AGPs) are not only important molecules in plant growth and development but also in industrial use. We would like to identify and characterize the enzymes involved in the glycosylation of AGPs in order to improve the basic understanding of these important glycoproteins, as well as provide tools for the modification of AGPs.
Our group has been investigating a glycosyltransferase (GT) from CAZy family 31 for its role in AGP biosynthesis. Biochemical assays using heterologously expressed protein suggested galactose transfer activity onto oligosaccharide composed of β-1,3-galactan. Beta-1,3-galctan is a backbone structure of AGP glycan and therefore the result indicated the enzyme as an AGP galactosyltransferase. The glycosydic linkage made by the enzyme onto β-1,3-galactan is currently under investigation.

Since mammalian GTs in the biosynthetic pathway are often organized as protein complexes (metabolons), we attempt to identify the other GTs as well as unknown interaction partners in the AGP biosynthetic pathway by analysing protein complexes of the above mentioned GT. *In silico* co-expression analysis indicates that some putative nucleotide sugar transporters are specifically coexpressed with the GT and we suspect a possible formation of metabolon of these transporters and the GT. We will characterize the transporters by heterologous expression in *N. benthamiana* and in *S. cerevisiae*, and will investigate protein-protein interaction by free flow electrophoresis, analysis on a native gel, bimolecular fluorescence complementation (BiFC) and Fluorescence resonance energy transfer (FRET).

## Elucidating Genes Conferring Natural Plant Resistance to Insects

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**Background:** Many plants have developed defense compounds that make them resistant against attacks from herbivorous insects. To study natural plant resistance to insects, we have developed a model system based on the wild crucifer winter cress, which is closely related to the reference plant *A. thaliana* and the crop *Brassica napus* (oil seed rape), and is resistant to a range of insect species.

**Results:** An unbiased LC-MS ecometabolomic approach based on a segregating population of a cross between resistant and susceptible types of winter cress identified four triterpenoid saponins as the main anti-insecticidal compounds against flea beetles. Pyrosequencing-derived transcriptomic datasets of the resistant and susceptible winter cress types were mined for oxidosqualene synthases (OSCs), cytochromes P450 (P450s) and family 1 glycosyltransferases (UGTs) that are considered key candidate enzymes for saponin biosynthesis. A genetic map of winter cress was created and showed division into eight linkage groups. Genome regions bearing genes for saponin and glucosinolate production, hairiness, and flea beetle resistance in winter cress were found. In parallel, the first UGTs putatively involved in *in planta* glycosylation of saponin aglycones were cloned from winter cress. Heterologous expression confirmed high specificity of these candidate UGTs towards saponin aglycones and investigation of their *in planta* expression pattern indicates co-regulation with saponin distribution in different plant organs.

**Conclusions:** As indicated by an ecometabolomic approach as well as QTL co-localization, triterpenoid saponins act as defensive compounds against flea beetle herbivory in winter cress. Current approaches to develop molecular markers based on synteny of winter cress and *A. thaliana* will further improve QTL definition and thereby facilitate identification of candidate genes involved in resistance to herbivory.
Saponin aglycone glycosylating UGTs from winter cress have been cloned and biochemical characterization studies indicate their involvement in saponin biosynthesis in planta.

### Cytochrome P450s involved in Thapsigargin biosynthesis

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Thapsigargin is a complex sesquiterpene lactone that has so far only be found within seeds and roots of plants belonging to the genus *Thapsia*. This guaianolide is currently undergoing clinical trials as a drug for the treatment of prostate cancer. Even though the content of thapsigargin is rather large in the plant (1-1.5 % of the dry weight), natural sources will not be able to meet future demands. Since large-scale synthesis of the compound is not possible because of its complex chemical structure, an alternative was desired. Presently thapsigargin's biosynthetic pathway is being elucidated by our group to enable an alternative production.

Although studies on sesquiterpene biosynthesis in plants of the family Apiceae have just started, we are already able to propose an enzymatic pathway starting from farnesyl pyrophosphate. Several steps in this pathway are believed to be carried out by cytochrome P450's. 454 and Illumina sequencing data of *Thapsia garganica* root and fruit cDNA lead to the cloning of 8 CYP450 candidate genes. These candidate genes share sequence similarites of 25-71 %. Among those are P450s belonging to new subfamilies, but all align nicely with other P450's shown to have sesquiterpenes as substrate. We have set out to characterize these P450s in regard of substrate specificity and their role in thapsigargin biosynthesis. To ensure optimal expression of these enzymes, a native *Thapsia* NADPH cytochrome P450 reductase was cloned and stably integrated into our yeast strain.

## Characterization of a family GH17 glycoside hydrolase highly co-expressed with a family GT31 glycosyltransferase in Arabidopsis

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CAZy glycoside hydrolase family 17 is one of the plant dominant GH families, and includes glucan 1,3-β-glucosidase, glucan endo-1,3-β-glucanase and lichenase. In Arabidopsis there are 51 genes in this family and *in silico* analysis suggests that several members are highly coexpressed with a glycosyltransferase from CAZy family 31, which is involved in type II arabinogalactan biosynthesis. We are interested in the relationship between these enzymes with respect to type II arabinogalactan biosynthesis. We chose the glycoside hydrolase (GH17) which shows the highest coexpression profile for further investigation.

This GH17 is expressed in the embryo and shoot apex in Arabidopsis. The protein sequence consists of an N-terminal signal sequence, a GH17 catalytic domain, CBM43 domain, and a site for GPI-anchoring on the C-terminus. The protein sequence between the catalytic domain and the CBM domain also contains S<u>P</u>S<u>P</u>SSSP sequence which is a potential motif for attachment of arabinogalactan side chains. We are interested in whether this GH17 is an acceptor-peptide for type II arabinogalactan attachment and/or it is involved in the modification of type II arabinogalactan in the cell wall. We are investigating these possible functions by analysis of Arabidopsis knockout mutants, and hydrolase assays using recombinant protein expressed in *N. benthamiana* and *E.coli.*

**Employing Translational Biology and Transport Engineering to eliminate Glucosinolates from *Brassica napus* Seeds**

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Glucosinolates are a diverse group of defence compounds characteristic of cruciferous plants, which include the economically important *Brassica* crops (e.g. rapeseed, cabbage and broccoli), as well as the model plant *Arabidopsis thaliana.*

Rapeseeds (*Brassica napus*) are an excellent source for oil used in the industry, and the remaining “seedcake” after oil-pressing has a high nutritional value, which would make it ideal for fodder. However, rapeseeds contain toxic glucosinolates. These compounds have hampered use of the otherwise very protein-rich seedcake in fodder. Intensive breeding programmes are thus directed towards reducing the seed load of glucosinolates.

We wish to employ translational biology to eliminate glucosinolate accumulation in rapeseeds by blocking transport to the seed. Two glucosinolate transporters, ATGTR1 and AtGTR2, have been identified in *Arabidopsis*, and the double knockout mutant *Atgtr1/Atgtr2* has been shown to be devoid of glucosinolates. Knocking out orthologues provides a novel approach for reducing seed glucosinolate content in rapeseed.

*B. rapa* is considered a valuable intermediate model crop in translational biology from *Arabidopsis* to *B. napus*. *B. rapa* TILLING (Targeting Induced Local Lesions IN Genomes) mutants for a candidate glucosinolate transporter have been obtained from RevGenUK. Biochemical characterization of the corresponding native transporter in Xenopus oocytes demonstrated glucosinolate uptake activity. Mutant lines harbouring non-functional glucosinolate transporter proteins will be analyzed for accumulation of glucosinolates in seeds.

**The genomic complement of purple acid phosphatase phytases in the *Triticeae***

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The endogenous phytase activity present in mature cereal grain is preformed during grain development and constitutes an important quality parameter for the utilization of phosphorus and minerals in food and feed. Cereals belonging to the *Triticeae* tribe are known to possess a significant preformed phytase activity in the mature grains, however varying significantly between the individual species. After multiple steps of chromatography, the phytase activity elutes as one peak, indicating that it consists of either one enzyme or more than one very similar enzymes. Recent work in our group** has demonstrated that these enzymes are purple acid phosphatase phytases (PAPhy's) encoded by a few highly conserved mRNA's expressed either during grain filling (*PAPhy_a's*) or germination (*PAPhy_b's*).

In the present study, 15 genomic *PAPhy* sequences from wheat, barley, rye, einkorn and *Aegilops taushii* were isolated and analyzed. Gene phylogeny and chromosomal mapping using aneuploid wheat and barley lines unravelled that the *PAPhy_a* and *b*'s constituted a set of paralogues located on the *Triticeae* chromosome 5 and 3, respectively. Cross-reference with sequenced and assembled grass genomes showed that this *PAPhy* duplication was shared only by the *Triticeae* sister tribe *Brachypodium*. In silico analysis of the *Triticeae PAPhy* promoters revealed a conserved 150 bp core promoter with two TATA-boxes. Upstream of this, the *PAPhy_a* and *b* promoters are without significant similarities. In this diverged section, *PAPhy_b* promoters contain elements typical of gibberellic acid induced germination related hydrolases. *PAPhy_a* promoters in contrast possess elements known from storage protein promoters.

****Dionisio G, Madsen CK, Holm PB, Welinder KG, Jørgensen M, Stoger E, Arcalis E, Brinch-Pedersen H.** Cloning and Characterization of Purple Acid Phosphatase Phytases from Wheat (*Triticum aestivum* L.), Barley (*Hordeum vulgare* L.), Maize (*Zea maize* L.) and Rice (*Oryza sativa* L.). *Plant Physiol. 2011a,* Jan 10.[Epub ahead of print]

### The biosynthetic genes in the cyanogenesis defense pathway are clustering in plant genomes

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The genomic clustering of unrelated genes encoding enzymes of the same chemical defense pathway has been observed in a number of plant species. Here we report the clustering of the core biosynthetic genes in the cyanogenesis defense pathway in three plant genomes, allowing their comparison. Cyanogenic glucosides are α-hydroxynitrile glucosides, which are synthesized by the sequential action of two cytochrome P450s, and a UDP-glycosyltransferase. Upon plant tissue damage, for example by insect feeding, the cyanogenic glucosides are hydrolyzed by specific β-glucosidases resulting in the release of hydrogen cyanide. The *cyanogenesis deficient1* locus in *Lotus japonicus* is identified as a partial deletion of the *CYP79D3* gene encoding the cytochrome P450 enzyme responsible for the first step in hydroxynitrile glucoside biosynthesis. The paralogous *CYP79D4* gene on the same genomic contig is suggested to have a more restrictive role in hydroxynitrile synthesis during early seedling development. This contig also contains the *CYP736A2* gene and the UDP-glycosyltransferase *UGT85K3* gene, completing a putative functional biosynthetic pathway. These three genes (*CYP79D3*, *CYP736A2* and *UGT85K3*) were confirmed to encode a cyanogenic glucoside biosynthesis cluster by their transient expression in tobacco which resulted in the ectopic synthesis of cyanogenic glucosides. Genomic co-localization of the biosynthetic genes in cyanogenic glucoside production was also observed in the cassava (*Manihot esculanta*) and sorghum (*Sorghum bicolor*) genomes. The β-glucosidases required for degradation of cyanogenic glucosides and release of cyanide gas do not co-localize with the biosynthetic genes in these genomes suggesting that the inheritance of a functional defense cassette was not a selection pressure for the clustering of the genes. We propose that the prevention of accumulation of toxic intermediates is the most likely explanation for the genomic clustering of cyanogenic glucoside biosynthesis genes.

**A single amino acid difference determines substrate specificity of β-glucosidases in hydroxynitrile glucoside metabolism**

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Cyanogenic glucosides (α-hydroxynitrile glucosides) are defense compounds found in a large number of plant species. The glucosylation is required for the stabilization and storage of these compounds. Upon tissue disruption, for example by chewing insects, the cyanogenic glucosides come in contact with β-glucosidase enzymes. These remove the glucose moiety resulting in degradation of the unstable α- hydroxynitrile and the release of hydrogen cyanide gas. The model legume *Lotus japonicus* produces the cyanogenic glucosides linamarin and lotaustralin derived from the amino acids Val and Ile. It also produces related non-cyanogenic γ- and β-hydroxynitrile glucosides called rhodiocyanosides.

Two closely related β-glucosidases, BGD2 and BGD4, play a role in hydroxynitrile metabolism in leaves. Mutant analysis showed that only BGD2 was involved in leaf cyanogenesis. Biochemical analysis confirmed that BGD2 is able to hydrolyze both cyanogenic glucosides and rhodiocyanosides, while BGD4 efficiently degrades rhodiocyanosides. Using sequence comparison and 3D-modelling we predicted key amino acid residues responsible for the difference in substrate specificity. The role of these amino acids was experimentally tested using site directed mutagenesis of the BGD4 sequence. Modeling predicted that Gly186 in BGD2, a Val in BGD4, was likely to be important for specificity as it determined the shape of the aglycone binding pocket. Changing the Val in BGD4 to a Gly enabled the modified BGD4 enzyme to use lotaustralin as substrate. Therefore a single amino acid change can be responsible for altering β-glucosidase specificity.

While leaves of mutants in *BGD2* were acyanogenic, flowers retained their cyanogenic potential. A related flower expressed β-glucosidase was identified based on the sequence requirements to hydrolyze cyanogenic glucosides and is being characterized presently.

**Functional and Biochemical Investigation of Sesquiterpene Synthases from *Thapsia***

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Terpenes comprise the largest group of plant secondary metabolites. The vast diversity and bioactivity of the terpene family make them highly valuable for a range of industrial and medicinal applications. Thapsigargin is a promising sesquiterpene produced by *Thapsia garganica*, and is currently undergoing clinical trials as a prostate cancer drug. Sesquiterpenes are a Terpene subgroup of C15 structures which have farnesyl pyrophosphate (FPP) as precursor. The first committed step in sesquiterpene biosynthesis is catalyzed by sesquiterpene synthases (STSs), and Thapsigargin biosynthesis is believed to proceed via an STS-catalyzed guaiene intermediate.

Two STS candidates, TgSTS1 and TgSTS2, have recently been isolated from *T. garganica* L. roots by our group. Preliminary observations indicated that TgSTS1 and TgSTS2 produce delta-cadinene and guaiene, respectively (by SPME and Pentane-ether extraction). In this study, targeted amino acid substitutions were performed in TgSTS1, according to corresponding residues in the 90% identical TgSTS2. As reference model, the structurally-characterized tobacco epi-aristolochene synthase (TEAS) was used to identify amino acids in and around the active site with potential roles in determining the product specificity. Five TgSTS1 mutants were created by site-directed mutagenesis and subsequently cloned into appropriate expression vectors.

Yeast expression studies with head space SPME provided stable and high sesquiterpene yields. Of the 4 STS1 mutants also analyzed, 3 showed a relative decrease in delta-cadinene production and increase in C5-C7, hydroxylated, and guaiene sesquiterpenes. The current work provides the initial steps in functional analysis of TgSTS1 and TgSTS2, with the future prospect of characterizing these enzymes and designing high yield guaiene producers.

## Towards production of Gum Arabic-variants in yeast

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Gum Arabic is a widely used food additive due to its emulsifying and stabilizing activity of oil, protein and fat in liquid phase. In addition to the properties as functional food ingredient, Gum Arabic has beneficial properties for human health as a prebiotic and immune modulator and by lowering glucose and cholesterol levels in blood. Gum Arabic therefore contributes remarkable nutritional value in our foods.

Gum Arabic is an exudate of acacia trees growing naturally in Africa with Sudan as the main producer. Variations in climate and political turbulence result in problems with unpredictable quality and supply. Recombinant production of Gum Arabic is an approach to ensure a stable supply with well-defined properties. To achieve this goal, it is essential to understand biosynthesis and structure/function relationship of Gum Arabic.

Gum Arabic glycoprotein (GAGP) is the main component of Gum Arabic. GAGP has many repeats of a 19 amino acids long GAGP-motif and it is heavily glycosylated by type II arabinogalactan. Marcia Kieliszewski and co-workers at Ohio University expressed the repetitive GAGP motif in tobacco BY2 cells and produced a glycosylated product with emulsifying and stabilizing activity comparable to native Gum Arabic. Therefore the production of recombinant Gum Arabic-variant by expression of GAGP-motif is a promising approach. In their system, the glycosylation relies on the endogenous enzymes present in the BY2 cells. However, since the glycan structure is most important for the emulsifying activity, we want to have control over the glycosylation step by identifying and characterizing each individual enzyme responsible for building the entire glycan, and we plan to reconstitute the pathway in yeast. The type II arabinogalactan is composed of a β-1,3-galactan backbone substituted with complex side chains containing arabinose, galactose, rhamnose and glucuronic acid, and its biosynthesis is poorly understood. Based on the linkages and sugars present in the type II arabinogalactan, at least 10 glycosyltransferases are needed to build the entire polymer, none of which have been identified. Our pursuit of identifying the glycosyltransferases is a close collaboration between KU and the Joint BioEnergy Institute in California. We have a collection of more than 700 glycosyltransferase genes from Rice and Arabidopsis in Gateway entry vectors and we are developing methods for screening expressed proteins for activities related to synthesis of type II arabinogalactan.

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**Chemical synthesis of AGP backbone motifs**

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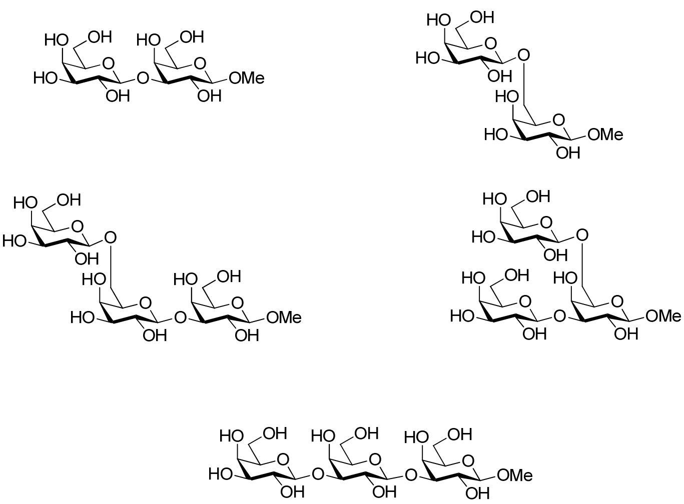
Gum Arabic is natural plant exudates in response to mechanical wounding of Acacia trees in Africa and is widely used as an emulsifier in the food industry. Gum Arabic is an arabinogalactan-protein and is composed of a linear β-(1-3) galactan backbone carrying short β-(1-6) galactan side chains.[1] This backbone is considered to be essential to emulsifier function. Motifs of this galactan backbone with side chains have been synthesised. Using a new strategy we have synthesised Methyl β-Gal- β-(1-3) Gal, Methyl β-Gal- β-(1-6) Gal, Methyl β – [Gal- β-(1-6)]- Gal –β- (1-3)-Gal, Methyl β –Gal – β-(1-3)-[Gal- β-(1-6)]- Gal, Methyl β –Gal – β-(1-3)-[Gal- β-(1-3)]- Gal. These motifs can be used to study the biosynthetic pathway of Gum Arabic arabinogalactan-protein.



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## Novel member of the CYP71AJ subfamily involved in the biosynthesis of furanocoumarins

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The Apiaceae plant family constitutes approximately 3,700 species including some very well-known species like the vegetables (e.g. carrot, celery, and parsnip), and the condiments (e.g. parsley, dill, and coriander). The plant Thapsia garganica L. is a member of this family and is currently undergoing a thorough investigation in order to clarify the biosynthetic pathways that lead to pharmaceutical interesting compounds. Transcriptomic 454 and Illumina sequencing data of root total RNA has led to the identification of a novel cytochrome P450 designated CYP71AJ5. The other biochemically characterized members of this enzyme subfamily are involved in the biosynthesis of furanocoumarins, CYP71AJ1-3 being identified as psoralen synthases (biosynthesizing linear furanocoumarins) and CYP71AJ4 being identified as an angelicin synthase (biosynthesizing angular furanocoumarins). Angelicin is found to be a substrate for CYP71AJ5, thus providing the next step in the biosynthesis of angular furanocoumarins.

Furanocoumarins, however, have never been identified in Thapsia garganica L. only hydroxycoumarins e.g. 6-methoxy-7-geranyloxycoumarin. We have therefore taken on various approaches to unravel this conundrum, i.e. metabolic scans on crude extracts from leaf and root material from this species using LC-MS and HPLC, searching for close psoralen synthase and angelicin synthase orthologous genes in genomic DNA of this species, homology modeling and substrate docking on CYP71AJ5 to find other substrate candidates, and functional yeast expression of CYP71AJ5 testing with other compounds that could be putative substrates.

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## Mutant study of cyanogenic glucoside and related compounds in barley

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The only known cyanogenic glucoside in barley (*Hordeum vulgare*) is epiheterodendrin. Upon enzymatic hydrolysis of the glucosidic bond, this α-hydroxynitrile glucoside can release hydrogen cyanide (HCN). In general, the release of HCN is known to function as a defence mechanism against plant-feeding pests. Barley lacks the enzymatic activity of a cyanogenic glucoside-cleaving β-glucosidase and does not release HCN. Therefore the biological role of epiheterodendrin, and its closely related non-cyanogenic β- and γ-hydroxynitriles, is unclear. These five barley hydroxynitrile glucosides are all derivatives of the amino acid leucine, and they all accumulate in the leaf epidermis.

We used a HCN-release assay applying additional almond-derived β-glucosidase, to identify low-cyanogenic barley lines. As screening material, we used the epidermis of the first leaf from a fast-neutron mutagenized barley population in the cultivar Golden Promise. We screened 3,600 individuals and identified 156 initial candidates, of which three candidate mutant lines have been confirmed. A low content of all five hydroxynitrile glucosides was confirmed by LC-MS. In order to search for the responsible mutations, the proposed biosynthesis genes will be sequenced. If these genes are intact, a map-based cloning approach will be initiated.

The role of hydroxynitrile glucosides in barley, which represent the major proportion of the sugar content in epidermis cells, will be studied. We will especially focus on the response of the mutant lines towards the epidermis restricted biotrophic fungus *Blumeria graminis*, which causes powdery mildew, a disease with high impact in agriculture. Furthermore, it will be of major interest to characterize the role of hydroxynitrile glucosides in plant development as carbohydrate and/or nitrogen source, using our iso-genic barley lines with high and low content of hydroxynitrile glucosides.

### Effect of nanocoating with Rhamnogalacturonan-I on surface properties and osteoblasts response

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Long-term stability of titanium implants are dependent on a variety of factors. Nanocoating with organic molecules is one of the methods used to improve osseointegration. Therefore, the aim of this study was to evaluate the in vitro effect of nanocoating with pectic rhamnogalacturonan-I (RG-I) on surface properties and osteoblasts response.
Three different RG-Is from apple and lupin pectins were modified and coated on amino-functionalized tissue culture polystyrene plates (aminated TCPS). Surface properties were evaluated by scanning electron microscopy, contact angle measurement, atomic force microscopy and X-ray photoelectron spectroscopy. The effects of nanocoating on proliferation, matrix formation and mineralization, and expression of genes (real-time PCR) related to osteoblast differentiation and activity, were tested using human osteoblast-like SaOS-2 cells.

It was shown that RG-I coatings affected the surface properties. All three RG-I induced bone matrix formation and mineralization, which was also supported by the finding that gene expression levels of alkaline phosphatase, osteocalcin and collagen type-1 were increased in cells cultured on the RG-I coated surface, indicating a more differentiated osteoblastic phenotype. This makes RG-I coating a promising and novel candidate for nanocoatings of implants.

### Polyphosphates in plants

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Phosphate is essential for all living organisms. In bacteria, fungi and lower plants such as moss and algae, phosphate can be stored in the form of polyphosphate, which consists of a linear polymer of ten to several hundreds of phosphate residues linked by high-energy phosphoanyhydride bonds. It is involved in various metabolic pathways. Polyphosphate can serve as P storage and an energy reservoir, and will chelate heavy metal ions such as $Ca^{2+}$. In bacteria, Poly P was shown to play a role in stress responses, gene regulation, motility and virulence. The polyphosphate kinase (PPK1) can catalyze both the synthesis of polyphosphate from ATP and the degradation of polyphosphate (while making ATP) and has been mostly studied in bacteria. Putative PPK1 have been identified in the genome of *Physcomitrella*, a moss whose whole genome has been sequenced, but no such enzymes have been found in higher plants yet. One of our aims is to understand the role of polyphosphate in plants, especially in lower plants, using *Physcomitrella* as a model organism and to engineer higher plants expressing the PPK1 to create a new form of P storage. These plants may serve in the phytoremediation of excess phosphate. Various methods for polyphosphate quantification exist, but the PPK assay which uses the recombinant *E.coli* PPK1, is one of the most sensitive and most convenient to use. In order to analyze polyphosphate levels in plants we have successfully cloned and purified the recombinant *E.coli* PPK1 using a poly His tag.  To further understand the metabolism of polyphosphate in plants, two putative PPK1 genes (PPK1A and PPK1B) were isolated from *Physcomitrella*. PPK1A and PPKB encode proteins of 90 and 98 kDa, respectively, and contain the recognized PPK1 domains that are present in *E.coli* PPK1. In order to biochemically characterize the proteins and confirm their function we have expressed the recombinant proteins in *E.coli* and purification is under way. Tobacco transient expression was used to localize the plant PPK1s at the cellular level. Arabidopsis plants overexpressing *Physcomitrella* PPK1s are currently being produced for analysis of polyphosphate content, phosphate and carbon metabolism.

### Changing the biomass composition of *Brachypodium distachyon* -more fructans for a more efficient biomass conversion

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Fructans are non-structural polysaccharides consisting of either β-2,1- or β -2,6-linked linear and branched fructose chains that act as a carbohydrate reserves as an alternative or in addition to starch. In some species and tissues they may account for up to 80% of the total dry weight. Fructans are commonly found in many of the important biofuel crops such as Switchgrass, Perennial ryegrass and Miscanthus.

Plant biomass is the largest available resource for the production of renewable liquid fuels the may replace fossil fuels in the future. However, the conversion of lignocelluloic biomass into biofuel is difficult due to the recalcitrance of these structures and the efficiency in terms of yield and cost is still too poor to make it feasible at a larger commercial level.

We intend to shift the carbon flux of the photosynthetic cells of the grass model species *Brachypodium distachyon* in order increase the proportion of soluble carbohydrates. The plants will be reprogrammed by overexpressing the biosynthetic enzymes of the fructan biosynthesis pathway and thereby changing the composition of the biomass allowing for easier and more efficient degradation.

Fructans are synthesized from sucrose by specialized fructosyl transferases, which can be classified depending on their preferential donor molecule. The S-type enzymes use sucrose while the F-type prefers fructan as fructosyl donors.

We have identified three putative fructosyl transferases from *Brachypodium*, which all have the chacteristics typical seen for S-type enzymes. These have been cloned into a Gateway based Binary Vector especially designed for transgene overexpression and cereal transformation (Himmelbach et al., Plant physiology, 2007, vol 145; 1192-1200) and we currently generating the transgenic plants.

Alongside, we have also cloned genes encoding fructan exohydrolases in *Brachypodium* and are currently working on cloning an endohydrolase from *Bacillus subtilis*, in order to obtain new enzymes suitable for degrading the fructans stored within the leaves.

**Engineering controlled mammalian type O-Glycosylation in plant cells**

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Human mucins are large heavily *O*-glycosylated glycoproteins (>200 kDa), which account for the majority of proteins in mucus layers that *e.g*. hydrate, lubricate and protect cells from proteases as well as from pathogens. *O*-linked mucin glycans are truncated in many cancers, yielding truncated cancer specific glyco-peptide epitopes, such as the Tn epitope (GalNAc sugar attached to either Serine or Threonine), which are antigenic to the immune system. In the present study, we have identified plant cells as the only eukaryotic cells without mammalian type O-glycosylation or competing (for sites) O-mannosylation. Machinery and target proteins for mammalian mucin type O-glycosylation were introduced into plants and initiation of mucin type O-glycosylation (Tn) demonstrated thus providing a first proof-of-concept of developing plant based platforms for production authentic and cancer specific human type O-glycoproteins in plants, which may *e.g.* be used as cancer-specific vaccines.

## The localisation of food reserves in marama bean (*Tylosema esculentum*) - a prospective African food legume

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Marama bean (*Tylosema esculentum*) is a wild perennial legume species and a prospective new crop in Southern Africa because of its exceptionally high nutritional value. The species is native to the Kalahari Desert and neighbouring sandy semi-arid regions of Southern Africa. Mature seeds are consumed roasted or cooked. The legume is also used fresh at the green immature stage.

Mature marama beans store large quantities of lipids and proteins, it is rich in dietary fibers, vitamins, minerals and essential amino acids. It contains no cyanogenic glucosides and no potent allergens as found in peanut. Despite the potential of marama bean as a healthy nutritive crop for developing countries, the species has not been domesticated and is thus not listed with other legume oilseeds such as peanut and soybean. Not much is known about the localisation of nutrients in the seeds and the nutritional value of immature seeds has not been investigated before.

In our study, proximate analysis indicated that mature marama seeds are rich in protein content (32%) comparing favorably with oilseeds. The protein level was high already at the immature edible stage (21%). The building up of storage proteins was followed in the electron microscope where protein bodies were closely associated with ER. At maturity, the protein-aggregates fused to form spherical bodies that were embedded in a droplet lipid matrix within the cell of the storage cotyledons.
The immature seeds had a low content of lipid (1.5%), whereas mature seeds had an exceptional high lipid content (40%), almost twice that of soybean. In mature seeds, lipid bodies encircle the protein bodies and line the plasma membrane, they do not seem to fuse. Histochemistry, scanning electron microscopy and confocal microscopy were applied as well in this study of accumulation of nutrients. The content and distribution of protein, lipid and carbohydrates in immature and mature marama beans make this underutilised nutritive legume a prospective crop plant and interesting for food processing applications.

## Glycosytransferases involved in arabinosylation of cell wall extensins

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Extensins are a group of ancient hydroxyproline rich cell wall glycoproteins that are found in some chlorophyte algae (such as Chlamydomonas), where they constitute the main wall building block, as well as in higher plant cell walls, where they constitute a relatively minor component of particular importance to wall assembly. The GlycosylTransferase family 77 (GT-family-77) *rra1-2* (Egelund et al. 2007) and *xeg113* (Gille et al. 2009) Arabidopsis, mutants have been suggested to be arabinosyltransferases involved in arabinosylation of extensins. We have now isolated extensins from these mutants and a new GT-family-47 putative extensin mutant, and obtained detailed structural information of the mutant extensin glycans. Based on the obtained structural information, suggested sites of action of the corresponding wild type enzymes in the extensin glycan synthesis are presented.

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## New insights in the vitamin D biosynthesis in plants

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Vitamin D is critically important for the development, growth, and maintenance of a healthy skeleton of vertebrates from birth until death. However, vitamin D deficiency is also a major health problem since vitamin D insufficiency increases the risk for development of osteoporosis, cancer, cardiovascular diseases, diabetes, and reduces immune defense. Plants containing vitamin D could have the potential to serve as a source of this important vitamin in feed and food. However, it is surprising and remarkable so little is known about to what extent plants produce D vitamins.
Some solanacean plant species, such as *Solanum glaucophyllum*, *Solanum lycopersicum* and *Nicotiana glauca* and the grass *Trisetum flavescens* have been reported to produce vitamin D or to show vitamin D-like effects on animals fed with them.
Due to the UV-b derived conversion of the 7 dehydrocholestrol (DHC) into vitamin D we performed light treatment on the species investigated and our procedure of extraction, purification and GC-MS and APCI-MS based methods revealed clear changes in the vitamin D and 7-dehydrocholesterol content after UV-b light exposure.
To investigate the biosynthesisof vitamin D in plants, we have explored the enzymes involved in the conversion of 2,3 oxydosqualene into cholesterol.
We have carried out functionality assays and localization investigation of three Arabidopsis thaliana enzymes involved in the late steps of cholesterol biosynthesis: sterol-C5(6)-desaturase, 7-DHC (dehydrocholesterol) reductase, and 24-DHC isomerase/reductase, that are expected to be upregulated in vitamin D producing plants.
Results obtained by *Nicotiana benthamiana* transient overexpression and A.thaliana stable transformation of these enzymes tagged with yellow fluorescent protein (YFP), revealed their endoplasmic reticulum (ER), plasma membrane (PM) localization and also the accumulation in particles, the latter is known for fungi but has never been reported in plant.
Moreover, we have isolated from *S. lycupersicum* three new genes; two of them are coding for enzymes showing a clear 7-DHC reductase activity and the last we propose is coding for a lanosterol synthase (LAS), which is present in chordate and yeasts but is not supposed to be functional in plant.
The presence of a functional LAS enzyme will provide evidence to hypothesize a new pathway leading to vitamin D, alternative to the well known pathway via cycloartenol.
In addition to that, the existence of two different enzymes performing the same 7-DHC reductase activity point in the same direction and future work will be directed towards a possible correlation with the biosynthesis of vitamin D by looking for the subcellular localization and substrate specificity of each of the two enzymes.

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### The role of DWARF5, $\Delta^{5,7}$-sterol-$\Delta^{7}$-reductase, in vitamin D biosynthesis in plants

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Vitamin D is a fat soluble steroid compound which is known to promote the absorption and metabolism of calcium and phosphorus in vertebrates along with a variety of other important functions. Vitamin D deficiency is an immense problem and it can cause conditions as osteoporosis, rickets, autoimmune and cardiovascular diseases, cancer and reduced immune defense.

Vitamin $D_3$ is known to be produced from photo conversion of provitamin $D_3$ (7-dehydrocholesterol (7DHC)) in the skin of vertebrates after exposure to UV B light.
The presence of vitamin D compounds has been established in a few plant families - including *Solanaceae* – and this represents an alternative source of vitamin D. Interestingly, the metabolic pathway leading to the formation of vitamin D in plants is basically unknown. The $\Delta^{5,7}$-sterol-$\Delta^{7}$-reductase (7-dehydrocholesterol reductase (7DHCR)) is known to catalyze the conversion of 7DHC into cholesterol in vertebrates while in plants a similar enzyme (DWARF5) converts the $\Delta^{5,7}$-sitosterol (7-dehydrositosterol (7DHS)) into sitosterol - a precursor for the brassinosteroid biosynthesis. Since the $\Delta^{5,7}$-sterols have a similar structure and can be processed by the same enzyme it should also be possible that 7DHS can be converted into vitamin D (vitamin $D_5$) by exposure to UV B light.

To verify if this conversion is occurring in plants *A. thaliana dwarf5* mutants have been exposed to UV B light. This *A. thaliana* line is accumulating $\Delta^{5,7}$-sterols (mainly 7DHS) due to a mutation in the $\Delta^{7}$-reductase and it shows a dwarf phenotype due to the down regulation of the brassinosteroid biosynthesis. *S. lycopersicum* (tomato) and *S. glaucophyllum* were also exposed to UV B light to see if any change in the composition of sterols was occurring. By blocking the 7DHCR/DWARF5 in vitamin $D_3$ producing plants 7DHC will also accumulate if present.

Two cDNA sequences highly homologous to *A. thaliana DWARF5* were isolated from *S. lycopersicum.* The whole cDNA sequences were amplified from *S. lycopersicum* and cloned into yeast and plant expression vectors. Complementation studies performed on yeast and GC-MS sterol characterization of the transformants showed a changed sterol profile which in both cases imply a lower level of ergosterol compared to the wild type and the accumulation of new compounds due to the $\Delta^{5,7}$-sterol-$\Delta^{7}$-reductase activity on ergosterol and its $\Delta^{5,7}$-sterol precursor.

The sequences fused to a GFP tag will be used to transform *S. lycopersicum*, *S. glaucophyllum* and *A. thaliana* wild type and *dwarf5* mutant plants.

To investigate the accumulation of 7DHC/7DHS a 200 bp highly polymorphic region of each cDNA sequence was amplified and used for virus induced gene silencing (VIGS) experiments. A chimeric probe was also designed on a highly conserved region to silence both genes at the same time. The VIGS experiment will be performed on *S. lycopersicum*, *S. glaucophyllum* and *N. benthamiana* which subsequently will be analyzed by Real Time PCR to assess changes in the gene transcript level and by GC-MS for modification in the sterol composition.

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### Biosynthesis of Triterpenes in *Tripterygium wilfordii*

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*Tripterygium wilfordii*, also known as ‘Thunder God Vine’, is a plant commonly used in traditional Chinese medicine and has been shown to produce a number of pharmacologically active sesqui-, di- and triterpenes. In particular, the triterpene celasterol has been shown to be an anti-inflammatory agent and have beneficial effects in the treatment of Alzheimer’s disease. The first committed step of celasterol biosynthesis is the cyclisation of oxidosqualene by a triterpene synthase (TTS) to form a friedelin-type triterpene backbone. Friedelin-type tritepenes are unique amongst the triterpenes as they are the product of the maximum number of rearrangement possible on the oxidosqualene substrate resulting in a ketone in contrast to an unsaturated alcohol. High-throughput sequencing of the *T. wilfordii* leaf transcriptome identified two highly expressed TTS’s present in the leaf. Phylogenetic analysis of these TTS’s indicate that they are more closely related to TTS’s involved in specialised metabolism and not general metabolism indicating these may be candidate genes for biosynthesis of friedelin-type triterpenes. Furthermore, members of the cytochromes P450 family CYP88, known to use terpenes as substrates, where also identified and may provide insight into further enzymes required for celasterol biosynthesis. Efforts are currently underway to biochemically characterise these candidate genes in yeast.

## Vanillin Biosynthesis pathway in *Vanilla planifolia*

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This research project is aimed at elucidating the biosynthetic pathway for vanillin glucoside in the vanilla orchid (*Vanilla planifolia*). Vanilla is the world's most widespread flavor and therefore one of the most popular plant natural products with an estimated annual worldwide consumption of over 2000 tons. Vanillin (3-methoxy-4-hydroxybenzaldehyde) is the main flavor compound in vanilla and is one of the key additives to food products, beverages, perfumery, and an intermediate in the pharmaceutical industry (Havkin-Frenkel and Belanger, 2011). Production of natural vanillin from pods of *V. planifolia* is a laborious and slow process.

Vanilla pods are matured, 9 to 10 months after hand pollination. Harvested pods are cured for several months to initiate enzymatic processes that break down flavor precursors and develop final flavor compounds. Production of 1 kilogram (kg) of vanillin requires approximately 500 kg of vanilla pods, corresponding to pollination of approximately 40,000 flowers. Currently, it is only 1% of the global production of vanillin that is derived from vanilla pods. Vanillin can also be produced synthetically using different fossil hydrocarbons. Biotechnologically produced vanillin is an alternative to the synthetic vanillin and can be obtained using microorganisms and cell culture processes (Palma et al., 2009, Hansen et al., 2009).

As vanillin is toxic in high concentrations to living cells, the glucosylated derivative, vanillin glucoside, is formed in plant cells. Vanillin glucoside is only found in the inner part of the vanilla pod (Odoux et al., 2003). Although vanillin glucoside seems to be a simple molecule, biosynthesis pathway of vanillin still remains to be revealed. It is speculated that the vanillin glucoside is a product of phenylpropanoid pathway from L-phenylalanine. Early studies have suggested, that ferulic acid or phydroxybenzaldehyde act as main precursors for vanillin glucoside biosynthesis (Havkin-Frenkel and Belanger 2011).

This study has taken three major approaches to elucidate the vanillin pathway. Namely, a genomic approach in which we have obtained the *V. planifolia* transcriptome from pod tissue. These sequences will be used for cloning of candidate genes, expressions and functional studies in yeast. Secondly, a biochemical approach to investigate intermediate compounds that are involved in biosynthesis of vanillin. For this purpose, C14 and C13 isotope labeled feeding assays were carried out with both L-phenylalanine and p-hydroxybenzaldehyde. Biochemical results confirm that vanillin glucoside is only formed in the inner part of the pod. It is also possible that the vanillin pathway includes several different conjugates and derivatives from Lphenylalanine rather than the single precursors from phenylpropanoid pathway (these data is yet to be confirmed). Thirdly, a proteomic approach in which soluble proteins from the inner part of the vanilla pod were extracted and will be analyzed by tandem mass spectrometry to obtain a profile of the proteins.

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## A new step in glucosinolate and camalexin biosynthesis

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Among the most well studied defense-related compounds in plants is the group of sulfur-rich secondary metabolites known as glucosinolates. After a decade of glucosinolate research in the Arabidopsis post-genomic era, the biosynthetic pathway is well understood and most biosynthetic genes are known. A notable exception is the step involving the incorporation of reduced sulfur, where the identity of the donating thiol has not been established. An indication of the involvement of glutathione as direct sulfur donor has come from *de novo* engineering of benzylglucosinolate into *Nicotiana benthamiana* (Geu-Flores *et al.* 2009). Another well-studied sulfur-containing plant defense compound in Arabidopsis is the phytoalexin camalexin, for which and the sulfur donor very recently has been established to be glutathione (Su *et al*. 2011).

The involvement of glutathione in these pathways would require further involvement of a glutathione conjugate processing enzyme. Here we demonstrate that an Arabidopsis mutant down-regulated in two novel γ-Glu peptidases (GGP1 and GGP3) shows altered glucosinolate levels and accumulates high amounts of related glutathione conjugates. Furthermore, we show that the double mutant is impaired in its production of camalexin and accumulates high amounts of the known camalexin intermediate GS-IAN upon camalexin induction. Finally, we show that GGP1's and GGP3's cellular and subcellular localization matches that of known glucosinolate and camalexin enzymes and that purified recombinant enzymes can both turn over the glucosinolate-related glutathione conjugate GS-B as well as GS-IAN. Our results demonstrate that glutathione is the sulfur donor in the biosynthesis of glucosinolates, and that GGP1 and GGP3 are cytosolic enzymes processing glutathione conjugates in the biosynthesis of both glucosinolates and camalexin.

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**Co-occurrence of cyanogenic glucosides and glucosinolates?**
**Two species – two cases**

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Occurrence of cyanogenic glucosides and glucosinolates are considered mutually exclusive within the same plant species. Papaya (*Carica papaya*) and garlic mustard (*Alliaria petiolata*) are rare exceptions to this rule.

The occurrence of phenylalanine-derived benzylglucosinolate and prunasin in papaya raises intriguing questions about the P450s involved and possible crosstalk between the glucosinolate and cyanogenic glucoside pathways. One hypothesis is that a single CYP71/83 is able to catalyze conversion of the oxime substrate to the cyanohydrin for cyanogenic glucoside synthesis and the *aci*-nitro compound-derived thiohydroximate for glucosinolate formation. Another possibility is that two different CYP71/83s are competing for interaction with a common upstream CYP79. An alternative route of prunasin formation could utilize desulfo-benzylglucosinolate-derived phenylacetonitrile as substrate for a CYP71-like-catalyzed conversion into the cyanohydrin. These questions will be addressed in biosynthetic studies and by heterologous expression and functional characterization of CYP83 and CYP71s present in the biosynthetic active tissues.

Garlic mustard is a glucosinolate producing plant releasing cyanide from an as-yet-unidentified compound (Cipollini and Gruner, 2007). The primary glucosinolate in garlic mustard is homomethionine-derived sinigrin. High amounts of the cyanoallyl glucoside alliarinoside are also present. We hypothesize that alliarinoside is biosynthesized from homomethionine through a pathway involving CYP79-catalyzed aldoxime-forming reactions followed by CYP71-catalyzed cyanohydrin formation. Dehydration of the cyanohydrin followed by oxidation by a flavin monooxygenase and the 2-oxoacid-dependent dioxygenase AOP3 or a homologue catalyze conversion of the methylthio group of 4-(methylthio)-but-2-enenitrile into a hydroxyl group. Hence, in addition to its well-established glucoside-metabolizing role in secondary modifications of glucosinolates, we suggest that AOP3 may also use aglycones as substrates.

An intriguing question is whether the hydroxynitrile may also be glucosylated hereby forming a novel homomethionine-derived cyanogenic glucoside, which could be the source of cyanide in garlic mustard. We are chemically synthesizing reference compounds in the suggested pathway for use in feeding assays and determination of metabolites by LCMS.

Papaya and garlic mustard are interesting gateways for elucidating the bifurcation and subsequent independent evolution of the pathways involved in cyanogenic glucoside and glucosinolate formation.

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### ROS signalling – Specificity is required

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The production of reactive oxygen species (ROS) increases in plants under stress. ROS can damage cellular components, but they can also act in signal transduction to help the cell counteract the oxidative damage in the stressed compartment. $H_2O_2$ may induce a general stress response, but it does not have the required specificity to selectively regulate nuclear genes required for dealing with localized stress, e.g., in chloroplasts or mitochondria. We here argue that peptides deriving from proteolytic breakdown of oxidatively damaged proteins have the requisite specificity to act as secondary ROS messengers and regulate source-specific genes and in this way contribute to retrograde ROS signalling during oxidative stress.
(This is a new project funded by FNU)

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**CY5 and CY5-like proteins are important at an early stage of chloroplast biogenesis**

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Plant chloroplasts evolved from a cyanobacterial ancestor after a single endosymbiotic event, which was followed by an extensive reduction of the plastid genome size. This has caused a limiting coding capacity of the plastid genome and most of the chloroplast proteins that have a structural or regulatory function are encoded in the nucleus and synthesized in the cytosol as precursors.

The CY5 and CY5-like proteins are nuclear-encoded homologous soluble proteins predicted to reside in the thylakoid lumen. Deficiency in either CY5 or CY5-like proteins leads to a seedling-lethal phenotype and homozygous knockouts of these proteins result in mutants with a pigment deficient phenotype that can only be grown on media supplemented with sucrose.

HPLC analysis of the chlorophyll biosynthesis pathway intermediates showed that the mutant plants are able to synthesise all the chlorophyll precursors. However, there is a restriction in the accumulation of Mg Protoporphyrin monomethyl ester (MgPME). This can be explained by the decreased level of the enzyme – MgProtoporhyrin IX methyltransferase, which is a membrane associated enzyme that catalyse the conversion of Mg Protoporphyrin IX to its methylester.

Ultrastructure analysis showed that mutant plants do not have fully differenciated chloroplasts. In the young CY5 and CY5-like mutant plants the initial thylakoid membrane formation is observed. However, further thylakoid formation is arrested at an early stage.

Expression level of investigated nuclear genes, coding for chloroplast proteins, is not affected. In contrast, both mutants have severely reduced levels of plastid encoded polymerase (PEP) dependent plastid transcripts. In contrast, nuclear encoded polymerase (NEP) dependent plastid transcripts are not negatively affected.

Additionally, analysis of protein levels in the mutant plants was performed. Nuclear encoded components of the photosynthetic complexes are barely detectable whereas nuclear encoded proteins with functions not related to photosynthesis are present in amounts comparable to wildtype. The fact, that some chloroplast encoded proteins are detected, suggets that plastidic translation machinery is present and functional.

To conclude, deficiency in CY5/CY5-like has a very profound impact on plant fitness and many processes in the chloroplasts suggesting that both proteins play an important role in early stages of chloroplast biogenesis, probably at a specific step in thylakoid biogenesis. Most likely, lack of CY5/CY5-like proteins affects the activity of the plastid encoded polymerase (PEP) and thereby preventing normal thylakoid formation.

## Construction of a synthetic light-driven enzymatic supra-metabolon

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Photosystem I (PSI) from plants, algae and cyanobacteria utilizes energy harvested from sunlight to mediate light-driven electron transport across the thylakoid membrane and produce reducing equivalents for the metabolic reactions of the photosynthetic organism. Cytochrome P450s constitute a very large and highly versatile superfamily of membrane-bound enzymes that catalyse a wide variety of different reactions, the most frequent being highly stereo- and regiospecific monooxygenations. In this project we are aiming at coupling PSI directly to a cytochrome P450 (P450) to develop a system in which the enzymatic reaction of some P450s is driven directly by the energy of solar light. As photosynthetic host organism we use the cyanobacterium *Synechococcus* sp. PCC 7002, which can be transformed, is fast growing and is tolerant to high light intensities.

In nature, PSI transports electrons from plastocyanin or cytochrome $c_6$ on the lumenal side to ferredoxin (Fd) or flavodoxin (Fld) on the stromal side of the thylakoid membrane. The electrons supplied by PSI may via the soluble electron carriers Fd or, during iron deficiency in some algae and cyanobacteria, Fld be donated to ferredoxin $NADP^+$ oxidoreductase (FNR), which reduces $NADP^+$ to NADPH. *In vivo*, the electrons required for the reactions of the P450s are taken from NADPH through the NADPH-cytochrome P450 oxidoreductase (CPR) enzyme. Our recent results from *in vitro* experiments indicate that the delivery of electrons to the P450 may not have to involve the production and oxidation of NADPH, but that direct electron transfer from PSI via Fld or Fd to a P450 adjacent to the PSI may be achievable.

A supra-metabolon containing PSI and a cytochrome P450 provides the opportunity for direct utilization of solar energy for production of complex chemical products. In this synthetic enzymatic supercomplex, the energy of the photons harvested by PSI will be utilized to mediate electron transport to the cytochrome P450 enzyme. To facilitate highly efficient electron transfer, the fusion protein complex has been designed to keep the interacting proteins in close proximity to each other. Thus we are aiming at linking the P450 directly to one of the small subunits of PSI containing one transmembrane domain. The P450 is fused to a PSI transmembrane domain so it can integrate directly into the PSI complex. Ultimately, the aim is to generate a system in which the fusion protein is stably expressed in the thylakoid membrane of *Synechococcus* and utilized to carry out the desired enzymatic reactions *in vivo*.

## The NAC transcription factors of barley

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It is now 15 years ago the first NAC transcription factor was described in the literature (Souer et al. 1996), since then a number of plant species have been fully sequenced revealing the NAC gene family to be one of the largest families of transcription factors in plants (Shen et al 2009). The NAC genes characterized so far have regulatory functions in a broad range of plant developmental processes and tolerances to both biotic and abiotic stresses. This makes the NAC family highly interesting target genes for plant researchers and breeders.

As part of a larger project on the identification of *Hordeum vulgare* (barley) leaf senescence regulators, we have attempted to characterize for the first time all presently available barley NAC genes (HvNACs).

By searching the NCBI barley EST database using the tBLASTn function, with all known NAC genes from Brachypodium and rice as input, in combination with in-house cloning and sequencing, we have produced a list of 48 barley NAC gene sequences (HvNACs). The full length coding sequences was obtained for all genes except 8. It was possible to design gene specific qRT-PCR primers for 46 of the NAC genes, which were used for their individual characterization. Using these primers we investigated the tissue specific expression patterns over a range of barley organs (young flag leaf, senescing flag leaf, young ear, old ear, milky grain, late dough grain, roots and developing stem) as well as their response to two different stress hormones (ABA and JA).

From these data we have identified not only putative regulators of leaf senescence (HvNAC005, HvNAC027 and HvNAC029), but also possible regulators of secondary wall synthesis (HvNAC033, HvNAC034 and HvNAC039), lateral root formation (HvNAC022) and seed development (HvNAC017, HvNAC018, HvNAC019 and HvNAC024).

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### Using the moss *Physcomitrella patens* as a platform for production of commercially attractive terpenoids

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This project aims at obtaining high level production of novel terpenoids in the moss *Physcomitrella patens*. *P. patens* is known to produce substantial amounts of the diterpene *ent*-kaurene, a common gibberellin precursor in eukaryotes, fungi and bacteria. Viable *P. patens* lines lacking *ent*-kaurene have been generated through targeted knock out by homologous recombination. These *ent*-kaurene free lines could constitute a great platform for *in planta* characterization of novel terpene synthases predicted to be involved in biosynthesis of high-value terpenoids. In addition to this, metabolic engineering will be performed to optimise *P. patens* strains for high terpene yields, a prerequisite for commercial production.

## Genetic engineering of myrosinase into *Nicotiana tabacum*

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Myrosinases are isoenzymes present in Brassicales, which catalyze the hydrolysis of a group of defence compounds called glucosinolates. The breakdown products released from this reaction, typically isothiocyanates, thiocyanates and nitriles, are biologically active and toxic. The glucosinolate-myrosinase defense system, also call the “mustard bomb”, has been partially transferred into tobacco. In order to complete this system, we propose to engineer the myrosinase gene into benzylglucosinolates-producing tobacco plants.

To evidence the activity of myrosinase in tobacco plants, we have expressed it in the *Nicotiana benthamiana* transient expression system along with the six genes of the benzylglucosinolates (BGLS) pathway. Biochemical analysis showed the presence of benzylisothiocyanates (BITC) in low amounts and also, detoxification products in the form of BITC conjugates, which were predicted to be formed due to the high toxicity of BITC.

Based on these results, we proposed to localize myrosinase in the apoplast of benzylglucosinolate-producing transgenic tobacco plants to prevent the hydrolysis of BGLS and avoid the production of detoxification products from BITC. Thus, glucosinolates will be hydrolyzed only after cellular disruption, for example insect attack. The gained experience will be used to generate transgenic potato plants that express the binary glucosinolates-myrosinase system in order to improve pest and disease resistance and reduce pesticide use in potato production.

## Iron Biofortification of Modern Wheat Cultivar

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Iron deficiency anemia (IDA) is a public health problem and is primarily due to poverty in the developing world which leads to a simple, undiversified diet that largely is based on stables, such as wheat and rice. One of the key targets in the international alliance HarvestPlus is to boost the iron content of these stables to improve the living conditions of the poor people. As member of HarvestPlus we are working to improve the iron content quanti- and qualitatively in the wheat grain. Therefore, wheat grain has been subjected to work through endosperm-specific expression of the ferritin protein as an iron storage complex. Primary evaluation of Bobwhite cv. has approved that endosperm expression of wheat's own ferritin, works as a sink for iron and accumulates two to three folds more iron in the endosperm. To bring the bioavailable iron on the people's tables, modern cultivars were applied for co-bombardment using *Bar* and ferritin cassettes. This, allows the possible out segregation of the antibiotic resistance gene and paves the way to meet biosafety and breeding criteria. We have created transgenic plants by a cisgenic approach. The genomic sequence including the coding part and the 3' untranslated part of the wheat's own most active homoeoallele of the *TaFer1* gene was inserted under the control of the wheat high molecular weight glutenine 1Dx5 promoter. Modern CIMMYT wheat lines were screened for transformability and two lines showing more than 1% transformation efficiency were used. Endosperm specific expression of the introduced ferritin gene was confirmed by real-time PCR and mineral analysis confirmed that lines with elevated iron content in the endosperm were obtained. Currently T1 plants produced seed flour has been subjected for bioavailability test as in-vitro by intestine Caco-2 cells.

The project also approaches to bypass the normal deposition of iron in the protein storage vacuole of the aleurone by tissue-specific down-regulation of vacuolar iron transporter, *Vit*1. Our hypothesis is that down-regulation of vacuolar iron storage will allow excess iron from the aleurone, symplastically to be translocated into the endosperm and stored. We have cloned the *Vit1-1* and *Vit1-2* genes from wheat and have made constructs for aleurone specific regulation. We have also found aleurone specific barley Ltp2 promoter in a same expression pattern in wheat. At present, transgenic plants are in the process of tissue specific expression analysis.

The combination of the ferritin and the *Vit1* approaches opens the opportunities to change iron storage from aleurone to the edible endosperm in cereal grains.
The work was supported by HarvestPlus, 2033 K Street, NW, Washington DC 20006-1002, USA

**Detailed insight into hordeins expression in high protein cultivars as a first stage to improve feed quality**

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Barley (*Hordeum vulgare*) is a very important nutrition source for monogastric animals like extensively produced pigs. Barley grain provides a high amount of starch and vitamins but is also characterized by unfavourable amino acid composition what force the need of supplementation before exploiting as a feed.

Hordeins, the main storage proteins of barley grain, are mostly responsible for unfavorable amino acid composition. Hordeins consist of gene families and their proportion in different lines may differ substantially. Detailed knowledge about their expression could be a starting point in creating novel cereal cultivars with high protein concentration and better amino acid composition.

Six barley cultivars with high protein content (Netto, PR3440, Kontiki, Fairytale, PR3528 and IC364) and Golden Promise as a control were selected for analysis of hordeins expression at four stages of grain development. All the known hordein coding sequences collected from commonly available databases (NCBI, HarvEST) were classified into groups and subgroups (B1, B3, C, D and Γ). Specific primers were designed with Primique and AlleleID softwares and checked with qRT-PCR first on the control cultivar Golden Promise followed by the gene expression analysis of the collected material from the high protein cultivars. According to the standard curve prepared for actin, the concentration of cDNA in all samples was evaluated and amount of individual transcripts was measured.  Amino acid profiles and protein content in all samples were analyzed using UPLC method.

It was noticed that there are big differences in quantity and quality of storage proteins transcripts in consecutive developmental phases and among selected cultivars. The data about mRNA expression, amino acids and total protein concentrations were plotted and analyzed using two chemometrics methods: PCA and PLS. Obtained models give an impression about correlations present in and among these two phenomena.

## Metallothionein in barley plants

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Metallothioneins (MTs) are small cysteine-rich proteins with a molecular weight between 5-10 kDa that are able to bind 10-20 weight % metal ions. The first MT was discovered in the 1950´s, when extracted from horse kidneys and since then these cysteine rich proteins have been found in a range of organisms including crop plants like barley and wheat. MT's form thermodynamically stable complexes with the $d^{10}$ element family (e.g. Cd and Zn) through the cysteine residue and it has been shown that MT's in plants are used to control the cytosolic Cu, Zn, and Cd homeostasis. It has been found that MT transcripts vary both in a tissue specific manner and during plant ontogeny and respond to a range of stimuli (e.g. excessive metal ion concentrations, heat, cold and salt). In major crop plants as barley and wheat biological engineering an increased level of MT could be the solution when aiming at an increased Cd tolerance, Zn storage capacity in the grain or fluxes of essential trace elements into the grain.

Only a few MT's have been purified from plant tissues and characterized. The reason is that MT's are sensitive to oxidation and are present in very low concentrations. Expressing MT genes in E-coli and yeast has made it possible to identify several plant MT's using mass-spectrometry. The coordination complex with wheat MT has been studied using electrospray ionization mass spectrometry (ESI-MS), but the sensitivity of this type of mass-spectrometer does not allow identification and quantification in low concentrations. Elemental analysis using hyphenated ICP-MS with its much higher sensitivity and higher matrix tolerance has been used to separate and quantify MT's and MT metal complexes in animal science, but not yet in plant science.

We have studied barley MT´s with special focus on the grain. MT3 and MT4 genes were expressed in E-coli and yeast. Yeast strains were used for expression studies and E-coli strains were used for producing large amounts of protein grown under elevated concentrations of Zn, Cd and Cu. The purified complexes were analysed on size exclusion chromatography (SEC)-ICP-MS using a method developed recently in our group, which allows simultaneous peak quantification of sulfur (S) and the elements Zn, Cd and Cu. This made it possible to determine the coordination complexes between the MT and metals under physiological growth conditions. The MT's were further characterized using ESI-TOF-MS allowing identification of the native MT and MT-Zn complex.

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## Implementation of biochemical screening to improve baking quality of Barley

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Barley (*Hordeum vulgare*) is mostly used in feed and malt production but has the ability to provide humans nutritional benefits. The current wheat based “barley” breads can unfortunately not exceed more than 20% barley flour mixed into the dough due to poor leavening properties. Therefore the objective is to develop barley varieties with good baking properties. The poor leavening properties of barley can be attributed, at least partially, to the physical properties of the storage proteins.

Studies based on small scaled baking trials and protein pattern analysed by SDS-PAGE on a large number of high protein barley cultivars suggested differences in baking quality and correlation between baking quality and D-hordeins.

The amino acid composition was measures using the newly developed AccQ Tag Ultra Amino acid (AA) derivatisation system designed for the Acquity UPLC. Obtained results from the total AA composition and the hordein pattern indicated that there are genetic variations not only in the distributions of the hordein polypeptides but also in the relative proportions of the storage proteins affecting the AA compositions. The free AA composition in the grain may also provide us the opportunity to give a forecast of the taste of the bread, as the AA composition is known to control certain aspects of the taste.

We uses a $MS^E$ approach on a time of flight instrument coupled to a UPLC and in gel digestion to identify and characterize the different D-hordeins responsible for baking quality. Digesting the proteins with chymotrypsin results in approx 60% sequence coverage in Biopharmalynx but higher sequence coverage is expected using both chymotrypsin and trypsin as overlapping peptides are produced in the optimal mass range. Sequencing the digested peptides also revealed post translational modifications of the identified hordein which needs further investigation.

Our results strongly indicate genetic variations in the relative proportions of the storage proteins affecting the amino acid compositions and baking quality.

### Effects of elevated atmospheric $CO_2$ on protein quality/quantity and Zn content of barley grain

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Among all metals Zn is required by the largest number of proteins. In plants, Zn deficiency leads to reduction of photosynthesis, decreased starch and protein formations, reduced auxin and amino acids levels and increased permeability of bio-membranes, which enhance inorganic P uptake. In humans it is estimated that nearly half of the world's population, primarily children and women in the developing world, suffer from zinc deficiency as they live on a diet that mostly is based on cereals and other starch-rich staples that have a quite low Zn content and with poor zinc bioavailability.

The rise of atmospheric $CO_2$ concentration during 21st century could lead to higher level of photosynthetic assimilates, improved water and nitrogen use efficiency as well as increased yield among the majority of plant species, especially those (eg., wheat) that fix $CO_2$ via the $C_3$ photosynthetic pathway. However, since the increases of yield is primarily in the form of starch there will be a "dilution" of other nutrients like minerals and proteins in the grain with severe consequences for the nutrition of human and livestock Although the nutritional short-comings could be met by using higher amounts of nitrogen and mineral fertilizer it would increase nitrogen and mineral load (in particular for zinc in areas with intensive pig production) so this strategy may not be warranted.

Alternative approaches to increase grain Zn content are to create transgenic lines that accumulate more Zn or to exploit natural variation in zinc content. Transgenic barley lines with a reduced C-hordein content showed not only an altered amino acid composition but a 2-3 fold increase in the grain zinc concentration. From bioinformatics studies we have also identified protein families which potentially might bind more zinc. The expression levels of these proteins in different types of materials with different zinc content are currently being assessed by quantitative PCR. In summary, it is the intention to generate barley that is genetically modified for improved zinc content through manipulation of protein quality/quantity.

Finally, we have found in a collection of barley cultivars (<300) with high grain protein content that there is a substantial genetic variation in grain Zn concentration in these germplasms. We will use this collection to further explore the potential link between protein quality/quantity and zinc content under elevated atmospheric $CO_2$ level.

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## Improving the nutritional quality of the barley and wheat grain storage proteins by antisense technology

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Prolamins are the predominant storage proteins in barley and wheat grains, accounting for 50 to 80% of total seed protein. However, the prolamins are not optimal feed for monogastric animals as they have a low content of certain essential amino acids such as lysine, threonine and tryptophan. As a result, a considerable amount of research is focused on improving the quality and quantity of seed storage protein both by traditional plant breeding and by modern genetic engineering technology.

In our research program we are trying to enrich the nutritional quality of barley and wheat grains using genetic modification with antisense or the more drastic RNAi suppression technology and study the change in protein pattern under different environmental conditions. We have five antisense and 12 RNAi C-hordein lines of barley (RNAi lines are under characterisation) and wheat RNAi lines (gamma and alpha gliadins) are also available from Germany and UK. We have grown them under different N regimes (high, medium and low N) in semi-field conditions.

Previously five different antisense C-hordein lines of barley have been characterized in our laboratory. The analyses revealed that the lysine, threonine and methionine content was increased with up to 16%, 13% and 11% while SDS-PAGE electrophoresis and reverse phase HPLC revealed a relative decrease in the amount of C-hordeins and relative increases in the content of other storage proteins.

Wheat omega-gliadin is highly homologues to the C-hordein of barley. We plan to construct wheat omega RNAi lines using RNAi technology. The cloning of the omega gliadin from wheat is in progress.

Finally, the agronomic properties and nutritional values of the genetically modified barley and wheat will be evaluated.

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## Cloning and Characterization of Purple Acid Phosphatase Phytases from Wheat (*Triticum aestivum* L.), Barley (*Hordeum vulgare* L.), Maize (*Zea maize* L.) and Rice (*Oryza sativa* L.)

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Barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) possess a significant phytase activity in the mature grains. Maize (*Zea mays* L.) and rice (*Oryza sativa* L.) possess little or virtually no pre-formed phytase activity in the mature grain and depend fully on *de novo* synthesis during germination. Here it's demonstrated that wheat, barley, maize and rice all possess purple acid phosphatase genes which expressed in *Pichia pastoris* gives fully functional phytases (PAPhys) with very similar enzyme kinetics. Preformed wheat PAPhy was localized to the protein crystalloid of the aleurone vacuole. Phylogenetic analyses indicated that PAPhys possess four conserved domains unique to the PAPhys. In barley and wheat, the *PAPhy* genes can be grouped as *PAPhy_a* or *PAPhy_b* isogenes (barley: *HvPAPhy_a* and *HvPAPhy_b1*, *HvPAPhy_b2;* wheat: *TaPAPhy_a1*, *TaPAPhy_a2* and *TaPAPhy_b1, TaPAPhy_b2*). In rice and maize only the "b" type (*OsPAPhy_b* and *ZmPAPhy_b*, respectively) were identified. HvPAPhy_a and HvPAPhy_b1/b2 share 86% and TaPAPhya1/a2 and TaPAPhyb1/b2 share up to 90% (TaPAPhy_a2 and TaPAPhy_b2) identical amino acid sequences. In spite of this, *PAPhy_a* and *PAPhy_b* isogenes are differentially expressed during grain development and germination. In wheat it was demonstrated that "a" and "b" isogene expression is driven by different promoters (~31% identity). *TaPAPhy_a/b* promoter reporter gene expression in transgenic grains and peptide mapping of TaPAPhy purified from wheat bran and germinating grains confirmed that the *PAPhy_a* isogene set present in wheat/barley but not in rice /maize are the origin of high phytase activity in mature grains.

**Heterologous expression and purification of barley (*Hordeum vulgare* L.) cysteine protease in yeast**

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The mobilization of protein during germination of barley seeds is essential and Cysteine Proteases accounts for more than 90 % of the total proteolytic activity in the degradation of barley seed storage proteins [1]. Cysteine proteases exist as pro-enzyme until activated through reduction of the active site cysteines and via removal of the pro-domain. The complement of cysteine proteases is comprehensive and for detailed studies of the individual components of this complement, a fast and efficient eukaryotic expression platform is highly desirable.

The barley key cysteine protease, endoprotease B2 (HvEPB2) was cloned, inserted into the *Pichia pastoris* expression vector pPICZ Aα and electrotransformed into *Pichia pastoris* strain KM71H. Heterologous protein production was induced with 2% MeOH and after 4 days, the supernatant was harvested. HvEPB2 was purified from the supernatant by $Ni^{2+}$-affininty chromatography and the purified fractions were analyzed via SDS-PAGE, western blotting for confirming the presence of HvEPB2 and via activity assaying. Purified HvEPB2 incubated with Osborne fractionated barley seed storage proteins revealed after SDS-PAGE a significant degradation of the storage proteins after 24 hours of incubation.

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### Characterization of N-type glycosylation sites and glycan structures of Purple Acid Phosphatase Phytases from Wheat (*Triticum aestivum* L.)

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Wheat (*Triticum aestivum* L.) possesses preformed phytase activity in the grain that is essential to make phosphate available to cell metabolism and in food and feed (*Brejnholt S.* et al., 2011). Cereals contain the purple acid phosphatase type of phytases, PAPhy (*Dionisio G.* et al., 2011a). Mature wheat grain is dominated by TaPAPhy_a which, after chromatographic purification, has been characterized by extensive peptide and glycopeptide sequencing by mass spectrometry. Seven N-linked glycosylation sites were found. Three of these sites were dominated by variant forms of the XylMan3FucGlcNAc2, i.e. the HRP-type of glycan. Complex-type glycans with one or two additional GlcNAc were observed, however in trace amount only. The mature protein is ca. 500 residues in size and appears to be truncated at the N- and C-termini (*Dionisio G.* et al., 2011b).

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## Phosphate transporters in *Pisum sativum*: functional studies using VIGS

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Phosphorus is an essential element, which is required for plants to grow and in animal and human nutrition. Plant growth is phosphorus limited in most soils and mineral fertilizers are therefore needed to maintain acceptable crop yields. However, current fertilization practice needs to be re-evaluated as reserves of phosphate rock are expected to become depleted within the next 5-6 decades.

Breeding of crop plants has been performed under high fertilization and it is therefore likely that there is a potential to breed more nutrient-efficient crops. Phosphate (Pi) can be taken up by most plants via two pathways, either through a direct pathway (DP) via the root or through symbiosis with arbuscular mycorrhizal fungi (AMF), the mycorrhizal pathway (MP).

We work with phosphate-uptake pathways in pea plants with focus on the MP-mediated Pi uptake. Our initial screening for PT genes in pea resulted in identification of one PT gene, which is expressed at low Pi "*PsPT1*" and one which is only expressed in AMF-colonized roots "*PsPT4*". The presence of more PT-genes was indicated by genomic Southern analysis.

Pea is not easily transformed and we therefore used virus-induced gene silencing (VIGS) for functional analysis of a phosphate transporter (PT) gene. VIGS was combined with the use of physiological isotope tracer uptake studies to discriminate between Pi uptake via MP and DP.

Silencing of the *PsPT4* resulted in reduced root Pi uptake via MP. Furthermore, AMF colonization was reduced in *PsPT4*-silenced roots. These results confirm the assumption that we have isolated a phosphate transporter involved in MP-mediated Pi uptake and show that the silencing levels obtained through VIGS were sufficient to interfere with the activity of *PsPT4.*

## Importance of individual glutamine synthetase isogenes for nitrogen metabolism and nitrogen use efficiency in *Arabidopsis*

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Plants have a fundamental dependence on inorganic nitrogen (N), which is usually taken up in the form of nitrate and ammonium. In current agriculture, crop plants are supplied with N fertilizer, but a substantial portion of this is lost from the soil and leached into the groundwater, rivers and oceans (1). Improving nitrogen use efficiency of crop plants, especially at low N input, is essential to ensure sustainable food production in the future.

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme of plant N metabolism, as GS catalyses the assimilation of ammonium in higher plants (2). Ammonium is assimilated into glutamine and glutamate through a consecutive reaction of GS and glutamate synthease (GOGAT), the so-called GS/GOGAT cycle. There are two types of GS isoenzymes in plants. They localize in different compartments: GS1 is located in the cytosol and GS2 in the plastid/chloroplasts. GS1 is the major form of GS in plant roots, and the ammonium taken up from the soil is directly converted to Gln by its reaction. Molecular biological studies have identified a number of genes encoding GS1 from various plant species (3-7). In Arabidopsis, five genes for GS1, GLN1; 1-5 are encoded in the genome (8). The multiplicity of cytosolic GS1 genes in higher plants does not simply provide functional redundancy (9). Rather, specific properties of each individual isogene and encoded protein are envisaged in terms of individual expression patterns as well as distinctive kinetic properties and ammonium responsiveness (10). The exact functional roles and physiological diversities of the individual GS1 isogenes in the model plant Arabidopsis have not been well characterized.

In this present PhD project, we will use single and multiple knockout mutants to study how each of the *Gln1* genes contributes to processes determining NUE. We will study the response of the mutants to various levels and forms of N in terms of shoot biomass, root biomass, seed biomass, N distribution between tissues, ammonia and nitrate levels and amino acid content. In addition, we aim to assign specific functions to the isogenes, for example to find out which one is responsible for reassimilation of ammonium during senescence, during which GS2 is degraded in the early phases. We will use $^{15}N$ to monitor N remobilization in single and multiple mutants.

Using wt plants, we want to draw a map of the expression patterns of the individual GS1 isogenes in terms of their organ- and cell-specific expression in shoots during the life cycle of the plant. This will be carried out using promoter-reporter constructs as well as quantitative real-time PCR analysis.

Furthermore, we want to over-express GS1 isogenes constitutively, in specific organs and at specific developmental times in order to increase the NUE of Arabidopsis.

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## Changing expression of cytosolic glutamine synthetase (GS1) to alter nitrogen use efficiency (NUE) in barley (*Hordeum vulgare* L.).

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Production of crop plants with increased nitrogen use efficiency (NUE), through conventional breeding or biotechnology, is a major research area in plant nutrition. N metabolism in both model and crop plants has been found to be complex and to differ between plant species due to e.g. tight regulation of enzymes involved and interconnection with the carbon network. Accordingly, it is a challenging task to alter this N network in order to increase NUE (Good *et al.* 2004).

Glutamine synthetase (GS) has been identified as a key enzyme involved in N metabolism and is crucial for NUE in e.g. rice and maize (Cai *et al.* 2009; Martin *et al.* 2006). GS catalyses the initial step in ammonium assimilation, i.e. the ATP dependent amination of glutamate to form glutamine. Two major isoforms of GS exist. GS2 is located in the plastids and assimilates N produced mainly from photorespiration, while GS1 is located in the cytoplasm and assimilates N from primary uptake. It has been found that one gene encodes GS2, while several genes encode GS1 isoforms (e.g. five in *Arabidopsis thaliana* and at least two in barley). The differential regulation of the multiple isoforms of GS1 enables a detailed control of expression, depending on developmental and environmental cues (Bernard *et al.* 2009).

In the present project, the role of GS1 will be investigated in barley (*Hordeum vulgare* L. cv. Golden Promise). Barley is one of the most important cereals on a worldwide basis, and is closely related to wheat, which has a much more complicated genome. Project activities involve molecular and physiological characterization of barley constitutively over-expressing *GS1a*. Transgenic 35S::GS1a lines were produced by Agrobacterium-mediated transformation. The regenerated transformants have initially been screened for the presence of the resistance marker *hptII* and subsequently examined by RT-qPCR to identify lines with few T-DNA inserts and a high *GS1* expression. The selected T2 lines have been grown in the green house and will be compared to wildtype at three different developmental stages and at two different nitrogen levels with regards to total N content, ammonium, nitrate and amino acid composition, GS enzyme activities, grain protein content and total biomass.

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### Effect of foliar nitrogen application on yield, nitrogen content and leaf scorching in field-grown wheat cultivars

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Wheat covers about 25% of the agricultural area in the EU and is the dominating cereal crop. In total, about 2900 million kg mineral fertilizer N is annually applied to wheat crops, corresponding to about 30% of the fertilizer N use in EU. On average, about 30–50% of the applied mineral N is recovered in the harvested grain during the first season. Higher values, exceeding 60%, have been reported for winter wheat in Denmark and in the UK.

Improvement of the use efficiency of mineral N fertilizer is a major goal with respect to sustainable intensification of wheat production. Foliar N fertilizer constitutes a direct and immediately available N source for the crop. As such, the combination of early application of N to the soil at sowing and later top-dressings might be a way to refine fertilization strategies to better match plant demand. However, foliar fertilization might result in leaf burning and a negative effect on plant growth and crop yield. Using winter wheat grown in climate chambers and the field, we are investigating the molecular-physiological processes which control the use efficiency of foliar applied N or are involved in the processes causing leaf burning.

A key enzyme in N metabolism is glutamine synthetase (GS), which assimilates mineral N into organic compounds and is important for controlling grain size and grain numbers in maize (Martin et al. 2006 Plant Cell 18, 3252-3274).

Experiments in the glasshouse have shown that foliar N applied as 0-40% Urea-Ammonium-Nitrate (UAN) was absorbed into the leaf within 1-6 hours and resulted in ammonium accumulation in the leaves to concentrations as high as 400µmol/gDW. In the same timeframe, GS activity rapidly declined, particularly in the 20-40% UAN treatments, which is likely a consequence of feed-back regulation caused by the accumulation of the reaction product glutamine and a simultaneous decline in the substrate glutamate. After 3 days, leaf burning was observed in plants treated with 20-40% UAN.

Based on field experiments, we have selected 6 modern genotypes with contrasting characteristics in their growth response to N. In the summer of 2010, these winter wheat cultivars were grown in a field experiment at Højbakkegård with different levels of nitrogen applied to the soil and to the foliage. Biomass data and total N content data has been obtained.

Of the total N applied to the crop (soil+ foliar), 80% was recovered in the straw and grain at maturity. Foliar N and N applied to the soil were equally well absorbed by the plants. This indicates that absorbtion, assimilation and transport of N from the leaves were not rate-limiting steps in the utilisation of foliar N. The leaf burning which was observed in this field experiment did not affect crop performance and N use efficiency. The effect of foliar N application on grain yield and protein content depended on the level of N supplied to the soil. At low soil N, the foliar application of N at flowering resulted mainly in an increase in grain N concentration, indicating higher protein content. At high soil N, the foliar application of N had an impact on grain yield. Future experiments will explore the observed genotype-specific variation in foliar N use efficiency and tolerance to leaf burning.

## Seasonal variations of nitrogen status and turnover in three different vegetation types

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### Overview

There is a close relationship between plant-air ammonia ($NH_3$) exchange and plant nitrogen (N) metabolism. The direction and magnitude of $NH_3$ exchange vary between different vegetation types and with plant developmental stage in close interaction with external environmental factors. Information about seasonal changes in plant N status and turnover are therefore required when developing models to estimate $NH_3$ exchange in terrestrial vegetations. In the present study, three different plant species, *viz.* beech (*Fagus sylvatica*), Douglas fir (*Pseudotsuga menziesii*) and ryegrass (*Lolium perenne*) were selected as representatives of deciduous forest, evergreen coniferous forest and perennial grassland ecosystems, respectively. Seasonal changes of N pools in the three different vegetation types were measured together with different N-related parameters and carbon content at different leaf positions.

### Methods/Approach

The sampling was carried out once or twice a month in 1998-1999 and 2008-2009 for beech leaves (Soroe, NitroEurope level 3 field site, Denmark), 2008-2010 for fir needles (Speulderbos, NitroEurope level 3 site, The Netherlands), and 2008-2009 for ryegrass (Easterbush, NitroEurope level 3 site, Scotland). In the forest ecosystems, leaves and needles were collected from the top (representing sun leaves) and base (representing shade leaves) of the crown of each of 4 trees. Ryegrass samples were collected in four replicate 1 $m^2$ sub-plots and subsequently divided into green leaves, green stems, senescent leaves, senescent stems and litter. All plant samples were immediately frozen upon sampling and kept in this condition until lyophilized or oven-dried. Chlorophyll was extracted from frozen leaf material in methanol for beech and ryegrass or dimethyl sulfoxide for Douglas fir. Other analyses were carried out as described by Husted et al. (2000), Mattsson et al. (2009) and Wang et al. (2011).

### Results

Seasonal patterns in total N differed among the three vegetation types. Beech leaves showed steadily declining leaf N concentrations over the season, while in ryegrass and Douglas fir, total N remained relatively stable. Bulk tissue Γ ($NH_4^+/H^+$ ratio) was constructed as a simple indicator for a comparison of $NH_3$ exchange potential among different vegetation types. Among the three vegetation types, bulk tissue Γ values decreased in the order: ryegrass green leaves > beech leaves > fir needles. This is in agreement with the fact that green leaves of ryegrass had the highest total N followed by beech leaves and fir needles during the major part of the growth season. The largest value of bulk tissue Γ was present in senesced materials of ryegrass, which previously have been demonstrated to act as an $NH_3$ source (Mattsson et al. 2009). The inter-specific and seasonal differences in Γ values did not match the corresponding patterns of bulk tissue $NH_4^+$, which in all three species showed distinct seasonal peaks (data not shown). The carbon content showed very limited seasonal variation, remaining at 46-49%, 47-50% and 39-42% on a dry matter basis of Douglas fir needles, beech leaves and ryegrass green leaves, respectively. The C content in stems and senescent tissues of ryegrass was lower than in green leaves and attained larger seasonal variation, decreasing down to about 31% during winter. In conclusion, our results demonstrate clear differences between vegetation types in N parameters and in the bulk tissue Γ values.

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### Suppression of *Fusarium graminearium* growth and toxin production by differently structured starch types

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Native starch granules possess different enzymatic degradability. Here we investigate the growth behavior and excreted amylolytic activity of *F. graminearum* cultivated on different types of native starch granules including normal barley (A-type crystalline polymorph), potato and *Curcuma zedoaria* (B-type crystalline polymorph), cassava (C-type crystalline polymorph) and high amylose maize (A+V-type crystalline polymorphs). *F. graminearum* grew poorly on B-type starches and the accumulation of biomass was similar to the control cultivated under carbohydrate starvation. In comparison three to five fold higher accumulation of fungal biomass was observed for growth on the A, C and A+V-type starches. Fungal amylolytic activity was induced by all types of native starch granules. B-type starches were most resistant to Fusarium exo-amylases and also induced the highest amylolytic activity. *F. graminearum* appears to respond to resistant starch types by a massive induction of amylolytic activity. This induction is possibly mediated by starch degradation products including maltose and malto-oligosaccharides. Glucose acted as a classical catabolite repressor.

## Effector candidates in biotrophic plant pathogens.

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Biotrophic plant pathogens like rust and powdery mildew fungi live in intimate contact with the hosts and are dependent on their ability to express and transfer effector proteins into the hosts. A major role of effectors is to inhibit host defence mechanisms and keep the plant cell alive; another role might be to re-direct the flow of nutrients towards the fungus.

A transcriptome analysis of barley epidermal strips highly infected with powdery mildew fungus (*Blumeria graminis f.sp. hordei, Bgh*) revealed a large super-family of effector candidates, some of which are highly expressed in the haustoria. They all have a common amino acid motif, which we named “YxC”, close to the N-terminal (Godfrey et al. 2010).

Careful annotation and analysis of the newly sequenced *Bgh* genome and comparisons with the draft sequences of two other powdery mildew fungi, *Erysiphe pisi* and *Golovinomyces orontii,* show that *Bgh* in total has more than two hundred YxC-effector candidates and that they are restricted to *Blumeria* (Spanu et al. 2010).

Analyses of gene sequences from the rust fungi *Puccinia graminis f.sp. tritici* and *P. triticina* show that the YxC-motif is also present in these cereal pathogens. This suggests that the YxC-motif is more connected to the hosts than to the pathogens. Therefore, we predict that the YxC-motif has a function in the host, likely in transport of the effectors into the host cytoplasm equivalent to the RXLR-motif in Oomycetes.

We will further discuss how we try to assign function to some of the effector candidates.

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## Development of a screening method for R gene specificity in wheat

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Crops and their wild relatives contain several hundred genes that confer disease resistance (R genes). This pool of genes represents a vast resource for breeding resistant crops, but the identification of R genes is lengthy and laborious by classical genetic approaches. Thus, the lack of knowledge about R genes, and the pests individual R genes may provide resistance against, is a major bottleneck in plant breeding.

Recently our cooperators from KU found that R genes mutated in specific domains act as dominant negatives conferring susceptibility, when introduced into plants resistant to the considered pathogen due to the presence of the wild type R gene. Based on this discovery and previous work of *Arabidopsis*, a strategy to match R genes to specific pathogens has been designed in wheat.

In wheat cultivar 'Bobwhite S-26' we will establish proof-of-concept using the wheat yellow rust system. The yellow rust R gene *Yr10* will be transformed into Bobwhite, and transgenic plants tested for resistance against an appropriate avirulent *P. striiformis* isolate harboring the *AvrYr10* gene. Regenerated transgenic plants will be re-transformed with a mutated form of *Yr10*. The host-pathogen phenotype resulting from inoculation with the appropriate pathogen isolate will be recorded, with susceptibility of the plants as anticipated outcome. The presence or absence of *Yr10* will be confirmed with a *P. striiformis* wild type *AvrYr10* isolate and a corresponding*Yr10*-virulent mutant isolate.

For the screening strategy, we will identify an inventory of wheat R genes via deep transcriptome sequencing. A Bobwhite-specific library of expressed NB-LRR genes will be generated. Via deep sequencing, publicly available DNA sequence information will work as scaffolds for establishing correct NB-LRR cDNA contigs. We plan to screen ~100 R genes from Bobwhite cultivar.

Transgenic Bobwhite lines with mutated forms of R genes will be inoculated with selected avirulent isolates of *P. striiformis*. Susceptibility will indicate that a mutated form of the R gene that confers resistance to a particular isolate is present in the transgenic plant, and in this way the R gene can be identified.

Based on the test system developed in Bobwhite S-26, the long term aim of the project is to develop a screening strategy to identify R genes in any wheat variety showing an R gene-based resistance phenotype against specific pathogen isolates. The identified R genes can subsequently become exploited in breeding for disease resistance.

## Effects of climate change on plant health

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In our future climate the mean annual temperature and concentrations of atmospheric $CO_{2,}$ and tropospheric ozone will increase substantially. Therefore, it is important to understand how these factors, individually or in combination, may influence plant growth and plant-pathogen interactions. Recent studies indicate that $CO_{2,}$ and temperature can be beneficial for plant growth but their combinations non-beneficial. However, very little is known regarding the effects of multi-factorial climatic change on plant–pathogen interactions and our current study aims to address this.

We investigate possible effects of multi-factorial climate change on disease resistance in barley towards the biotrophic fungus *Blumeris graminis* sp. *hordei* (powdery mildew) and the hemibiotrophic fungus *Bipolaris sorokiniana*. Elevated temperature or ozone concentration, but not $CO_2$ resulted in increased basal resistance against powdery mildew. However, elevated $CO_{2,}$ either in combination with increased temperature (2 factor) or increased temperature and ozone (3 factor) dramatically compromised basal resistance.

For *B. sorokiniana* elevated $CO_2$ resulted in less disease symptoms, elevated temperature resulted in increased disease symptoms, whereas elevated ozone concentration had no effect. Elevated temperature in combination with increased $CO_2$ (2 factor) and in combination with increased $CO_2$ and ozone (3 factor) also lead to increased *B. sorokiniana* disease symptoms. However and surprisingly, there were no correlation between fungal growth and the observed increase in disease symptoms, as biomass measurements of *B. sorokiniana* only showed no increased in fungal growth in any of the treatments except when temperature was elevated as single factor. We are currently analyzing histological, metabolic and physiological changes to explain the differences. Based on our preliminary results, we conclude that combination of factors can result in unpredictable disease response and only by investigating muliti-factorial changes it will be possible to predict effects of climate change on plant health.

## Plant genes required for barley-powdery mildew biotrophy--functions of effectors in barley cells

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Biotrophic pathogenic fungi (eg. powdery mildew fungi) rely on specialized organs (haustoria), which they make inside the plant cells. A specialized plant membrane is generated around the haustorium, which thereby become separated from the plant cytosol.

By an unknown mechanism, effector proteins produced by the barley powdery mildew fungus (*Blumeria graminis f.sp. hordei, Bgh*) (Godfrey et al., 2010) are transferred to the plant cell in order to re-direct nutrient or suppress the plant defense mechanisms.

In an approach towards generating disease resistant plants. my project aims at two main points. The first is to figure out how effectors are transferred into the cytosol, and subsequently we want to disturb the transferring mechanism in order to stop the function of the effectors. The second is to identify barley proteins that interact with a few of the most highly expressed Bgh effector candidates using yeast 2-hybrid.

Such interactors are expected to include proteins of different functional categories. They can be proteins that bind the special motif which is common in these effector candidates. They can also include defense signaling proteins; proteins involved in transferring effectors and proteins mobilizing nutrients to the fungus.

The role of identified target proteins will be further analyzed by biochemical, molecular, cellular and genetic methods.

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Godfrey, D., Böhlenius, D., Pedersen, C., Zhang, Z., Emmersen, J., Thordal-Christensen, H. Powdery mildew fungal effector candidates share N-terminal Y/F/WxC- motif (2010) BMC Genomics 11: 317-330

## Multiplex PCR based on multiple gene targets: An effective tool in the diagnosis of members of the genus *Xanthomonas* in culture and plant tissue

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Polymerase chain reaction primers were designed for the specific detection of *Xanthomonas* species based on three gene targets namely; *GumD* (Xan 7), TonB dependent receptor (TBR3) and 16S–23S internal transcribed spacer (ITS) sequences. The PCR protocol was validated with pure cultures of reference strains by testing 40 *Xanthomonas* from 24 different species as well as 15 non-xanthomonad strains. Uninoculated cabbage, rice, and tomato plants as well as host plants inoculated with pathogenic *Xanthomonas* were tested to validate the application of the PCR assays in plant diagnosis.

The developed primers were highly specific as they amplified the targeted genes *GumD, TonB and 16S-23S ITS* sequences of xanthomonads giving amplicons of 402, 239 and 254 bp, respectively. In tests conducted with pure cultures, the primers were run in multiplex with 16S bacterial ribosomal RNA primers, while for testing of DNA of xanthomonads from plants, the PCR targets were multiplexed with M26S primers from the plant mitochondrial 26S ribosomal RNA gene. The ITS primers effectively amplified DNA of all 40 tested *Xanthomonas*, while the TBR3 primers detected only 39 of the strains of xanthomonads tested showing two bands. The use of TBR3 primers did not result in the amplification of DNA from *X. theicola* giving only one band as it was the case in the tests with non-xanthomonads strains. Xan 7 primers differentiated *Xanthomonas* spp. into two groups by detecting only strains members of group 2 of xanthomonads and not the distant xanthomonads placed in group 1 as revealed by several phylogenetic studies.

The results from the present study indicate that the *Xanthomonas*-selective PCR primers were highly specific and may represent a powerful tool for detection of *Xanthomonas* populations and screening of these bacterial pathogens from plant material; the multiplex PCR assays offer an alternative to the culture-based and laborious methods for the routine diagnosis of these important plant pathogens. The PCR can also be used to indicate the status of bacterial strains isolated from various plant hosts as members of one of the two main groups of the currently described *Xanthomonas* spp.

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## Investigating the role of Zearalenone lactonohydrolase during fungal-fungal interactions

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The fungus Clonostachys rosea is antagonistic against many plant parasitic fungi including certain mycotoxin-producing *Fusarium* species. Among other mycotoxins, F. culmorum and F. graminearum produce zearalenone (ZEA), inhibiting growth of other fungi. Previously, it has been shown that C. rosea is able to detoxify ZEA through the enzyme zearalenone lactonohydrolase (ZHD101), which cleaves the lactone-ring of ZEA resulting in less toxic derivatives. However, the exact role of ZHD101 during fungal-fungal interaction is not known. We hypothesize that ZHD101 allows C. rosea to withstand the toxin and to antagonize *Fusarium* species effectively. We use C. rosea strain IK726 to examine the evolution, regulation, function and subcellular localization of ZHD101.

The enzyme ZHD101 is encoded by the intron-free 795 base-pair gene named zhd101. BLAST searches showed no similarity of zhd101 to any fungal sequences, but only towards bacterial hydrolases. In combination with codon-usage analysis this suggests that C. rosea zhd101 was acquired through horizontal gene transfer from bacteria. Sequencing of 10 additional C. rosea isolates revealed that zhd101 is highly conserved with few nucleotide changes. We have used RT-qPCR to show that expression of zhd101 is inducible by ZEA – the expression was significantly increased after exposure to the toxin in comparison with control. Interestingly, zhd101 expression was reduced after supplement with arginine, aspartate, glucose, malate and sucrose, suggesting a connection with nutrient starvation. The Δzhd101 and zhd101::GFP mutants were generated recently through Agrobacterium-mediated transformation to unveil the role of the gene. The Δzhd101 mutant grew significantly slower than either its wild type or the zhd101::GFP mutants on the medium containing the toxin, supporting the suggested role for ZHD101 in detoxifying ZEN during interactions with *Fusarium* species. The Δzhd101 mutant will undergo further testing to determine whether it affects the antagonistic ability towards F. culmorum and F. graminearium in various bioassays. In addition, ZHD101 is predicted to lack a signal peptide for secretion, implying that the protein has an intracellular localization. At present, the zhd101::GFP mutant is now being investigated localization of the ZHD101 during parasitic interaction with *Fusarium* species. using fluorescence microscopy.

## Plant hemoglobin gene expression adjusts *Arabidopsis* susceptibility to *Pseudomonas synringae* and *Botrytis cinerea* though scavenging of nitric oxide

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NO has earlier been shown to influence ethylene production during *Pseudomonas syringae* elicited hypersensitive response in tobacco. In this work *Arabidopsis* plants with silencing or null mutation of hemoglobin genes (*glb1* and *glb2*) and transgenic lines over-expressing Glb1 and Glb2 demonstrated a causal link between NO generation, hemoglobin-dependent NO scavenging, the production of ethylene and resistance to *Botrytis* or *Pseudomonas.*

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### Plant genes required for barley-powdery mildew biotrophy--functions of effectors in barley cells

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Biotrophic pathogenic fungi (eg. powdery mildew fungi) rely on specialized organs (haustoria), which they make inside the plant cells. A specialized plant membrane is generated around the haustorium, which thereby become separated from the plant cytosol.

By an unknown mechanism, effector proteins produced by the barley powdery mildew fungus (*Blumeria graminis f.sp. hordei, Bgh*) (Godfrey et al., 2010) are transferred to the plant cell in order to re-direct nutrient or suppress the plant defense mechanisms.

In an approach towards generating disease resistant plants. my project aims at two main points. The first is to figure out how effectors are transferred into the cytosol, and subsequently we want to disturb the transferring mechanism in order to stop the function of the effectors. The second is to identify barley proteins that interact with a few of the most highly expressed Bgh effector candidates using yeast 2-hybrid.

Such interactors are expected to include proteins of different functional categories. They can be proteins that bind the special motif which is common in these effector candidates. They can also include defense signaling proteins; proteins involved in transferring effectors and proteins mobilizing nutrients to the fungus.

The role of identified target proteins will be further analyzed by biochemical, molecular, cellular and genetic methods.

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## Unraveling plant regulatory networks: NAC transcription factors in disease resistance

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NAC proteins are transcription factor family unique to a plants. Specific NAC proteins have key roles gene in cross talk between different pathways, and are involved in processes as diverse as flower development and reproduction, hormones, biotic and abiotic stress responses, light responses and senescence. We have demonstrated previously that barley HvNAC6 and its orthologue Arabidopsis ATAF1 have a role in regulating effective penetration resistance towards the barley powdery mildew fungus, *Blumeria graminis* f.sp. *hordei,* and that there is an interaction with abscisic acid: ABA levels are negatively associated with penetration resistance (Jensen et al. 2007;Jensen et al. 2008).

Our current studies aim to assess further functions of HvNAC6 and two main activities have been initiated. In collaboration with Per Gregersen and Michael Wagner at Faculty of Agricultural Science at Aarhus University, we have prepared stable transgenic barley plants that express RNAi to *HvNAC6* in order to silence this gene. At present we are testing these plants and the experiments will include examination of responses of these plants to the hormone ABA and pathogens with different life styles *i.e.* biotrophic, like the powdery mildew fungus, *Blumeria graminis*, that need to obtain nutrients from the tissues of living plant cells, and necrotrophic, like the spot bloch fungus, *Bipolaris sorokiniana*, that kills host cells to feed.

A second pillar of our studies is to investigate the transcriptional regulation of the *HvNAC6* gene. We obtained genomic sequences including the *HvNAC6* promoter region from a BAC library available at IPK in Gatersleben, Germany. *In silico* analysis of this promoter and comparison with the orthologues in Arabidopsis *ATAF1* and rice *OsNAC*6 demonstrate the presence of similar putative regulatory elements including myc, ABRE, and W boxes. We are preparing stably transformed transgenic plants for the *HvNAC6* promoter linked to the reporter genes GUS and GFP to study the localisation and expression of *HvNAC6* on a tissue and organ level under different biotic and abiotic stress treatments.

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**Cell wall acetylation is important for cuticle permeability and resistance against *B. cinerea***

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The cell wall is one of the most important structural components of plants. The wall defines cell shapes, provides strength to withstand the turgor pressure and serves as a physical barrier against invading pathogens.

Previous studies on a subset of cell wall mutants indicate that the cell wall polysaccharides contribute to defence not just as mechanical barriers but also as sensors for incoming infections. We have recently identified an acetylation mutant of *Arabidopsis thaliana*, *reduced wall acetylation 2 (rwa2)*, defective in cell wall acetylation. Detailed cell wall analyses identified that *rwa2* has 20% reduction in acetylation of cell wall polymers. The reduction in cell wall acetylation has a profound effect on the plant surface as *rwa2* has collapsed trichomes and a more permeable cuticle. In addition, *rwa2* showed enhanced resitance to the necrotic fungal pathogen *Botrytis cinerea*. These data suggest an intricate link between the cell wall, cuticle and pathogen responses. Exact molecular mechanisms responsible for the permeable cuticle and pathogen response are currently under investigation and will be presented.

## Improving the Hardiness of Tropical Species

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Reduced growth of chilling sensitive plants subjected to reduced temperatures may be ascribed to reduced photosynthesis and decreasing water and nutrient uptake. Many ornamental plants come from sub-/tropical areas and require large amounts of energy for greenhouse production in northern latitudes, and less energy demanding production forms for such crops are therefore desirable.

Two different strategies for producing more chilling tolerant transgenic plants are being combined in this project; (1) altering the chloroplast membrane lipid composition to decrease photoinhibition, and (2) optimisation of water and nutrient uptake.

High levels of unsaturated fatty acids in chloroplast membranes, and thus greater fluidity at low temperatures, have been shown to result in a faster stabilisation of the photosynthetic machinery after chilling-induced photoinhibition (Sui *et al.*, 2007). The level of unsaturated fatty acids in plant membranes is primarily determined by the substrate selectivity of the enzyme glycerol-3-phosphate acyltransferase (GPAT) (Upchurch, 2008), and this project will investigate the effect of overexpression of a chilling tolerant GPAT enzyme in two chilling sensitive model plants, *Kalanchoë blossfeldiana* and *Exacum affine*.

One of the most significant physiological consequences of root chilling stress is changes in plant water status. Most water absorbed by plant roots passes through water canals belonging to a well conserved family of membrane proteins, the aquaporins. Previously it has been demonstrated that both the regulation and the number of active aquaporins is crucial for plant sensitivity to abiotic stresses such as chilling, drought and salt (Peng *et al.*, 2007), and a greater abundance and/or activity of aquaporins may facilitate an improved plant recovery after chilling (Aroca *et al.*, 2005).

Transgenic plants with both a chilling tolerant GPAT gene and overexpression of aquaporin genes have thus greater potentials to improve chilling tolerance than either of the genes alone.

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## Genetic Structure and Association Mapping in a Population of Common and Synthetic Hexaploid Wheat

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A total of 1792 DArT markers (Stodart et al. 2007) that cover the whole wheat genome were used to study the genetic structure of a collection of 192 wheat accession. Out of the 192 accessions used in this study, 68 are common (hexaploid) wheat and 124 accessions are synthetic hexaploid wheat. Synthetic hexaploid wheat presents an important source of resistance genes and enriches the genetic base of common wheat that suffered from a genetic bottleneck during the formation of common hexaploid wheat. Additionally, the wheat collection was genotyped with 81 SSR markers covering the whole wheat genome. Comparison between SSR and DArT marker typed showed a high correlation between the genetic distances that produced based on both marker type with r= 0.72. A clear separation was found between the syntactic and common wheat accessions based on both DArT and SSR data. Among the synthetic wheat accessions included in this study, 12 accessions were produced by crosses between emmer wheat (*Triticum dicoccon*) and *Aegilops squarrosa* (syn. *A. tauschii*) while the rest of synthetic wheat accessions were produced by crosses between rivet wheat (*T. turgidum*) and *A. squarrosa*. Based on SSSR and DArT, a clear separation was found between the synthetic wheat of emmer- and rivet-origin.

Association mapping or linkage disequilibrium is a QTL mapping method that takes advantage of historic disequilibrium to link phenotypes to genotype (Gupta et al. 2005). By using a model that controls the genetic structure by the SSR data, we performed an association analysis between the DArT data and resistance against yellow rust, powdery mildew and Fusarium head blight. This analysis resulted in the detection of alleles present in the population that confer resistance to the diseases included in the study. The advantage of this method is that it in the same step provides knowledge of resistant genotypes in the given pool. Other markers than the DArT marker can be added in order to provide the breeder with appropriate tools to efficiently use these resistances in their breeding programs or to get an even higher resolution. Further, the availability of the complete wheat sequence will provide the opportunity to physically localize all the DArT markers used in this study (as their sequence is known) which is a very advantageous starting point for the isolation of the genes found in this study.

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### Characterization of *Brachypodium* CAD genes and proteins towards improved cellulosic bioethanol production

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Lignin is a major recalcitrant factor in the production of bioethanol from lignocellulosic biomass. The object of this project is to isolate and clone genes involved in the biosynthesis of lignin in grasses to facilitate reduction of recalcitrance of temperate grasses. We present the cloning of five cinnamyl alcohol dehydrogenase (CAD) genes from the *Brachypodium distachyon* genotype Bd21-3 and their differential expression in different plant tissues. Of the five isolated CAD genes two (*BdCAD1* and *BdCAD3*) was successfully expressed in *E. coli* as His-tagged proteins. One of these proteins BdCAD1 exhibits 86% identity to CAD2 proteins from maize and sorghum. Phylogenetic analyses of known CAD proteins further group BdCAD1 together with CAD4 and CAD5 from dicot Arabidopsis. This branch of CAD proteins has been implicated in the lignification of vascular tissues. Mutations in maize and sorghum CAD2 proteins have been linked to the *brown midrib phenotype* which is associated with reduced lignin content and increased digestibility. BdCAD3 exhibits 53% identity on protein level to BdCAD1 but does not group with the same branch of CAD proteins.
The his-tagged expressed proteins was purified to >95% and characterized by different physical-chemical techniques. Furthermore, their ability to convert coniferyl aldehyde to coniferyl alcohol and vice versa was investigated under different pH-values and at different temperatures.

Keywords: biofuel; lignin; cellulosic; cinnamyl alcohol dehydrogenase; CAD; *Brachypodium distachyon*; protein expression; protein purification

**Phenotypical and Genotypical Characterisation of Compact *Kalanchoë* Lines Produced by a Non-GMO Transformation Strategy**

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Chemical growth regulators are widely used in the ornamental plant industry to obtain compact growth, a feature which is favoured by the consumers. *Kalanchoë* is the most important ornamental plant produced in Denmark with more than 40 million plants produced yearly. Transformation of ornamentals using unmodified strains of the naturally occurring soil bacterium *Agrobacterium rhizogenes* is a non-GMO strategy to breed compact potted plants and an environmentally friendly alternative to chemical growth retardants. We have previously transformed *K. blossfeldiana* 'Molly' with a strain of *A. rhizogenes* which harbours four *rol* and two *aux* genes. The transformed *Kalanchoë* plants (T1) had compact phenotypes and some lines exhibited improved tolerance towards ethylene. F1 lines were generated by crossing the commercial *Kalanchoë* cultivar 'Sarah' with selected T1 lines. Subsequently, F2 populations were produced by self-pollination of individual selected F1 lines. A number of F1 and F2 lines were analysed genotypically and phenotypically. The presence of *rol*-genes was confirmed in all F1 and several F2 plant lines exhibiting dwarfism. Screening of F1 showed that the *rol*-genes were inherited together. Besides decreased plant height, several F1 and F2 plants containing *rol*-genes exhibited changes in plant diameter and number of branches as well as flower diameter and time and duration of flowering. Screening of F2 populations derived from self pollination of F1 lines containing *rol*-genes indicated a shift towards a shorter mean plant height within the entire plant population compared to a control population of plants without *rol*-genes. Furthermore, two populations containing *rol*-genes also exhibited earlier flowering compared to the control population. As the presence of the *rol*-genes also results in improved quality in the progenies from crosses, this type of breeding is a valuable biotechnological method applicable in commercial breeding programmes.

## Function of laccases in cell wall biosynthesis

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Laccases are multicopper oxidases capable of polymerizing monolignols. Histochemical assays have shown temporal and spatial correlation with secondary cell wall formation in both herbs and woody perennials. However, in plants laccases constitutes a relatively large group of isoenzymes with unique substrate specificities and expression patterns.

As part of the strategic research centre Bio4Bio, the present project deals with laccase functions in relation to cell wall formation in grasses based on a study of the model species Brachypodium distachyon. Thirty-one isozymes have been retrieved from the recently published genome. Histochemical assays show high correlation between lignification and laccase-like activity in this species. Temporal and spatial correlation between these processes will be further studied by analysis of gene expression based on quantitative PCR and fluorescent in situ hybridization. Specific isozymes that show high correlation with the process of secondary cell wall formation will be further studied in a reverse genetic study in which candidates will be knocked out using RNA interference.

Phenotypes of knock-out mutants are to be described in relation to cell wall degradability in order to reveal possible gains for saccharification of grass-derived feedstocks in bioethanol production and industrial processing.

## Genetic markers for flowering in perennial ryegrass

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Perennial ryegrass (*Lolium perenne L.*) is the principal forage grass utilized in Danish agriculture and underpins the beef and dairy sectors. It is characterized as having high digestibility, high nutritional value, and high productivity during vegetative growth. However, at the reproductive growth stage there is a significant reduction in digestibility due to an increase in the stem to leaf ratio. Thus, the controlled inhibition or delay of flowering would result in a significant increase of forage quality. Traditional breeding programs have been successfully breeding new varieties of perennial ryegrass for decades. However, it can take more than ten years to develop a new variety and involves intensive phenotyping at all stages of selection. It is envisaged that with the aid of molecular markers, breeding programs can be accelerated and gains increased. Our goal is to develop and validate functional molecular markers suitable for marker-assisted breeding for delayed flowering time in perennial ryegrass. We are particularly interested in the changes in the transcriptome of perennial ryegrass during the induction of flowering, which we are investigating using an RNA-Seq approach. Candidate genes will be converted to molecular markers and mapped in an existing mapping population previously characterized for flowering time and vernalization response.

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**Association mapping for accumulation of phytic acid in the barley grain**

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Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) is the primary storage compound of phosphorus in seeds. Although the biosynthesis is not fully understood, several genes have been shown to be involved in phytic acid accumulation[1)]. To investigate the importance of the different genes involved, we are examining the natural variation in the level of phytic acid and free phosphorus in barley grains.

A collection of 233 spring barley cultivars was grown in two replicates in Halle, Germany as a part of the ERA-PG EXBARDIV project[2)]. The level of phytic acid and free phosphorus in the grain is quantified colorimetrically. 762 SNP markers from the barley OPA 1 Illumina Oligonucleotide Pool Assay are used for the association analysis to study the pathway of phytic acid and to confirming known and new candidate genes important for the content of phytic acid in cereal seeds

In human nutrition, phytic acid acts as an anti-nutritional factor for, in particular, Fe and Zn uptake and thus potentially contributes to the 'hidden hunger' caused by mineral malnutrition. In animal husbandry, the main problem caused by phytic acid in the grain is that phosphate bound in phytic acid cannot be hydrolyzed by monogastric animals. A full understanding of the phytic acid pathway will help solving the problems that high levels of phytic acid create in food and feed consumption.

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## Cisgenic barley for animal feed

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Genetic transformation is currently met with substantial scepticism among the general public. One major concern is the mingling of genetic material between species. We have initiated a collaborating project with different groups from University of Copenhagen, the Danish Agricultural Advisory Service and Sejet Plant Breeding based on the Cisgenesis concept. Cisgenesis implies that the plants are transformed only with its own or very closely related genetic material and that the final Cisgenic plants have to be free of any foreign genes. The Cisgenesis concept allows for the introduction of extra gene copies of a particular gene whereby a particular trait can be accentuated. Transgenic crops generated by the Cisgenesis concept are accordingly very similar to those generated by conventional breeding.
In our part of the project we are focusing on barley phytases as candidate genes for Cisgenesis. Recently, Dionisio et al. (2011) have cloned and characterized phytases belonging to the purple acid phosphatases (PAPs) in barley. We have isolated the genomic PAP-clone of the isoform expressed during grain filling including 2.3 kb of the promoter region and 600 bp of the terminator region using a genomic barley lambda library. The clone has been inserted into a Cisgenic *Agrobacterium* vector where both the gene of interest and the selection gene are flanked by their own T-DNA borders in order to promote integration of the two genes at unlinked places in the plant genome. $T_0$-plants show increases in the phytase activity of mature seeds from 1350 in wild type to 7500 FTU/kg in $T_0$-plants. We have identified two Cisgenic $T_1$-lines without selection gene and vector backbone but with one additional genomic clone of the phytase gene. Lines homozygous for the additional cisgene show 2-3 fold increases in phytase activity. The integration pattern of the cisgenes are currently investigated.

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## Cloning and TILLING of wheat ITPK genes

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ITPKs (Inositol tris/tetrakisphosphate kinase) are involved in the stepwise phosphorylation of Ins3*P* to Ins$P_5$s and play a key role in biosynthesis of phytic acid (PA) Thus maize mutants defective in an ITPK gene contained 30-50% less PA in the seed compared to the wild type. The number of ITPK genes seems to differ among species as four have been identified in both Arabidopsis and soya bean, while six have been identified in rice and one has been identified in both maize and barley.

In order to design primers for cloning of the wheat homoeologs, the raw sequences of Chinese spring (5x genome coverage, http://www.cerealsdb.uk.net) and the Wheat Estimated Transcipt server http://www4.rothamsted.bbsrc.ac.uk/whets) were mined for wheat sequences with high similarity to OsITPK-5. The amino acid sequence of the three cloned wheat homoeologs were found to be 97-98% identical to each other, while identity to the corresponding barley, rice and maize genes were 96, 78 and 78%, respectively. Genome specific primers have been identified for one of the wheat homoeologs, and have been used for TILLING (Targeting Induced Local Lesions in Genomes) to identify a number of mutations for the gene in an established TILLING population of hexaploid wheat. In addition this gene has been physically mapped to the long arm of wheat chromosome 1D.

This work is part of the iKORN project funded by the Strategic Research Council.

### Large-scale development of gene-associated SNP markers for linkage mapping in perennial ryegrass (*Lolium perenne* L.)

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Single Nucleotide Polymorphisms (SNPs) are increasingly becoming the DNA maker system of choice due to their extensive occurrence in the genome and the ability to be used in highly multiplexed genotyping assays. Although needed in high numbers for genome-wide marker profiles and genomics-assisted breeding strategies, a surprisingly low number of validated SNPs are currently available in perennial ryegrass. The advent of next generation sequencing opened up the opportunity for efficient and high throughput *in silico* SNP discovery in absence of a reference genome sequence. However, the percentages of false discovery rates depend on the method of SNP identification and range between 5 and 50% (Ganal et al. 2009). Moreover, the highly complex genome structure of outbreeding species with a high proportion of paralogous genes might limit the success rate of highly multiplexed GoldenGate SNP genotyping assays.

A reference set of 9,399 *Lolium perenne* L. genes was assembled with 802,156 high quality cDNA reads sequenced with the Roche 454FLX Titanium sequencing system and used for *in silico* SNP discovery. Out of more than 1,200 SNPs fulfilling highly stringent assembly and detection parameters, a total of 768 SNP markers were selected for GoldenGate genotyping on 181 individuals of the perennial ryegrass mapping population VrnA, which has been previously evaluated for important agronomic traits.

A total of 692 (90%) of the 768 SNPs tested were successfully called. Of these, 96 (14%) did not reveal a clear cluster separation. An additional 83 (12%) were monomorphic. A total of 513 gene-associated SNPs were available for linkage mapping, out of which 495 (64% of the total 768 SNPs on the array) were successfully mapped in the VrnA population. The current VrnA map contains a total of 837 DNA markers (ranging from 87 on linkage group 5 to 165 on linkage group 4, on average 120 per linkage group) spanning 731 centi Mogan (cM) with an average marker interval distance of less than 0.90 cM. The map represents the genome location of 723 ESTs including carefully selected candidate genes controlling important agronomic traits.

Here, we describe an approach for using next generation sequencing data for SNP discovery and the successful design of a 768plex Illumina GodenGate genotyping assays in a complex genome. The SNP markers presented here represent the first large-scale *Lolium* SNP set which is publicly available. Moreover, the high density transcriptome map provides an important tool for the assignment of candidate genes to QTL, map-based cloning and functional genomics in the most important temperate grassland species *Lolium perenne* L.

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## Molecular characterization of cytoplasmic male sterility (CMS) in perennial ryegrass (*Lolium perenne* L.)

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Perennial ryegrass (*Lolium perenne* L.) is a key grassland species in temperate climates. As an out breeding crop, ryegrass is currently being bred as population and synthetic families, thereby not fully exploiting the genetically available heterosis. Thus, hybrid breeding has the potential to increase biomass yield, improve nutritional value and tolerance towards abiotic and biotic stress.
Cytoplasmic male sterility (CMS) is an efficient tool to control pollination for hybrid seed production. In order to identify the causative polymorphism of the CMS phenotype, a cytoplasmic male sterile plant and the corresponding fertile maintainer genotype was established in the glasshouse. The mitochondrial DNA has been isolated from crude mitochondrial preparations of young leaf tissue of four months old ryegrass clones. Mitochondrial DNA was isolated, checked for contamination of genomic DNA, quantified and sequenced using 454 next-generation sequencing technology, resulting in approximately 800,000 high quality single reads.

Here we report on the sequencing and the assembly of the mitochondrial genome from perennial ryegrass. Moreover, the assembly and annotation of the male-sterile and fertile mitochondrial genomes will enable to identify the causative polymorphism of CMS phenotype in perennial ryegrass.

**Towards a yeast cell factory platform for production of sulphur rich secondary metabolites from plants.**

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Glucosinolates (GLs) are sulphur containing secondary metabolites produced in plants from the Brassicaceae family, as a part of their natural defense against herbivores and microorganisms. In human nutrition, GLs are mostly known for their sharp taste characteristic of plants such as mustard, radish and wasabi. Epidimiological studies have indicated an inverse correlation between cancer incidence and GLs consumption. Therefore, there is an increasing interest to use GLs as possible dietary supplements or pharmaceuticals. To achieve this application, GLs are to be produced by a cheap reliable source. As a highly complex molecule, chemical synthesis is difficult and expensive, and isolation from their natural source is also typically low yielding. Recently, production of benzyl- and indole-glucosinolate has been reported in tobacco upon transient expression of six biosynthetic genes in the GL pathway. Introduction of the biosynthetic genes to yeast by metabolic engineering enables production of GLs through large scale fermentations on inexpensive sugars. In this work we established a yeast technology platform validating 15 integration sites on three separate chromosomes in the vicinity of essential genes in an isogenic CEN.PK113-11C strain. This feature enables the construction of the first glucosinolate producing yeast cell factory, by stable chromosomal integration of the entire biosynthetic pathway using identical promoters and terminators.

## 454 pyrosequencing based transcriptome analysis of *Zygaena filipendulae* with focus on genes involved in biosynthesis of cyanogenic glucosides

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An essential driving component in the co-evolution of plants and insects is the ability to produce and handle bioactive compounds. To study the molecular mechanism behind the co-adaption in plant–insect interactions, we have investigated the interactions between *Lotus corniculatus* and *Zygaena filipendulae*. They both contain cyanogenic glucosides which liberate toxic hydrogen cyanide upon breakdown. Moths belonging to the Zygaena family are the only insects known able to carry out both *de novo* biosynthesis and sequestration of cyanogenic glucosides from their feed plants. The ratio and content of cyanogenic glucosides is tightly regulated in *Z. filipendulae*, and they play several important roles in addition to defense in the life cycle of Zygaena. The transfer of a nuptial gift of cyanogenic glucosides during mating has been demonstrated as well as the possible involvement of hydrogen cyanide in male attraction and nitrogen metabolism. The biosynthetic pathway for cyanogenic glucoside biosynthesis in *Z. filipendulae* proceeds using the same intermediates as in the well known pathway from plants, but none of the enzymes responsible have been identified yet. A genomics strategy founded on 454 pyrosequencing of the *Z. filipendulae* transcriptome was undertaken to identify some of these enzymes in *Z. filipendulae*. Promising gene candidates for biosynthesis of cyanogenic glucosides was identified, and the suitability of *Z. filipendulae* as a model system for cyanogenesis in insects is evident. Conclusively, pyrosequencing is an attractive approach to gain access to genes in the biosynthesis of bio-active natural products from insects and other organisms, for which the genome sequence is not known.

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**Classification, naming and evolutionary history of glycosyltransferases from sequenced Rhodophyta and Chlorophyta genomes**

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The Archaeplastida consists of three lineages, Rhodohytae (red algae), Plantae and Glaucocystophyceae. The Plantae can then be divided into two lineages, namely the Chlorophyta and the Streptophyta (which includes Embryophyta aka plants). Members of the Rhodophyta, Chlorophyta and the Streptophyta rely on a carbohydrate based or a highly glycosylated protein based cell wall. In order to elucidate possible evolutionary link between the three lineages regarding to their polysaccharide biosynthesis, comparative genomics work was initiated. Fully sequenced genomes from Rhodophyta (Galdieria), Chlorophyta (Volvox, Chlamydomonas, Micromonas, Osterococcus) and the well defined CAZy database on glycosyltransferases (GT's) containing known GT's from Genbank including Physcomitrella (moss), Selaginella (spikemoss) and higher plants were applied in the analysis.

The number of GTs found in the Chlorophyta and Rhodophyta species are generally much lower then what is observed in Embryophyta. Especially three aspects of the physiology of Embryophyta increase the number of GTs in their genomes; (1) Cell wall biosynthesis, the more complex cell walls of Embryophyta require a larger number of GTs for biosynthesis (2) a richer set of protein glycosylation and (3) glycosylation of secondary metabolites, a large proportion of GT1 is involved in secondary metabolite biosynthesis.

Looking into more specific aspect of polysaccharide biosynthesis distinct characteristics could be observed in (1) N-linked protein glycosylation, Volvox and Chlamydomonas have a different mannosylation and glucosylation pattern (2) O-linked glycosylation, clear orthology could be observed in extensin glycosylation between Chlamydomonas, Volvox and embryophytes (3) GPI anchor biosynthesis, which is apparently missing in Rhodophyta and truncated in Chlorophyta and (4) cell wall biosynthesis, the Embryophyta have unique cell wall related polymers not found in Rhodophyta and Chlorophyta.

## Homology model of a complex between NADPH-dependent thioredoxin reductase (NTR) and thioredoxin from barley

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Thioredoxins (Trx's) are protein disulfide reductases that regulate the intracellular redox environment. Furthermore they participate in a large number of cellular processes including DNA synthesis, oxidative stress response and apoptosis. They are present in several isoforms in plants of which the cytosolic Trx h isoforms are reactivated by specific NADPH-dependant thioredoxin reductases (NTRs) [1].

NTRs catalyze the reduction of a redox-active disulfide bond in Trx using NADPH as reductant and FAD as cofactor. NTR consists of two domains, the FAD and the NADPH binding domains, and can shift between two conformations; the flavin oxidizing (FO) or the flavin reducing (FR) conformation. To obtain the latter, one domain has to rotate 66° relative to the other. Only by covalent binding of NTR to Trx can the FR be stabilized for characterization [2].

We have solved the structure of one isoform of NTR from barley (*Hv*NTR2) in the FO conformation [3]. Based on this structure, a model of the FR conformation in complex with a Trx from barley (*Hv*trxH2) has been built. The model indicate that the complex is stabilized by protein-protein interactions involving several amino acid residues that have been subjected to site-directed mutagenesis and currently are evaluated by enzyme kinetics.

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**Characterization of barley glutathione peroxidase, a thioredoxin-dependent antioxidant enzyme in seeds**

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Plants have evolved advanced redox control systems including the thioredoxin (Trx) and glutathione pathways as well as the ascorbate-glutathione cycle (1). These systems are involved in regulation of many cellular functions, like photosynthesis and sugar metabolism, and are important in critical developmental steps as seed maturation and germination, during which cells are subjected to harsh conditions. Accumulating evidence suggests that these systems are interlinked in a complex manner. Based on previous experiments, we selected glutathione peroxidase (Gpx) as a model target for further study (2). Gpxs have been shown to be Trx-dependent peroxidases in plants, and to efficiently reduce peroxides (3). Expression studies have shown that they are expressed in the seeds of several plant species, and thus likely play an important role in protecting the seed tissues, in particular the embryo, against oxidative stress.

Barley Gpx was expressed in *E.coli* and purified to homogeneity. Biochemical characterization showed that barley glutathione peroxidase is a strictly thioredoxin-dependent peroxidase able to eliminate different types of peroxides. Interestingly, the enzyme is also prone to inactivation by $H_2O_2$, and this could be part of a regulation mechanism. Structural aspects of the protein function, switching from a monomeric form to a dimeric form upon mild oxidation, is also investigated. Polyclonal antibodies against the protein were used to studies the proteins expression in seeds during germination and in different stress conditions.

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## Role of an enzyme in the regulatory network controlling glucosinolate biosynthesis

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In *Arabidopsis thaliana*, more than 30 different glucosinolates are formed from methionine as chemical defenses against a broad range of enemies. Biosynthesis of these compounds occurs through a compartmentalized, non-linear pathway and requires more than 30 enzymes as well as additional enzymes from other pathways, e.g. providing co-substrates. Relative and total levels of methionine-derived glucosinolates are characteristic for different accessions of *A. thaliana*, different plant organs and different developmental stages. Nevertheless, plants constantly adjust steady-state levels of individual glucosinolates in response to environmental cues which requires coordinate regulation of distinct subsets of biosynthetic genes.

Three closely related MYB transcription factors, *MYB28*, *MYB29*, and *MYB76* have been characterized as direct transcriptional regulators of genes involved in methionine-derived glucosinolate biosynthesis. Additionally, an enzyme catalyzing a late step in the pathway, AOP2, has been reported to increase glucosinolate accumulation. To investigate a potential role of *AOP2* in the regulatory network controlling glucosinolate biosynthesis, we studied the regulatory ability of *AOP2* in various mutants and transgenic lines with altered MYB expression levels. Our results show that *AOP2* functions as upstream regulator of certain MYBs without acting as transcription factor itself.

**Metabolite-independent feedback by a biosynthetic enzyme**

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Most enzymes involved in the production of aliphatic glucosinolates are identified. However, it is not yet understood, how glucosinolate production is regulated. Other pathways are found, where regulation is based on feedback by the product. A similar metabolite feedback could be the reason for that one of the late enzymes in the glucosinolate pathway - AOP3 - has been coupled to regulation, as glucosinolate levels in different *Arabidopsis thaliana* accessions are higher, when AOP3 is present. The regulatory abilities of AOP3 could be due the enzymatic functions and thereby the glucosinolates it depletes or produces.

To investigate if glucosinolates are involved in feedback regulation, substrate and product of AOP3 were fed to seedlings of an *A. thaliana* accession, where these glucosinolates are either present in small amounts or not at all. Furthermore, different versions of AOP3 were over-expressed in an *A. thaliana* accession not expressing AOP3 but producing the substrate of AOP3. Over-expression of AOP3 led to conversion of substrate into a product not normally present in this accession. By contrast over-expression of AOP3 with mutations in the active site led to a non-functional enzyme and thereby no glucosinolate conversion.

The observed effects on glucosinolate profiles led to the identification of a regulatory ability of AOP3, which is not connected to the substrate or product of AOP3. Taken together it seems that AOP3 has a feedback effect independent of enzymatic activity and metabolites.

### Validation of elite cultivar protein coding sequences in the draft doubled monoploid potato genome sequence by proteomics and transcriptomics

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The potato is the world's third most important food crop after rice and wheat and has great potential to be used not only for feed and food but also for energy production as bioethanol. In order to meet these future requirements the potato farming need to have as high a starch production per area as possible. In order to achieve this gold the negative affect on the potato harvest due to particular potato late blight and drought need to be further investigated and processed. Despite this big potential, the potato is in much lesser extent described in the scientific literature [1]. Two vital information sources is the genome and proteome say organism's genes and the expressed proteins. The Potato Genome Sequencing Consortium (PGSC) is an international consortium established to genome sequenced the complete potato DNA sequence (850 Mbps) which being finalized currently (www.potatogenome.net). However, genes coding for proteins (eg. enzymes, membrane proteins, transport proteins, neurotransmitters, and defense materials) is only approx. 3% of long DNA sequences that constitute a plant genome. Therefore, it is a challenge to determine the correct protein sequence. It is for that reason also important to investigate the potato proteome.

During the last five year we have accumulated very large Proteomic and Transcriptomics (Next Generation Sequencing) datasets from multiple potato elite cultivars (eg. Kuras, Bintje, Sarpo Mira) [2-5] and multiple tissues (e.g. leaf, tuber, amyloplast, vacuole). A part of this data is currently in public domain (www.solanumdata.dk). Based on 38 DM1-3 516R44 mRNAseq libraries of the DM v3 genome sequence and 17 RH89-039-16 libraries (818 mio. reads in total) a sequence data base was assembled by mapping to the DM v3 genome sequence followed by prediction of putative genes. The exon-intron structure of a large set of putative gene isoforms were curated by manually inspecting the reference assembly. In cases where a gene model had multiple transcripts, the longest transcript with validated exon-intron structure was chosen. The mentioned DM data base together with the PUT PlantGDB data base and the *Solanum tubersosum* Gene Index (StGI) data base (version 13) were utilized in order to investigate different potato datasets.

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### Gene expression analysis of starch metabolism using mRNAseq and the potato genome sequence

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Starch accumulation in potato tubers is a result of metabolic processes that are highly redundant with regards to gene isoforms and biological pathways. Starch synthesis takes place by direct incorporation of glucose-1-phosphate or via UDP-Glucose, while starch breakdown occurs via phosphorylytic or hydrolytic reactions. In plants, starch synthesis takes place not only in storage organs but also in leaves, where transient starch produced during the day is consumed during the night. Many genes of starch metabolism have been analysed in potato but completion of the genome sequence enables the creation of a more complete overview of gene isoform usage in starch metabolism. Using mRNAseq, the expression of individual gene isoforms of a doubled monoploid and a diploid breeding variant, have been analysed and mapped to the metabolic model of starch metabolism. An interestingly high degree of tissue specificity is observed where contrasting isoforms are utilized in tubers and leaves. E.g. five loci of starch phosphorylase exist; two tuber specific, two leaf specific, and one stolon specific. Furthermore, although several isoforms of a gene exist, their expression level and hence their likely biological importance differ widely. This is observed for example for starch synthase where one locus is 5-20x higher expressed than the other six. Knowledge of isoform usage is important when trying to optimize carbon flux into crop sink organs. Encouragingly a different set of genes are utilized in leaf and tuber starch metabolism, respectively, as this enables manipulating of carbon flux in tubers without severely affecting leaf carbon metabolism.

### The glycosyltransferase transcriptome of *Coleochaete scutata*

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The charophycean green algae (CGA) are the closest algal relatives of the embryophytes and their ancestors gave rise to the land plant lineage. Extant CGA consist of 6 clades and several thousand species with a diversity of morphologies, ranging from asymmetrical unicells to complex and erect multicellular bodies. The Coleochatales are an order of charophycean green alga (CGA) of particular importance for understanding the evolution of higher plant processes *and are* of one of the two groups of CGA that have been proposed as immediate ancestors of land plants. A survey of expressed *Coleochaete scutata's* glycosyltransferases (GTs) with probable roles in cell wall synthesis was conducted with the aim of enhancing our understanding of the requirements for constructing complex plant tissues, since cell differentiation, specialization and cell adhesion in tissues all depend on cell wall structure and metabolism. A GT-transcriptome analysis is presented and interpreted based on experimentally obtained data on cell wall architecture and composition. *Coleochaete scutata* cell walls contain many polysaccharides that are common to land plan walls including homogalacturonan, an XXXG-type xyloglucan, xylan, mannan, arabinogalactan proteins and extensin. However, *Coleochaete scutata* cell walls appear to lack the complex branched pectic domain rhamnogalacturon II and although some evidence for rhamnogalacturon I was found, if RGI is indeed present, it is at very low levels. Our study provides new insight into the evolutionary window during which the structurally complex walls of embryophytes originated and the significance of the advanced CGA during these events.

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