Plant Biotech Denmark
Annual meeting 2013
January 31 - February 1

Faculty of Science
University of Copenhagen
Cover photo: Localization of PAPhy_a expression in the developing wheat grain, ~18 DPA. In the embryo, GUS activity is restricted to the scutellum.
Work By Henrik Brinch-Pedersen and Claus K. Madsen, Molecular Biology and Genetics, Science and Technology, Aarhus University, Research Center Flakkebjerg, DK-4200 Slagelse
Programme

THURSDAY - January 31, 2013

9.00 - 9.30 Registration, coffee/tea and croissant

09.30 - 9.35 Welcome, by Preben Bach Holm, Head of steering committee, Plant Biotech Denmark

Session 1: Products/Signaling and cellular trafficking
Chair: Barbara Ann Halkier

09.35 - 10.20 Keynote talk:
**Structural basis for cellulose synthesis and membrane translocation**, by Associate Professor Jochen Zimmer, School of Medicine, University of Virginia, USA

10.20 - 10.40 Novel immune-glyco-arrays for the high throughput screening of glycoside hydrolase activities, by PhD Student Silvia Vidal-Melogsa, Department of Plant and Environmental Sciences, University of Copenhagen

10.40 - 11.00 Using genetically encoded biosensors to study plant cell signaling, by Postdoc Sisse K. Gjetting, Department of Plant and Environmental Sciences, University of Copenhagen

11.00 - 11.20 Coffee/tea and fruit

Session 2: Nutrition/Diseases
Chair: Michael Lyngkjær

11.20 - 12.05 Keynote talk:
**Endocytosis regulates immune receptors and signaling**, by Professor Silke Robatzek, The Sainsbury Laboratory, Norwich Research Park, UK

12.05 - 12.25 Phosphate transporter genes in mycorrhizal Brachypodium distachyon, by Postdoc Mette Grønlund, Department of Chemical and Biochemical Engineering, Technical University of Denmark

12.25 - 12.45 Barley vesicle trafficking regulators localise to the powdery mildew extrahaustorial membrane, by Postdoc Mark Kwaaitaal, Defence Genetics Lab, Department of Plant and Environmental Sciences, University of Copenhagen

12.45 - 13.30 Lunch
**Session 3: Breeding - quality and productivity/Synthetic - and systems biology**  
*Chair: Poul Erik Jensen*

13.30 - 14.15  
**Keynote talk:**  
*Towards a molecular blueprint for engineering C4 photosynthesis*, by Professor Andreas Weber, Center of Excellence on Plant Sciences (CEPLAS), Institute of Plant Biochemistry, Heinrich-Heine-University, Düsseldorf, Germany

14.15 - 14.35  
**De novo DNA sequence driven bulk segregant analysis of Water Use Efficiency (WUE) in potato without prior knowledge of molecular markers**, by PhD Student Kacper Piotr Kaminski, Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University and Department of Agroecology, Aarhus University

14.35 - 14.55  
**Drought priming during multiple vegetative growth stages in wheat improves drought tolerance during generative growth: insights from proteomics and physiology**, by PhD Student Xiao Wang, Department of Agroecology, Aarhus University and Department of Systems Biology, Technical University of Denmark

14.55 - 15.25  
*Coffee/tea, cake and fruit*

**Session 4: Poster Elevator talks**  
*Chair: Birte Svensson*

15.25 - 15.55  
3 minute talks based on abstracts (lecture hall)

15.55 - 18.00  
**Poster session in the Marble Hall – Wine/beer and snacks**

18.00 - 22.00  
**Dinner at Gumle, Thorvaldsensvej 40**
FRIDAY - February 1, 2013

Session 5: Current and future trends in plant production
Chair: Preben Bach Holm

9.00 - 9.40 Meeting the global challenges for agriculture: Sharing resources and free exchange of information, by Adjunct Professor Melvin Oliver, The Global Plant Council

9.40 – 10.10 Intellectual Property Right (IPR) in Plant Breeding, by Kurt Hjortsholm, Association of Danish Variety Owners and European Seed Association - Committee for intellectual property rights in plants

10.10 - 10.40 Coffee/tea, cinnamon roll and fruit

10.40 - 11.10 Do we have a food crisis and how do the market economic mechanisms function? Senior Advisor, Henning Otte Hansen, Institute of Food and Resource Economics, University of Copenhagen

11.10 - 11.40 Safety assessment and regulation of new plant breeding technologies, by Professor Joachim Schiemann, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Biosafety in Plant Biotechnology, Quedlinburg, Germany

11.40 - 12.20 Delivery of pheromones and other semiochemicals for food security by GM, breeding and companion cropping, by Dr Michael Birkett, Biological Chemistry and Crop Protection Department, Rothamsted Research, UK

12.20 – 13.00 Lunch

Session 6: Technologies
Chair: Søren K. Rasmussen

13.00 - 13.30 Most recent applications of new Free Flow Electrophoresis techniques, by Dr Gerhard Weber, FFE service Gmbh, München, Germany

13.30 - 14.00 Genomic Prediction in Plants: How and When Does it Work? by Dr Marco C. A. M. Bink, Department of Biometris, Wageningen University and Research Centre, Wageningen, The Netherlands

14.00 - 14.30 Dynamic interaction of plant and environment for resource use efficiency - quantitative approaches to non-invasive plant phenotyping?, by Professor Ulrich Schurr, Forschungs-zentrum Jülich, Plant Sciences, Jülich, Germany

14.30 - 15.00 TALEs and TALENs for expression control and targeted genome engineering, by Dr Jens Boch, Institute of Biology, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

15.00 - 15.30 Coffee/tea and fruit
**Key Note Talk**

**Structural basis for cellulose synthesis and membrane translocation**

*Jochen Zimmer*

*School of Medicine, University of Virginia, USA*

Cellulose, the most abundant biological macromolecule, is an extracellular, linear polymer of glucose molecules. It represents an essential component of plant cell walls but is also found in algae and bacteria. In bacteria, cellulose production frequently correlates with the formation of biofilms, a sessile, multicellular growth form. Cellulose synthesis and transport across the inner bacterial membrane is mediated by a complex of the multi-spanning catalytic BcsA subunit and the membrane-anchored, periplasmic BcsB protein. The crystal structure of a complex of BcsA and BcsB containing a translocating polysaccharide reveals many aspects of cellulose biosynthesis. The structure demonstrates the architecture of the cellulose synthase, reveals how BcsA forms a cellulose-conducting channel, and suggests a model for the coupling of cellulose synthesis and translocation.
Novel immuno-glyco-arrays for the high throughput screening of glycoside hydrolase activities

Silvia Vidal-Melgosa¹, Henriette L. Pedersen¹, Malwina Michalak², Jørn D. Mikkelsen², William G.T. Willats*¹

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¹Department of Plant and Environmental Sciences. Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1. sal-1871 Frederiksberg C, Denmark, ²Department of Chemical and Biochemical Engineering. Technical University of Denmark, Søtofts Plads. Building 227, 2800 Kgs. Lyngby, Denmark.

A combination of genome sequencing, bioinformatics and molecular modelling has identified vast numbers of putative glycoside hydrolases (GHs) that have been deposited in the Carbohydrate Active enZymes data base (CAZy). However, our current ability to empirically test the activities of these enzymes lags significantly behind our ability to identify them, and it is estimated that no more than around 20% of the activities of the proteins within CAZy can be safely predicted (Gilbert, 2010). This imbalance is a hindrance to the commercial exploitation of GHs and to our understanding of their biological functions. There is therefore a pressing need for the development of new high throughput technology for GHs screening and here we present approaches based on combining the high-throughput capacity of carbohydrate microarrays with the specificity of monoclonal antibodies (mAbs). One significant advantage of this method compared to many existing techniques is that, because of mAb specificity, GH activities can be determined even when many enzymes and substrates are mixed together. This is significant because many commercial GHs, notably those used for biomass saccharification, are used as cocktails against substrates that are components of complex mixtures of polysaccharides, for example partially degraded plant cell walls.
Using genetically encoded biosensors to study plant cell signaling

Sisse K. Gjetting, Cecilie K. Ytting, Khalid Mahmood, Alexander Schulz and Anja T. Fuglsang

Section for Transport Biology, Dept. of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Live imaging approaches have become powerful tools for dissecting cellular dynamics in plants. Genetically encoded biosensors are non-invasive optical tools that can detect changes in analyte concentration with a high spatial and temporal resolution. They have been used extensively in mammalian cells to study a variety of ligands, but also plant cells have been targeted with these sensors, where they have been particularly useful in studying calcium fluxes (Allen et al., 1999; Haruta et al., 2008; Krebs et al., 2011). We have developed a new type of ratiometric pH sensor, pHusion, for studying pH dynamics in the cytosol (pHusion) or apoplastic (apo-pHusion) compartments of plant cells (Gjetting et al., 2012). Whereas the cytosol is highly buffered, pH in the apoplast is a highly dynamic part of cellular pH signaling, and tools to study apoplastic pH fluxes in detail are in demand. In combination with a flexible perfusion system we have been able to study live pH changes in young Arabidopsis root cells stably expressing apo-pHusion, in response to externally applied stimuli, such as the phytohormone auxin (Gjetting et al., 2012) and secreted peptide hormones of the RALF family (rapid alkalinization factor). These pH changes were specifically localized to the elongation zone of the growing primary root, indicating a role in root cell growth, possibly involving regulation of the plasma membrane H^+-ATPase (unpublished data).

Furthermore, a phosphate sensor, FLIPPi has been developed (Gu et al., 2006) and is currently being characterized in plants. Together these two types of sensors represent highly sensitive tools to study signaling of pH and phosphate in plant cells. In the future, these sensors may contribute to the expanding sensor toolbox with the overall aim to create combined flux maps of many metabolites in complex cellular networks in plants.

References:
Key Note Talk

Endocytosis regulates immune receptors and signalling

Silke Robatzek, Martina Beck, Gildas Bourdais, Fabio Gervasi, Malick Mbengue, Thomas Spallek, and Ji Zhou

The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK

All plant organs are vulnerable to colonisation and molecular manipulation by microbes. In our attempts to understand the full nature of the interactions that occur between a potential pathogen and its host, we are focussing on membrane biology in plant-microbe interactions. Immune receptors constitute recognition sites to detect invading pathogens and to trigger defenses, and the activity of these receptors depends upon the dynamic membrane trafficking network. The plasma membrane receptor FLAGELLIN SENSING 2 (FLS2) confers plant immunity through perception of bacterial flagellin (flg22). Following elicitation, FLS2 is internalized into vesicles. To resolve FLS2 trafficking, we exploited quantitative high-throughput confocal imaging for co-localisation studies and chemical interference. FLS2 localises to bona-fide endosomes via two distinct endocytic trafficking routes depending on its activation status. I will present our high-throughput confocal imaging pipeline to study endosomal pathways in plants and will discuss results from our current research, which addresses the molecular components regulating FLS2 endocytosis, and the intersection between FLS2 endocytosis and flg22 signaling. I will further describe recent results demonstrating that ligand-induced internalization of FLS2 represents a conserved common endocytic trafficking pathway. To advance our research, we developed a range of computational tools to extract meaningful data from bioimages and large image data sets, which I will briefly discuss for the detection of stomata closure and callose deposition in response to flg22. Altogether, high-throughput confocal imaging combined with functional studies allows us to tackle the dynamic cellular changes involved in the interaction between plants and microbes.

Research in S.R.’s laboratory is supported by the Gatsby Charitable Foundation and by a grant of the European Research Council (ERC).
Phosphate Transporter Genes in Mycorrhizal *Brachypodium distachyon*

Mette Grønlund, Signe Sandbech Clausen, Edith Hammer, Iver Jakobsen

Department of Chemical and Biochemical Engineering, Technical University of Denmark, P.O. Box 49, DK-4000 Roskilde, Denmark.

Arbuscular mycorrhizal (AM) fungi can induce growth depressions in e.g. grasses. We are testing two different hypotheses to explain the growth depressions: They could be caused by fungal drain of plant carbon and/or by phosphorus limitation. Two phosphate (Pi) uptake routes are present in AM plants: the mycorrhizal pathway (MP) and the direct pathway (DP). A shift to the MP may occur at the expense of function of the DP and so AM plants can become P limited when they shift to Pi uptake through MP, if the decrease of the DP is not fully compensated by MP uptake.

The model grass *Brachypodium distachyon* is used to study growth depressions by selected AM fungi. We are testing the above hypotheses by growing AM plants at elevated CO₂ and by analyzing gene expression of selected phosphate transporter genes, combined with physiological isotope tracer uptake studies to discriminate between Pi uptake via MP and DP.

Three putative DP phosphate transporter genes were identified in *B. distachyon*, these genes are down-regulated at high phosphate levels and in mycorrhizal plants. Split-root grown plants were analysed to study whether the interplay between DP and MP is systemic and how it is influenced by sudden increase in phosphate availability for one root half. We found differences in the regulation of two of the putative DP phosphate transporter genes in response to mycorrhiza and elevated CO₂. Furthermore, effects of elevated CO₂ on growth responses to increasing soil P levels different widely between *B. distachyon* and *Medicago truncatula*. 
Barley vesicle trafficking regulators localise to the powdery mildew extrahaustorial membrane.

Mark Kwaaitaal1 and Hans Thordal-Christensen1

Defence Genetics lab, Department of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Denmark.

Biotrophic powdery mildew fungi are fully dependent on their plant host for all stages of their life. After germination of the conidiophore, the fungus forms an appressorium that attaches to the leaf surface. This first interaction results in a plethora of basal plant defence responses, which result in the targeting of cellular defences towards the pathogen entry site. Secreted fungal effectors confer massive reprogramming of cellular signalling and vesicle trafficking to facilitate fungal invasion and growth. A successful breach of the cell wall, suppression of plant defences and invasion of the host cell triggers the formation of a specialised intracellular feeding structure, the haustorium. The membrane surrounding the haustorium, the extrahaustorial membrane (EHM), is of plant origin. It is believed to mediate fungal effector transport to the host cytosol and is essential for nutrient acquisition to sustain fungal growth. The molecular mechanisms leading to the formation of the EHM, the subcellular origin or the identity of this enigmatic membrane are unknown.

The model system used in our studies is the interaction between barley (Hordeum vulgare) and the powdery mildew fungus, Blumeria graminis f.sp. hordei (Bgh). Using confocal microscopy, we followed the subcellular localisation of fluorescent organellar markers and vesicle trafficking regulators during pathogen ingress at the single cell level. We observe a close association of the early secretory pathway with the fungal haustorium; however, the endoplasmic reticulum or Golgi compartments do not form a continuous entity with the EHM. Our studies resulted in the identification of two proteins, ExtraHaustorial Membrane-Associated 1 and 2 (EHMA1 and 2) controlling vesicle trafficking, that associate with the EHM. We confirmed their presence at the EHM by comparing their localisation with fluorescently labelled vacuolar and plasma membrane markers. Furthermore, diffusion measurements revealed that both proteins are membrane-associated. As EHMA1 and 2 are involved in the same vesicle trafficking pathway, they point to the likely origin of the EHM. Alternatively, they reflect the extensive cellular reprogramming fungal effectors enforce, which results in altered regulatory protein localisation.
Key Note Talk

Towards a molecular blueprint for engineering C4 photosynthesis

Andreas P.M. Weber

Center of Excellence on Plant Sciences (CEPLAS), Institute of Plant Biochemistry, Heinrich-Heine-University, Universitätsstrasse 1, D-40225 Düsseldorf, Germany, aweber@hhu.de

C4 photosynthesis involves alterations to the biochemistry, cell biology, and development of leaves. Together these modifications increase the efficiency of photosynthesis, and despite the apparent complexity of the pathway, it has evolved at least 45 times independently within the angiosperms. To provide insight into the extent to which gene expression is altered between C3 and C4 leaves, and to identify candidates associated with the C4 pathway we used massively-parallel mRNA sequencing of closely related C3 (Cleome spinosa) and C4 (Cleome gynandra) species. This analysis shows that 818 transcripts differ in abundance between these C3 and C4 leaves. This includes twenty transcription factors, putative transport proteins, as well as genes that in A. thaliana are implicated in chloroplast movement and expansion, plasmodesmatal connectivity, and suberin deposition. These are all characteristics known to alter in a C4 leaf, but which previously had remained undefined at the molecular level. Unexpectedly, we document a large reduction in transcripts encoding ribosomal subunits. Our approach defines the extent to which transcript abundance in these C3 and C4 leaves differs, provides a blueprint for the NAD-ME C4 pathway operating in a dicotyledon, and furthermore identifies potential regulators. We anticipate that comparative transcriptomics of closely related species will provide real insight into the evolution of other complex traits.

In this presentation, I’ll focus on the identification and functional analysis of metabolite transporters required for C4 photosynthesis, with specific focus on transporters residing in the chloroplast envelope membrane.

References


De novo DNA sequence driven bulk segregant analysis of Water Use Efficiency (WUE) in potato without prior knowledge of molecular markers.

Kaminski, K.P.\textsuperscript{1,2}, Sønderkær, M.\textsuperscript{1}, Kørup, K.\textsuperscript{2}, Andersen, M.N.\textsuperscript{2} and Nielsen, K.L.\textsuperscript{1}

\textsuperscript{1}Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University and \textsuperscript{2}Department of Agroecology, Aarhus University

Population analyzed during the experiments originated from a cross between 90-HAF-01 (S.\textit{tuberosum}\textsubscript{1}) and 90-HAG-15 (S.\textit{tuberosum}\textsubscript{2}xS.\textit{sparsipilum}), which was previously evaluated for total glycoalkaloid content (TGA).\textsuperscript{1} Responses of stomatal conductance to different combinations of light, humidity, CO\textsubscript{2} concentration on leaf surface as well as temperature were analyzed and their consequences on water use efficiency were evaluated during the climate chamber experiments in 2010 and 2011.

We have previously participated in development of a de novo sequence-based alternative to positional cloning (SHOREMap).\textsuperscript{2} Here we employ a new method that allows QTL mapping exclusively from genomic sequence reads and a single reference genome sequence.\textsuperscript{3} The procedure was applied to the population for which the low and high WUE groups in respect to dry matter / water use and photosynthesis / transpiration were identified. Four bulks (two of high and two of low WUE) and the parents were subsequently sequenced to \textasciitilde30x coverage. Non-random distribution of sequence read-based polymorphisms between the WUE pools and the parental pool delimited highly resolved regions on several chromosomes. Within these regions candidate genes were found, namely glutamate ammonia ligase and proline synthase, that are presently being analyzed.

Drought priming during multiple vegetative growth stages in wheat improves drought tolerance during generative growth: insights from proteomics and physiology

Xiao Wang, Marija Vignjevic, Dong Jiang, Susanne Jacobsen and Bernd Wollenweber

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2Aarhus University, Department of Agroecology, Research Centre Flakkebjerg DK-4200 Slagelse, Denmark
3Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Wheat is one of the major crops grown throughout the world with a primary use in human nutrition. Anticipated changes in global climatic variability leading to more frequent extreme conditions will require adaptations of crop species on an unprecedented magnitude in order to sustain agricultural production. Drought stress is one of the most severe abiotic stress forms, constraining both plant growth and productivity. The response of plants to stress involves both short-term molecular and physiological responses as well as long-term structural and morphological modifications. Little is known on the impact of the increased frequency of extreme climate events and the implications of environmental variability on quality parameters in wheat.

We tested the hypothesis that acclimation to drought-stress during vegetative growth could be “memorized” by the plants and could lead to tolerance against a stress event during reproductive growth. In order to investigate the effect and mechanisms of drought acclimation, the responses of the physiological (photosynthesis rate and activities of antioxidant enzymes) and proteomic parameters (protein expression in leaves) were analyzed and identified. The results indicate changes to both enzyme synthesis and enzyme activity which may contribute to drought tolerance mechanisms in wheat.
### Session 4: Elevator talks

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Meeting the global challenges for agriculture: Sharing resources and free exchange of information

Melvin Oliver, Adjunct Professor

The Global Plant Council

At the present time the world is home to more than one billion people who are going hungry and the malnutrition that they suffer contributes heavily towards disease and mortality (FAO). Predictions are that this situation can only get worse as the global population expands from the current 7.2 billion to 9.2 billion by 2050, the majority of which will occur in low or mid-income countries (World Bank). In order to provide the critical needs of food and nutrition global agriculture will have to produce at least twice as much food as we do today. This will also have to be accomplished in the face of a changing global climate and without an increase in land use at a time when usable land for agriculture is being lost to urbanization and soil degradation. It is critical that if we are to meet global food security and sustainability, plant scientists, in particular plant breeders must be able to access relevant phenotype and genotype data as well as relevant germplasm: wherever and whatever that germplasm may be. Although simple in concept, this is an extremely complex issue encompassing such topics as data sharing (genotypic and phenotypic), large data set access, infrastructure, biodiversity, germplasm collection and preservation, germplasm access and property rights, open access publishing, the private-public research interface, and many others. The Global Plant Council, a coalition of plant science societies of the world, has declared its advocacy for the global free exchange of free exchange of information, data, and resources (including germplasm) that are in the public domain. Several organizations and groups have recognized the need for promoting and facilitating such sharing activities and it is the purpose of this presentation to highlight what is underway on the global level, discuss what efforts are currently proposed, and to hopefully stimulate debate and support for such efforts.
New, improved and better varieties are the key for efficient and increased worldwide production of more food, feed, fibre and fuel in a sustainable way. Only with worldwide access to- and proper protection of newly bred varieties, can the plant breeding be financed and developed economically healthy. There are various ways to protect new varieties, but the most commonly used is the PVP –system (Plant Variety Protection) which is based on the UPOV-convention established in 1961. UPOV is an intergovernmental organization which provides and promotes an effective system of plant variety protection for the benefit of society, farmers and breeders. This system grants the breeder of a new plant variety an intellectual right-the breeder’s right. In short, the breeder’s authorization is required to propagate the protected variety for commercial purpose and normally the actual breeder collects a royalty for the use of the variety. For example, regarding the spring barley malt variety Columbus for sowing in Denmark 2013, the breeder collects 48 DKK per 100 kg certified seed sold. The royalties and conditions are very different around the world, reflecting the differences in farming practices, prices for input and output, and the genetic value of the specific variety. The breeder’s right can only be granted if the variety is new and distinct from existing commonly known varieties, is sufficient uniform and stable (DUS criteria) and has a suitable denomination. The breeder’s right does not extend to the purpose of breeding other varieties (breeder’s exception) and the protection is limited in time, normally 25 years. In the European Union, a PVP –community system based on UPOV principles has been in place since 1994. This PVP-system gives effect and protection in all 27 member states with one application. A European Common Catalogue, which combines above DUS-criteria with VCU criteria (Value for Cultivation and Use), further secures that new and improved agricultural varieties can be sold and commercialized everywhere in all 27 member states. Additionally, obligatory certification, of listed varieties, secures varietal identity, high quality of seed and safe food and feed for the benefit of consumers and growers in EU. This PVP-system has been very successful and gives the economic base for a strong development in the seed and breeding industry across Europe. In Europe, varieties are not patentable at all and essentially biological processes for production of plants neither. In recent years, increasingly more patents have been granted in the plant segment (obviously not plant varieties) and these patents have challenged the plant breeding industry drastically. Inventions in plants, above and below the varietal level, can be granted patent protection, if the normal patent obligations like novelty, non obviousness, industrial applicability etc. are fulfilled. Patented elements inside plant varieties have legally prohibited crossing and therefore new rulings for the grey zone between PVP and Patents must be agreed upon. In certain countries “a limited breeder’s exception” has already been introduced in national patent laws, as in the very recently agreed “Unitary EU-patent” where the "limited breeder’s exception" was also included. According to these new rules, the act of crossing varieties containing patents will be allowed, but marketing of any material (varieties e.g.) with the "patented elements included" needs agreement with the patent holder. To facilitate future plant breeding programs and selection of parents, there are currently European efforts to develop a patent web site, where breeders will have easy access to information about patented elements in varieties.

IPR in plants is a very complicated area and only few selected elements have been addressed. The overview is seen from a European perspective and interested are invited to look for further European and global information.
Do we have a food crisis and how do the market economic mechanisms function?

Senior advisor Henning Otte Hansen, Ph.D.

Institute of Food and Resource Economics, Faculty of Science, University of Copenhagen, DK  hoh@foi.ku.dk

During the last couple of decades, there have been several serious global food crises with severe economic, political and market-related consequences. Most recently during 2007-08 and again in 2010-11, crises occurred with rapidly increasing food prices. It is important to shed light on the content, form, causes and consequences of food crises in order to be better able to predict and prevent future food crises. The most recent food crises share many common features and it was largely the same economic and market mechanisms which were at work. With increasing pressure on agricultural and food markets and future changes in market structures, it is likely that food crises will be a recurring phenomenon.

The most recent 3-4 food crises have shown that food supply, food markets and food companies are vulnerable when external influences create the significant price increases that characterise food crises

The food crises have created a debate over whether the actually market works, whether unrestricted free market forces are sufficient to prevent future food crises, and whether there is a need for intervention in the form of reserve-building, regulation of speculation, support for local agricultural production, etc.

Limiting speculation may seem difficult, as there is always a gray area between speculation and normal hedging. Increased reserves, controlled by a super-national body, could stabilise prices and eliminate much of the incentive to speculate. Building and controlling international grain reserves as buffers against dramatic price fluctuations could be extremely effective, but it requires substantial political support from the most significant grain producing countries.

Supply has proved to respond quickly to price increases and the market adapts relatively quickly to a new equilibrium. Yet turmoil broke out in the short period of adjustment, resulting in many serious and adverse effects. Therefore, the task involves avoiding new serious food crises and limiting the most serious effects as much as possible.
Several new breeding techniques are currently under way which differ from transgenic techniques used in the last two decades. For some of them the regulatory framework in the EU might be no longer feasible, resulting in a legislative uncertainty concerning these techniques. A working group established by the European Commission (EC) in 2007 has been evaluating whether eight new techniques constitute GM techniques and, if so, whether the resulting organisms fall within the scope of the EU GMO legislation. These techniques are: Nuclease technology, oligo-directed mutagenesis, cisgenesis, RNA-dependent DNA methylation, grafting on GM rootstock, reverse breeding, agro-infiltration and synthetic biology. In 2012 the working group finished the evaluation and circulated its opinion for comments.

Recently, EC’s Joint Research Centre (JRC) published a study on “New plant breeding techniques - State-of-the-art and prospects for commercial development” (1). The study shows that some of these techniques should not necessarily be regulated under the GMO legislation and most of them can be handled by existing protocols for risk assessment and monitoring. In addition, EC has requested EFSA opinions on the adequacy of EFSA guidelines to perform risk assessment on plants developed through new biotechnologies and to address the risks irrespective of whether or not they fall under the GMO legislation. The EFSA working group envisaged to provide the opinions one by one, starting with cisgenesis and intragenesis (2) and Zinc Finger Nuclease 3 (3).

Meanwhile, several positions have been expressed concerning prospects for regulating new biotechnologies, including a position paper by the ETP “Plants for the Future” (4).

**Oligo directed mutagenesis** is a technique using short oligonucleotides as a tool to induce targeted alterations in the genome ranging from one to a few adjacent nucleotides. The alterations are caused by mispairing of nucleotides and the subsequent DNA repair by the host cell.

**Cisgenesis/intragenesis** technique means the integration of genes in a plant genome, a normal transgenic approach, but restricting the source of the genes to sexually compatible crossing partners of the recipient plant. By this approach only cisgenesis produces progeny which can arise by chance by a conventional breeding process (CBP), whereas intragenesis always produces progeny which cannot be achieved by CBP.

The aim of **RNA-dependent DNA methylation** is the induction of gene silencing via promoter methylation of target genes. Depending on the delivery method of the DNA which encodes for the silencing RNAs the expression can be transient only inducing epigenetic changes of the genome by the RNA silencing pathway.

**Grafting** with GM scions or rootstocks can be used for the transient expression of proteins or other effectors like RNAs to induce e.g. enhanced pathogen resistance. This technique can be performed using a non-GM scion onto a GM rootstock or vice versa.
The reverse breeding technique is able to reconstitute the parental lines starting with an elite F1 hybrid whose genetic material is unknown. Reverse breeding combines several other techniques. During this procedure a transgenic step can be included but the final plant lines are non-transgenic.

Synthetic genomics aims to synthesize large DNA molecules followed by transformation in a recipient structure. Concerning the application for plants, this technique is in a very early stage.

Zinc finger nuclease technique was the original term describing a new technique which uses an unspecific nuclease coupled to a sequence specific DNA binding element, the zinc finger. The restriction activity is directed to a certain locus in the genome of an organism by the zinc finger binding activity. After induction of a double strand break, the repair is done completely by the host cell. TALENs are important new tools for genome engineering. Fusions of transcription activator-like (TAL) effectors of plant pathogenic Xanthomonas spp. to the FokI nuclease, TALENs bind and cleave DNA in pairs.

The term Agro-infiltration (or Agro-inoculation) means that a plant or plant tissue is infiltrated with a liquid Agrobacterium sp. suspension. The Agrobacterium delivers T-DNA from which genetic material is expressed locally and transiently at high levels in the plant to produce a specific protein.

We will discuss recent developments and prospects for regulating the new biotechnologies in Europe including a short outlook into the overseas state-of-the-art.

(2) EFSA Panel on Genetically Modified Organisms. Scientific opinion addressing the safety assessment of plants developed through cisgenesis and intragenesis. EFSA J. 10(2) 2561 (2012).
(3) EFSA Panel on Genetically Modified Organisms. Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. EFSA J. 10(10) 2943 (2012).
Delivery of pheromones and other semiochemicals for food security by GM, breeding and companion cropping

Michael Birkett

Biological Chemistry and Crop Protection Department, Rothamsted Research, UK

Currently, food production relies on chemical synthesis, although often inspired by natural processes. However, in the face of climate change and world population increase, more sustainable approaches will need to deliver such chemistry. This will be via the seed so as to avoid high energy intensive seasonal inputs to agriculture. The most powerful tools will involve secondary plant metabolism, but delivered for sustainable agriculture via the seed by accessing biodiversity and using new breeding technologies and, more importantly, genetic modification (GM). Use of organic chemistry for structure determination via bioassay (phenotyping) guided fractionation provides novel opportunities from plant metabolism, which will be essential in devising the completely new tools necessary for the sustainable intensification of agricultural production. Evidence of the value of such approaches will be discussed in terms of exploiting plant metabolites, for example by delivery via new crops from natural diversity through to plant sources created by GM, including the induction of genes by external natural chemical elicitors.

Related references


High-throughput genome sequencing and SNP genotyping technologies, including genotyping by sequencing, are rapidly reducing the cost of high-density marker data in many plant species. Advances in the analysis of these data by statistical models for complex traits have led to the application of genomic prediction in plant and animal species. Genomic prediction relies on the strong assumption that every locus affecting the quantitative trait (QTL) is in tight linkage disequilibrium with one or multiple markers. These markers themselves are taken as explanatory variables in the statistical model and thereby bypassing the QTL modelling as in linkage mapping. Key to genomic prediction is a reference population for which full phenotype- and genotype information is available and that is used to construct an equation predicting the breeding values of unphenotyped individuals from marker genotypes. Based on these predictions, individuals can be selected even before phenotypes are available. The actual response to selection depends on four factors, i.e., selection intensity; prediction accuracy; genetic variance; and generation interval, where shortening the interval has been considered to be most effective to accelerate the accumulated response. Genomic prediction has been successfully applied to dairy cattle for several years and the application to major field crops such as maize is now underway. However, the use of genomic prediction in many other plant species has been impeded for multiple reasons. Firstly, complete genome sequences are often not available, which hampers the development of high-throughput SNP arrays. Secondly, a sufficiently-large reference population is required to establish a robust prediction equation. Thirdly, the costs of collecting accurate phenotypic records can be prohibitively expensive. Fourthly, practical implementation may be less straightforward as breeding is often focussed on varieties whose performance and stability must be tested across multiple years and environments and may dramatically increase the generation intervals. This presentation will cover the concepts of genomic prediction, the relationship to genome wide association studies, review initial results for several plant species, and discuss pending challenges and opportunities.
Dynamic interaction of plant and environment for resource use efficiency - quantitative approaches to non-invasive plant phenotyping?

Ulrich Schurr

Forschungszentrum Jülich, IBG-2: Plant Sciences, 52425 Jülich, Germany
u.schurr@fz-juelich.de

Resource use efficiency is key to improve sustainable agricultural production will thus be the basis of an urgently needed 2nd Green Revolution. In order to be efficient plants have to handle spatially and temporally varying resources and environments. In recent years we developed technologies to quantify the dynamics and the heterogeneity of plant structure and function as well as of environmental cues. These mostly non-invasive technologies are developed and implemented into concepts that allow novel insights in the dynamic characteristics of plants above- as well as belowground. The technologies include high-resolution analysis for mechanistic understanding (like MRI and PET for structure, growth and activity of roots and shoots), the high-throughput approach for analysis of large numbers of genotypes and environmental conditions as well as field approaches, which are the reference to indicate the relevance. The talk will provide an overview on recent developments in technologies as well as conceptual approaches as the basis for a quantitative understanding of plant-environment-dynamics and its application for plant breeding and plant management. The talk will report about recent technological developments in plant phenotyping as well as about infrastructure platforms that have been and will be established in our institute, in Germany and in Europe.
TALEs and TALENs for expression control and targeted genome engineering

Jens Boch

Martin Luther University Halle-Wittenberg, Institute of Biology, Department of Genetics, Weinbergweg 10, D-06120 Halle (Saale) Germany.

The DNA-binding domain of TALEs (transcription activator-like effectors) from plant-pathogenic Xanthomonas bacteria has become an important tool for the programmable and specific targeting of DNA. Natural TALEs function as transcription factors in plant cells to support pathogen colonization of host plants. TALE proteins bind to DNA via near-identical tandem repeats of 34 amino acids. Each repeat recognizes one base in the target DNA sequence via repeat-variable diresidues (RVDs). The simple and modular repeat architecture allows rearrangement of TALE repeats to generate artificial TALEs with virtually any tailored DNA-binding specificity\(^1\). Highly specific genome-editing TALE nucleases (TALENs) can be engineered for targeted mutagenesis in plants and a wide variety of other eukaryotic organisms. We analyzed specificities and activities of TALEs experimentally in a transient reporter system using Agrobacterium-mediated expression in planta. Different RVDs exist in nature, but the DNA-specificity of only a few of them is known, so far. I will present novel results on RVD specificity and efficiency\(^2\), as well as the design of programmable gene switches and programmable precision mutagenesis tools. TALEs and TALENs are versatile virulence factors for the pathogen and exceptional tools for biotechnology.

Free flow electrophoresis (FFE) is a powerful liquid based separation technique for many applications in life sciences. The separation is matrix-free, i.e. no solid components such as polyacrylamide or columns are used. The sample is flowing in a continuous laminar flow through an electric field perpendicular to the flow. The high voltage electric field causes the deflection of sample components in the aqueous buffer system. The sample is then separated into 96 fractions that are collected in a 96 well MTP plate.

FFE applications include separation of peptides, proteins, membranes, bioparticles, organelles and other charged or chargeable particles.  
The Free Flow Electrophoresis System has advantages over other separation processes:

• Separate samples in an analytical or preparative mode
• Continuous sample application, separation and collection
• Multiple samples can be run consecutively in the same mode
• Complex samples can be separated with high yield
• Matrix-free separation
• Separation in native and denaturing conditions
• Compatibility with many up and downstream processes like HPLC, PAGE, ELISA, MS
• Quick sample separation (less than 15 minutes) provides excellent throughput capacity

In this study we also introduce novel separation protocols for high resolution native separation of protein isoforms and the separation of nanoparticles (nanodiscs).
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- **Jensen et al.**  Mapping Lipid Specificity of P4-ATPases  
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- **Aguilar et al.** Searching for fungal effectors shaping the extrahaustorial membrane
- **Gjendal et al.** Impact of Climate change on emerging plant diseases and their threat to food security
- **Roelsgaard et al.** Barley: The correlation between powdery mildew infection and leucine-derived hydroxy nitrile glucosides
- **Nafisi et al.** Interaction between Arabidopsis and natural bacterial isolates
- **Stranne et al.** The role of plant cell wall acetylation on plant fitness
- **Kosawang et al.** ABC transporters are potentially evolutionary evolved to provide resistance to xenobiotics in the mycoparasitic fungus Clonostachys rosea IK726
- **Zhang et al.** Barley powdery mildew effector candidate CSEP0443 interacts with a RING finger E3-ligase-type protein

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- **Byrne et al.** Application of Genome Wide SNP Markers to Estimate the Extent of Linkage Disequilibrium in a Collection of Perennial Ryegrass Genotypes.
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- **Czaban et al.** Genotyping by sequencing of a training population for genomic selection in L. perenne
- **Paina et al.** An RNA-Seq approach to study vernalization and the induction of flowering in perennial ryegrass
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Within each category one abstract has been selected for oral presentation.

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Barbara Halkier, University of Copenhagen
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Selection committee for Signaling and cellular trafficking
Jens Stougaard, Aarhus University
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Selection committee for Synthetic- and systems biology
Kåre Lehmann Jensen, Aalborg University
Poul Erik Jensen, University of Copenhagen
Søren Bak, University of Copenhagen
Starch characterization in *Brachypodium distachyon*  

Vanja TANACKOVIC1*, Jan T. Svensson1, Alain Buléon2, Mikkel A. Glaring1, Susanne Langgård Jensen1, Massimiliano Carciofi1, Andreas Blennow1  

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*Brachypodium distachyon* was recently introduced as a model plant for temperate cereals (IBI 2010). In order to explore pre-domesticated and novel features of cereal starch metabolism, we aim to establish *Brachypodium* as a model plant.  

Bioinformatics analysis identified starch biosynthesis genes: 7 soluble starch synthases (SSs), 2 granule bound starch synthases (GBSSs), 4 starch branching enzymes (SBEs), 2 glucan- and 1 phosphogluca I- water dikinase (GWD, PWD). Transit peptides and putative carbohydrate-binding modules (CBMs) of the families CBM20, CBM45, CBM48 and CBM53 were identified.  

Grain starch micro structure, granule size, amylopectin chain length distribution, phosphate- and amylose content, as well as kernel starch, β-glucan and free sugar content from two lines, Bd21 and Bd21-3 were analysed providing data for comparing *Brachypodium* with barley (*Hordeum vulgare*). Wide-angle X-ray scattering (WAXS) and differential scanning calorimetry (DSC) revealed low crystallinity of *Brachypodium* starch granules as compared to barley. Transitory starch in leaves and stem of *Brachypodium* was higher than in barley.  

We demonstrate that *Brachypodium distachyon* is valuable to provide new insight into starch bioengineering in temperate cereals.
Evolution of the biosynthesis of cyanogenic glucosides in Lepidoptera

Mika Zagrobelny, Nanna Bjarnholt, Søren Bak, Birger Lindberg Møller

Plant Biochemistry Laboratory
Department of Plant and Environmental Sciences (PLEN)
University of Copenhagen, Denmark

An essential 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* (Fabaceae) and *Zygaena filipendulae* (Zygaenidae). They both contain the cyanogenic glucosides (also called α-hydroxynitrile glucosides) linamarin and lotaustralin 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 the same cyanogenic glucosides from their food plants.

The pathway for cyanogenic glucoside biosynthesis in *Z. filipendulae* proceeds using the same intermediates as the pathway from plants. In both plants and insects, convergent evolution has led to two P450 enzymes and a glucosyl-transferase acting in sequence to catalyze cyanogenic glucoside formation. Thus plants and insects have independently found a way to package a cyanide bomb to fend off herbivores and predators. The first committed enzyme of the biosynthetic pathway, CYP405, has close homologs in several species of butterflies and moths, some of which (Heliconiini butterflies) also contain cyanogenic glucosides. Furthermore the γ-hydroxynitrile glucoside sarmentosin, which is closely related to cyanogenic glucosides, probably share the first biosynthetic step with the cyanogenic glucosides. Sarmentosin is found in several species of butterflies and moths, and in a few species both types of compounds are found. For both types of compounds it has been hypothesized that some insect species can biosynthesize them. This implies that the ability to biosynthesize both types of compounds may have evolved in a ditrysian ancestor common to Papilionidae and Zygaenidae, or even earlier. The ability to produce and handle hydroxynitrile glucosides would render butterflies and moths capable of colonizing new food plants containing these same compounds, thus creating competitor-free niches.


Redox regulation of starch biosynthetic enzymes in *Arabidopsis thaliana*

**Katsiaryna Skryhan**, **Mikkel A. Glaring**, **Brian B. Nielsen**, **Morten M. Nielsen**, **Jose A. Cuesta-Seijo**, **Monica M. Palcic**, **Andreas Blennow**

1VKR Research Centre Pro-Active Plants, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark  
2Carlsberg Laboratory, Gamle Carlsberg Vej 10, 1799 Copenhagen V, Denmark.

Starch is the major and the most abundant storage carbohydrate in higher plants and it is also the most important energy source in the human and animal diet. Starch is composed exclusively of two structurally distinct α-D-glucose polymers, namely amylose and amyllopectin, which are stored inside chloroplasts (transient starch) or amyloplast (storage starch) in form of insoluble granules. Transient starch is synthesized during the day and is degraded over the night to meet up the ongoing energy requirements of plant metabolism in the dark [1]. The redox state of the plant cell seems to be the main factor by which photosynthesis and starch metabolism is coordinated. In the chloroplast, reducing equivalents produced during the day by photosynthesis are transported from photosystem I via the ferredoxin-thioredoxin system to the target enzymes. These proteins contain key cysteine residues that make disulfide linkage upon oxidation [2].

In our study we performed a comprehensive analysis of the redox sensitivity of known starch biosynthetic enzymes in leaf extracts of *Arabidopsis thaliana* using zymograms and enzyme assays. Our results confirmed that at least four starch biosynthetic enzymes are activated by reduction at physiologically relevant potentials - starch synthases SS1 and SS3, starch branching enzyme BE2 and isoamylase complex ISA1/2 [3]. We chose first three enzymes to be expressed as 6xHis-tagged proteins in *E. coli* cells. An analysis of purified recombinant proteins also confirmed its redox sensitivity. To identify which cysteines are involved in the formation of the regulatory disulfide linkage, we are currently generating site-specific mutants, one for each cysteine present in the sequence. Each mutant is carrier a single cysteine to serine substitution, and all of them will be expressed in *E. coli*, purified and tested for redox sensitivity by redox titration.

References:
De novo Biosynthesis of Cyanogenic Glucosides in Zygaena filipendulae

Joel Fürstenberg-Hägg, Mika Zagrobelny, Kirsten Jørgensen, Søren Bak and Birger L. Møller

Plant Biochemistry Laboratory, Department of Plant and Environmental Sciences & The VKR Research Centre “Pro-Active Plants”, University of Copenhagen, Denmark

Cyanogenic glucosides (CNglcs) are amino acid derived bio-active natural products, present in more than 2,500 different plant species. CNglcs are mainly used as defence compounds against generalist herbivores, due to their bitter taste and through the release of toxic hydrogen cyanide (HCN) and ketones or aldehydes upon tissue disruption. The larvae of the specialist insects belonging to the Zygaena family are able to both sequester the CNglcs linamarin and lotaustralin from their polymorphic food plants (Lotus) and biosynthesise these compounds de novo when encountering a plant with suboptimal ratio and/or content of CNglcs. In Zygaena, CNglcs are used mainly as defence compounds, but also for storage of reduced nitrogen, in mating communication and as nuptial gifts in imagines. The biosynthesis of CNglcs in the burnet moth Zygaena filipendulae follows the same pathway as the food plant Lotus corniculatus, with two multifunctional P450 enzymes and a glucosyl-transferase acting in sequence, and contains the same intermediates. Remarkably, the pathways are not the result of horizontal gene transfer but have evolved convergently in plants and insects.

We are studying Z. filipendulae to identify the site of de novo biosynthesis of the CNglcs linamarin and lotaustralin, the regulation of the pathway as well as the roles of these CNglcs during the life cycle. We have discovered that the regulation of the biosynthesis of CNglcs in Z. filipendulae larvae is related to the amount of CNglcs present in the food plant. Further, evidence is provided for the fat body and epidermal cell layer of the integument to be the site of biosynthesis in larva. By injecting radiolabelled precursors for the CNglcs, we surprisingly found that at least the female adult moths can biosynthesise CNglcs; a feature previously described only in Heliconini butterflies. In addition, male moths that are rejected from mating by females show lower levels of the biosynthesis gene transcripts. In adult moths, the biosynthesis genes are expressed in the abdomen, from where the CNglcs are transported mainly to the eggs for protection of the offspring as well as to the head and thorax. We are currently conducting time series analysis of the biosynthesis gene expression as well as concentrations of the CNglcs during the metamorphosis from larva to adult moth.

References
Grasses containing fructans may represent a rich source of carbohydrates for biofuel or other purposes. To realize this potential, enzymes for degrading both the $\beta2\rightarrow1$ and the $\beta2\rightarrow6$ bonds will be needed. To degrade $\beta2\rightarrow6$ we have expressed a gene encoding a suggested levanase, LevB, from *Bacillus subtilis* in *E. coli*, and we have obtained active enzyme preparations. The gene encoding LevB was cloned and expressed using the Gateway (Invitogen) recombinational cloning system. The gene was amplified by PCR and inserted in vectors to express both a His$_6$-tagged, and a combined MBP-His$_6$ tagged version of enzyme. The expressed proteins were obtained in soluble forms that can be partially purified by standard affinity chromatography techniques. We are presently characterizing the active His$_6$-tagged version of the enzyme with respect assay conditions and substrate preferences.
Characterization of a novel β-glucuronosyltransferase from Arabidopsis involved in the glycosylation of type II arabinogalactan polysaccharides

Adiphol Dilokpimol¹, Eva Knoch¹, Theodora Tryfona², Christian P. Poulsen¹, Jesper Harholt¹, Bent L. Petersen¹, Peter Ulvskov¹, Masood Z. Hadi³, Paul Dupree², and Naomi Geshi¹

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Arabinogalactan-proteins (AGPs) are a group of complex plant glycoproteins analogous to animal proteoglycans. AGPs are found on cell surface from red algae to flowering plants and subtle change of glycan structure is considered to play important roles in plant growth and development¹. AGPs consist of protein backbone, glycan (arabino β-1,3-galactan; type II AG) and in some cases also GPI anchor on C-terminus. The glycan part of AGP often occupies more than 90% of the entire molecule and considered as the major contributor of the function of AGPs. However, structure of type II AG is heterogeneous, depending on plant species, tissues and developmental stages, which makes it difficult to understand molecular function of sub-populations of type II AG. We try to address the question by studying enzymes involved in the glycosylation process of type II AG. Biosynthesis of AGPs occurs by post translational modification of protein backbone in secretory pathway, where the major glycosylation process occurs in Golgi apparatus catalyzed by glycosyltransferases (GTs). Type II AG is commonly composed of β-1,3-galactan backbone with substitution of β-1,6-galactan side chains at O6 position. The side chains are also substituted with other sugars such as Ara, Rha, and (4-O-me)-GlcA. At least 10 different GTs are involved in the glycosylation of type II AG, but little has been identified and characterized.

Recently, we identified four GTs from Arabidopsis thaliana which are involved in type II AG biosynthesis. They are co-expressed with each other based on the In silico database analysis². In this poster, we present biochemical characterization of one of the four GTs described above. This GT belongs to the family GT14 in Carbohydrate Active Enzyme database and has a topology of type II membrane protein, typically for a Golgi-localized GT. We expressed soluble catalytic domain of the GT in Pichia pastoris and characterized the encoding biochemical activity in vitro. We present evidence to show that the GT possesses a glucuronosyltransferase, which transfers GlcA to the terminal Gal of β-1,6-galactan side chain in type II AG via β-glycosidic linkage. Thus, we named the enzyme as AtGlcAT14A (Arabidopsis thaliana GlcA transferase family 14). This is the first report for biochemical characterization of a plant enzyme in family GT14 which possesses GlcAT activity involved in biosynthesis of type II AG.

References
Use of Cowpea Mosaic Virus-Hyper Translatable technology to elucidate functions of cyclases involved in saponin biosynthesis

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In the Brassicaceae, there are two native Barbarea vulgaris types; a pubescent P-type and a glabrous G-type. G-type plants are natural resistant against the crucifer pest Phyllotreta nemorum (flea beetle) while the P-type plants are susceptible. The resistance in the G-type is primarily caused by the presence of the triterpenoid saponin hederagenin cellobioside and also correlates with the presence of oleanolic acid cellobioside and other minor compounds. Saponins are derived from the sterol biosynthetic pathway in which 2,3-oxidosqualene is a key intermediate and act as a branch point between phytosterol and saponin formation. In the saponin biosynthetic pathway 2,3-oxidosqualene is cyclized by an oxidosqualene cyclase into β-amyrin, lupeol and other triterpenes backbone skeletons, oxygenated by cytochromes P450, and finally glycosylated by a family 1 UDP glycosyltransferase.

In B. vulgaris knowledge about genes involved in saponin biosynthesis is scarce. The focus of this study is to identify and characterize a cyclase, possibly involved in the production of saponins in B. vulgaris.

By studying the functions of Arabidopsis thaliana oxidosqualene cyclases and data mining of datasets from transcriptomic and genomic sequencing data LUP2 was found as a candidate oxidosqualene cyclase gene from B. vulgaris. BvLUP2 was cloned and transformed into Agrobacterium tumefaciens and finally infiltrated into Nicotiana benthamiana for transient expression using the Cowpea Mosaic Virus-Hyper Translatable technology (CPMV-HT). From Thin Layer Chromatography and GC-MS analysis it was observed that leaves infiltrated with BvLUP2 constructs produced the triterpenoid lupeol.

Based on Real-Time quantitative PCR it was shown that BvLUP2 has higher expression levels in P-type compared to G-type. Combining these findings we hypothesize that BvLUP2 belongs to the biosynthetic pathway of P-type, but not G-type, related saponins, of which functions yet are unknown.
Cytochromes P450 involved in thapsigargin biosynthesis

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Thapsigargin, a sesquiterpene lactone produced by the Mediterranean plant *Thapsia garganica*, is currently being developed as a new type of drug against solid tumor cancers (Genspera Ltd.). However, cultivation of *T. garganica* is difficult and chemical synthesis of thapsigargin is a cumbersome and expensive procedure. As a new system for production of thapsigargin the model plant *Physcomitrella patens* has been proposed due to its ease of genetic manipulation and industrial scale-ability. Before this production system can be fully developed the biosynthesis pathway of thapsigargin has to be elucidated.

The majority of information on sesquiterpene lactone biosynthesis comes from compounds found in the Asteraceae family, and the anti-malaria drug artemisinin is particularly well studied. *T. garganica* belongs to the Apiaceae family and sesquiterpene lactones found in this family have a different stereochemistry from those found in Asteraceae. The precursor of all sesquiterpene lactones, farnesyl pyrophosphate (FPP), was found by Pickel et al. (Biochemical Journal, 2012, 448, 261-271) to be converted to kunzeaol in *T. garganica* by the sesquiterpene synthase, STS2 (AFV09099.1). The structure of kunzeaol makes it a probable candidate for the first step in thapsigargin biosynthesis. Further hydroxylation and modification of the kunzeaol skeleton is expected to be performed by enzymes from the cytochrome P450 family. Cytochromes P450 from Apiaceae only share low sequence identity with cytochromes P450 from other plant families. However, because so far only cytochromes P450 from the 71 clade have been found to participate in biosynthesis of sesquiterpene lactones, *T. garganica* cytochromes P450 in this clade are promising candidates for characterization. Full length sequences of cytochromes P450 belonging to the 71 clade found in a sequencing dataset of the transcriptome of *T. garganica* root were selected as the primarily candidates to undergo expression and substrate analysis. The yeast strain EPY300, which has been modified to have an enhanced production of FPP, was chosen as an expression host. Each candidate cytochrome P450 was cloned into the pESC LEU2d yeast expression vector along with one of the native cytochrome P450 reductases, CPR1, and the sesquiterpene synthase, STS2. A variety of sampling methods are currently being tested including the use of volatile analysis using headspace SPME fibers and diethyl ether extraction followed by cyanide derivatisation and analysis by GC-MS. This will hopefully lead to the discovery of a cytochrome P450 able to hydroxylate kunzeaol into an alcohol, an acid or facilitate formation of an epoxide.
Identification and Characterization of Forskolin Biosynthetic Enzymes from *Coleus forskohlii*

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*Coleus forskohlii* is a plant used since ancient times in Hindu and Ayurvedic traditional medicine for treating a broad number of human health disorders. The main active compound is forskolin, a labdane type diterpene accumulating in the roots of coleus. A plethora of diverse pharmaceutical properties have been attributed today to forskolin, extending from antiglaucoma, anti-HIV or antitumor activities to treatment of hypertension and heart failure problems. The majority of forskolin properties are due to its ability to act as a potent activator of the adenyl cyclase enzyme leading to marked increase of the intracellular level of cAMP (3'-5'-cyclic adenosine monophosphate) in all mammalian *in vitro* and *in vivo* systems tested so far. Recently, a semisynthetic forskolin derivative has been approved for commercial use in Japan for treating cardiac surgery complications, heart failure, or cerebral vasospasm. The discovery of forskolin as direct activator of adenyl cyclase, with the ability to modulate the intracellular levels of cAMP and to elicit cAMP-dependent physiological cellular responses, has fuelled intensive pharmacological research during the last years. However, the exact biosynthetic pathway and the enzymes involved in forskolin biosynthesis have not yet been characterized.

Using next generation high throughput sequencing we generated a coleus root transcriptome library, mining of which resulted in the identification of a number of diterpene synthases (*CfdiTPSs*), the enzymes responsible for the construction of the forskolin backbone. The biosynthesis of the diterpenoid backbone in angiosperms requires the activity of two independent classes of diterpene synthases namely class I and class II. Phylogenetic analysis showed that four of the identified coleus diterpene synthases are related to the plant specialized metabolism and more specifically *CfdiTPS1* and *CfdiTPS2* belong to the class II while *CfdiTPS3* and *CfdiTPS4* to the class I enzymes. Following *in vivo* (transient expression in tobacco) and *in vitro* (enzymatic assays using recombinant proteins expressed in *E. coli*) functional characterization, we showed that *CfdiTPS2* in combination with *CfdiTPS3* or *CfdiTPS4* are involved in the synthesis of forskolin precursor molecules.

Further, we showed that forskolin is accumulating primarily in a specific cell type of the root namely root bark. Characteristic of those cells is the presence of single oil-body like subcellular structures, as it was detected microscopically. Purification and metabolites’ analysis of these oil bodies showed that forskolin is accumulated in those structures. Interestingly, the *CfdiTPS1*, *CfdiTPS2* and *CfdiTPS3* genes were found to be expressed highly in the root bark, supporting a particular role of these cells in forskolin production, while the *CfdiTPS4* followed a different pattern.

The identification of forskolin biosynthetic enzymes can facilitate the generation of biosustainable and cost-effective production platforms to supply commercially exploitable amounts of forskolin.
Biosynthesis of benzoxazinoids, Transcriptional regulation and cellular metabolism in rye

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Abstract:
Benzoxazinoids are a group of specialized plant compounds, important for defense against pathogens and weeds. These compounds also have some potential health promoting effects as i.e. anti-allergy, anti-inflammation or anticancer. The aim of this study was to draw a detailed overview on benzoxazinoid profiles during germination in rye, both on genetic and metabolic level.

Five BX genes (ScBX1-ScBX5) were analysed for their transcript levels at various stages of seed germination and during seedling development. Moreover, levels of 12 different benzoxazinoids were also estimated on LSMS/MS in similar samples. Our results showed that all BX genes are co-regulated during germination of rye seeds. Maximum transcript levels were found after 24hr of germination. Developing shoots have high level of BX transcripts compared to roots. Chemical contents of corresponding benzoxazinoids synthesized from these BX genes were elevated after 30hr of germination. BX gene transcription and internal plant metabolism controls the benzoxazinoids level at later stages. A detailed overview on these aspects will be discussed.

This study is the first of its kind, as no detailed information on BX gene transcription in growing embryo and individual tissues has been available so far. It will help us to understand the biosynthesis pathway of benzoxazinoids in rye and may help us to design pre-baking processes in bread making, with effects on the levels of potentially healthy compounds.
Targeted and untargeted approaches to identify protein-protein interactions

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Physical associations between proteins, so-called protein-protein interactions, are an essential aspect of all biological processes. There is an increasing awareness that we have to think in terms of the dynamic interactome as opposed to the classical one-protein-at-a-time approach. A crucial prerequisite for studying this dynamic interactome is to identify the proteins involved and their physical associations. We work on protein-protein interactions within transport, regulation and biosynthesis of secondary metabolites using glucosinolates as a model compounds. Here, we present our recently established protein-protein interaction platform which is founded on a cytosolic split-ubiquitin-based yeast two hybrid system (CytoY2H) optimized for transcriptionally active bait proteins (Möckli et al., 2007).

We have screened our normalized cDNA library from mixed tissues of Arabidopsis thaliana to identify interacting partners in regulons centered around a key regulator of glucosinolate biosynthesis. As a complementary untargeted approach to isolate and identify regulatory protein complexes in vivo, we are currently conducting tandem affinity purification-mass spectrometry experiments. By contrast, we chose a targeted CytoY2H approach to provide evidence for the proposed metabolon driving the core structure biosynthesis of glucosinolates. We have generated a “targeted prey library” consisting of more than 80 genes involved in or associated with glucosinolate biosynthesis which enable us to test any given bait for all possible binary interactions amongst all known players within a week using CytoY2H.

References:
Expression of therapeutic glycoproteins with plant specific allergenic glycoforms

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The incidence of atopic diseases such as asthma, allergic rhinitis and atopic eczema has increased substantially over the past decades, now affecting more than 25% of the European population. A large fraction of natural allergens originates from plants and a large fraction of these are glycoproteins.

While plant N-linked cross-reactive carbohydrate determinants (CCDs) generally are considered clinically irrelevant with regards to allergy, plant specific extensin type single arabinofuranoses $\beta$-linked to hydroxyprolines (O-linked) of the glycoprotein backbone (Hyp-Ara$_f^1$) have been implicated in the overall allergenicity of a number of allergenic glycoproteins including the major allergens Art v 1 and Lol p 1 of mugwort and ryegrass, respectively. Tolerance to a particular allergen may be achieved by continuous administration of the allergen(s), termed specific immunotherapy (SIT). Recent studies have revived the importance of including mixtures of allergens to ensure a consistent and well-balanced composition in glycoepitopes linked with allergens reflecting natural (authentic) exposure and sensitization conditions.

In the present study we explore the suitability of using Arabidopsis thaliana mutants deficient in their extensin type O-glycosylation machinery for controlled production of extensin type glycoepitopes, including the allergenic Hyp-Ara$_f^1$ epitope. Tagged versions of Art v 1 and Lol p 1 were expressed in the mutant backgrounds and are now awaiting purification and structural analysis of their glycoforms.
Linking chlorophyll biosynthesis to photosynthesis

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The production of chlorophyll in higher plants is closely regulated in accordance with the need for the pigment. This ensures both a sufficient supply of the light-absorbing pigments for a fully functional photosynthetic apparatus but also avoid the accumulation of free chlorophyll intermediates potentially leading to photo-oxidative damage.

Most steps in the biosynthesis of chlorophyll have been elucidated at the genetic and biochemical level, but the localization and the subunit composition of some of the biosynthetic enzymes is yet unclear. In particular the step leading to the formation of the fifth ring in the chlorophyll molecule is not fully characterized. This step is catalyzed by the so called aerobic cyclase. We have previously identified one catalytic subunit of this enzyme: CHL27 (Tottey et al., 2003). CHL27 contains two irons in its active site and in order to complete its catalytic cycle these irons need to be reduced from Fe3+ to Fe2+. So far no enzyme involved in this has been identified despite huge efforts from many researchers. This prompted us to search for alternative ways to reduce the two irons in CHL27. We have used specific inhibitors of electron transport and mutants affected in regulation of electron flow to show that this particular step in chlorophyll biosynthesis indeed is directly regulated by the photosynthetic electron transport chain. This has interesting consequences since there now is a direct connection between photosynthesis and the biosynthesis of photosynthetic pigments.

Engineering the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway in moss *Physcomitrella patens* for terpene production

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Genes of the MEP pathway enzymes in *Physcomitrella patens*, including DXS (1-deoxy-D-xylulose 5-phosphate synthase) and DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase), which are considered as the rate-limiting enzymes in the plastidial biosynthesis of the universal precursor IPP (isopentenyl pyrophosphate), were cloned and re-introduced into moss under the control of strong constitutive promoter. This was done in order to increase the precursor pool for exogenous terpene synthases introduced by homologous recombination.

*P. patens* is able to produce large amounts of the diterpenoid ent-kaurenes. In order to increase the amount of precursor available for terpenoid production, a knock-out of PpCPS/KS (copalyl diphosphate synthase/ent-kaurene synthase) moss line was created previously. The line will be utilized together with the up-regulation of MEP pathway to enhance terpenoids production.

Retarded moss growth may be encountered probably due to depletion of universal terpenoid precursors or toxicity of heterologous terpene product. Targeted product may also be decorated by a hidden array of endogenous enzymes in transformed moss. Pathway negative feedback and gene silencing is another layer of risk for metabolic engineering. These plant physiological features need to be further studied.
Sesquiterpene Synthases in *Thapsia garganica*

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Thapsigargin is a guaianolide only found in two members of the genus *Thapsia* (Apiaceae) [1]. Covalently linked to a peptide, thapsigargin has been converted into a prodrug targeted for the treatment of solid tumors. Phase 1 clinical trials have proven highly promising, and the drug is currently undergoing Phase 2 trials for the treatment of prostate cancer [2].

Since neither natural sources nor chemical synthesis can fulfill future market demands, biosynthesis in a heterologous host is an attractive alternative. A prerequisite for biotechnological production is knowledge about the enzymes catalyzing fundamental steps of the biosynthesis.

Thus, enzymes involved in thapsigargin biosynthesis are investigated, with the first step being a terpene synthase converting farnesyl pyrophosphate into a sesquiterpene backbone. Next generation sequencing data enabled us to identify two sesquiterpene synthases, TgSTS1 and TgSTS2. TgSTS1 catalyzes d-cadinene synthesis, and TgSTS2 mainly catalyzes formation of the unstable germacrenol derivative kunzeaol that is thought to be an intermediate in thapsigargin biosynthesis [3]. Cytochromes P450, flavin monooxygenases (FMO) and 2-oxoglutarate-dependent dioxygenases (2OGDD) genes are thought to be responsible for the further steps in thapsigargin biosynthesis, and are currently under investigation.

TgSTS1 and TgSTS2 need further characterization with respect to kinetics, substrate and product promiscuity in relationship to other variables like pH, temperature, and cofactors. Comprehensive study of these enzymes should provide insight about how activity and product profiles can be manipulated by altering abiotic factors in order to enhance future biotechnological production [4, 5]. Highly active or more specific enzymes may be used to provide a potential heterologous platform with optimized enzymes that can further limit the production costs of thapsigargin.

Yeast (*Saccharomyces cerevisiae*) strains fulfill our requirements for preliminary screening, and tobacco (*Nicotiana benthamiana*) provides simple screening for *in planta* analysis. Additionally, alternative means of production are being investigated. The moss *Physcomitrella patens* is being investigated as the anticipated factory for thapsigargin production. Currently, TgSTS1 and TgSTS2 are being investigated using moss as an expression host.

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Genotypic variation and effect of nitrogen fertilization on Si accumulation in wheat straw

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Plant feedstock such as crop residues can be used as a lignocellulosic biomass for bioenergy production through biochemical or thermochemical degradation. One of the problems of using plant feedstock is that they often contain relatively high amounts of silicon (Si), which inhibits degradation of biomass. In biochemical degradation, Si may reduce the efficiency of enzymatic saccharification of biomass while presence of Si together with alkali metals produces slag and corrosion and damages a reaction chamber in thermochemical degradation (Van Soest, 2006; Jenkins et al., 1998). Therefore understanding the mechanisms of Si accumulation in plants and the effect of Si on digestibility of plant biomass is important to improve efficiency of bioenergy production.

In this study, genotypic variation in the concentration of Si and other inorganic elements as well as enzymatic saccharification efficiency of wheat straw was first investigated in 20 different wheat varieties grown at two locations. The results showed that there were significant influences of both growth location and variety on accumulation of Si as well as sulphur and major alkali metals in the straw. Furthermore, a significant negative effect of Si on enzymatic saccharification efficiency was detected in part of the samples.

In addition we investigated how nitrogen (N) fertilization affects elemental composition of wheat straw, since use of N fertilization is important for agricultural production influencing on yield. Wheat straw, which had been grown with a different range of N supply in the field was analysed for the elemental composition and enzymatic saccharification efficiency. Interestingly Si concentration in the straw was significantly decreased as more N was supplied to the field, while concentrations of alkali metals were increased. The concentrations of major organic components such as cellulose, hemicellulose and lignin showed only minor changes. Enzymatic saccharification of the straw was reduced with higher N supply indicating the overall negative effect of N fertilization on the quality of lignocellulosic biomass for biochemical degradation.

Our study suggests that both growth condition and genotype influence on elemental composition including Si in straw. Selection of cultivars and further breeding for the trait of low Si content may improve straw quality and thus contribute to increasing efficiency of bioenergy production. It has been shown that differences in the abundance of Si transporter between rice varieties resulted in different accumulation level of Si in the straw (Ma et al., 2007). Modification of Si transporters involved in Si uptake and deposition can also help to regulate Si content in plants.

References:
Seven CAD-like Genes from *Brachypodium*

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Cinnamyl alcohol dehydrogenase (CAD) catalyzes the final step of the monolignol biosynthesis, the conversion of cinnamyl aldehydes to alcohols, using NADPH as a cofactor. Seven members of the cinnamyl alcohol dehydrogenase family were identified in the genome of *Brachypodium distachyon* and five of these were isolated and cloned from genomic DNA. RT-PCR revealed differential expression of the cloned genes, with BdCAD5 being expressed in all tissues and highest in root and stem while BdCAD3 was only expressed in stem and spikes. A phylogenetic analysis of CAD-like proteins place BdCAD5 on the same branch as *bona fide* CAD proteins from maize (*ZmCAD2*), rice (*OsCAD2*), sorghum (*SbCAD2*) and *Arabidopsis* (*AtCAD4, 5*). The predicted 3-D structures of both BdCAD3 and BdCAD5 resembled that of *AtCAD5*. However, the amino acid residues in the substrate binding domains of BdCAD3 and BdCAD5 are distributed symmetrical and BdCAD3 is similar to that of poplar sinapyl alcohol dehydrogenase (*PotSAD*). BdCAD3 and BdCAD5 were expressed and purified from *Escherichia coli* and both showed a temperature optimum of about 50 °C and a molar weight of 49 kDa. The optimal pH for the reduction of coniferyl aldehyde were determined to be pH 5.2 and 6.2 and for the reverse reaction, oxidation of coniferyl alcohol, the optimal pH were determined to be 8 and 9.5 for BdCAD3 and BdCAD5, respectively. Kinetic parameters for conversion of coniferyl aldehyde and coniferyl alcohol showed that BdCAD5 was clearly the most efficient enzyme of the two. These data suggest that BdCAD5 is the main CAD enzyme for lignin biosynthesis and that BdCAD3 has a different role in *Brachypodium*. All CAD enzymes are cytosolic except for BdCAD4, which has a putative chloroplast signal peptide adding to the diversity of CAD functions [1].

![Scheme 1](image)

**Scheme 1.** The conversion of coniferyl aldehyde to coniferyl alcohol by cinnamyl alcohol dehydrogenase using NADPH as co-factor.

Abstract: Plant cell walls are highly complex three dimensional matrixes, composed of diverse polysaccharides. Cell walls support the plant body and constrain cell shape but must also where required to be capable of controlled elongation to allow growth. There has been an intense research effort to understand the roles of plant hormones in regulating cell elongation, especially in response to tropic stimuli such as light and gravity that result in differential elongation and curvature. Much less well understood though are the presence roles of cell wall polysaccharides during cell elongation. Our model subjects are cotton fibre, Arabidopsis thaliana and pea plants. We induce differential elongation to samples either by environmental tropisms like gravitropism or by local application of plant hormone auxin. To determine cell wall composition in our samples, we utilize comprehensive microarray polymer profiling (CoMPP). Confocal microscopic techniques are also applied to study the structural changes in situ. Here we present some preliminary results from these studies.
Biological functions and turnover of cyanogenic glucosides in sorghum seeds

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Sorghum is a grass indigenous to the steppes and savannas of Africa and is due to its origin both more drought- and temperature tolerant than maize, soybeans, wheat and other commonly farmed crops. It is one of the most important food crops for millions of people in rural regions in the semi-arid tropics of Asia and Africa and is among the top 5 produces cereals in the world.

Despite these important benefits, sorghum has a severe downside. Sorghum is cyanogenic and produces and stores the alpha-hydroxynitrile glucoside dhurrin in vacuoles throughout the entire plant. Upon tissue disruption caused by foraging animals, the vacuole content is released and exposed to hydrolytic cleavage by endogenous β-glucosidases (dhurrinases) resulting in the release of the cyanohydrin (alpha-hydroxynitrile) and glucose moiety. The unstable cyanohydrin decomposes both spontaneously and by the action of an alpha-hydroxynitrile lyase to cyanide and a carbonyl compound. The released cyanide is highly toxic to herbivores and ensures that only minimal damage is inflicted upon the plant. The distribution and concentration of dhurrin in sorghum is both tissue specific and age related. The highest concentrations are found in the young plant in the first weeks after seed germination. After an initial peak in dhurrin, the concentration slowly decreases to a low and more stable concentration in the adult plant. In the adult plant the amount of dhurrin reaches a level tolerable to most herbivores.

From the onset of sorghum culture it has been known that the seeds of sorghum also contain cyanogenic glucosides. More specifically it is the developing seeds of sorghum that contain toxic amounts of dhurrin. In these stages the seeds would make attractive feeding objects for birds, where it not for the high amounts of dhurrin. Bird feeding studies have indeed confirmed that the immature (tannin-free) stage of the sorghum seed confers the highest resistance to bird predation. In the later stages of development and maturation, where dhurrin concentration is low, an increased tannin content ensures that the seeds are unattractive to birds.

Although it has been known for decades that developing sorghum seeds contain high amounts of cyanogenic glucosides and the mature seeds only trace amounts, the precise trajectory for the accumulation and turnover has never been investigated. In our studies we have shown that the developing seeds of sorghum show the same characteristic curve as the developing seedling. From a low initial concentration, dhurrin is accumulated until a peak is reached. Following the peak, dhurrin is rapidly degraded until the concentration reaches the barely detectable level of the mature seed.

The biosynthetic pathway responsible for the production of dhurrin from tyrosine has been known since its elucidation in the 80’s, but the turnover pathway still remains to be fully discovered. Through next-generation sequencing of sorghum seeds at different developmental stages, we hope that we are able to discover novel enzymes and verify already proposed ones in the turnover pathway.
A putative galactosyltransferase knockout mutant with a xylan phenotype

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The Glycosyltransferase family 31 (GT31) has been shown to contain functionally proven galactosyltransferases, and plant GTs classified in family GT31 are believed to be involved in the addition of galactose on ArabinoGalactan Proteins (AGPs), a group of highly complex glycoproteins in the cell wall. A specific GT31 from a subclade containing a domain of unknown function 604 (DUF604) was characterised in the present study. mAbs specific for Xylan and Homogalacturonan displayed reduced binding in the lower part of the stem in a T-DNA mutant of the GT, although the corresponding total monosaccharide composition appear unaltered. The soluble part of the GT was expressed heterologously in Pichia pastoris and Galactosyltransferase activity was obtained using the free sugar assay. Putative functions are discussed in relation the presented data.
The moss *Physcomitrella patens*: a new platform for pathway elucidation and industrial production of high-value terpenoid bioproducts

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The moss *Physcomitrella patens* is a promising organism for heterologous production of plant terpenoids. Full genome sequencing and biochemical characterization revealed a single functional diterpene synthase. Knockout of this gene using homologous recombination results in moss with a biochemical profile free of endogenous terpenoids. This provides a clean background for characterization of novel terpenoid pathways. Furthermore, unused pools of isoprenoid precursors could be redirected into production of heterologous pathways. The upstream pathways of isoprenoid biosynthesis have been characterized, and optimization of key bottleneck enzymatic steps may increase metabolic flux to downstream terpenoid products. We have used both stable chromosomal integration as well as transient expression to characterize terpene synthases from higher plants. *Physcomitrella* provides a robust and flexible platform for high-throughput characterization of unknown terpene synthases, as well as a heterologous expression host which can be scaled up for industrial production of high-value bioproducts.
Biological factors to improve quality and quantity of the biomass for the 21st century (B21st)

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The vision of the B21st project (Biomass for the 21st century) is to develop novel biomass resources and technologies to go beyond their current uses for heat, power and ethanol, towards future applications in chemicals and fuel for shipping and aviation. This large multisectoral project involves academic and industrial partners with expertise ranging from biological sciences to bioconversion and direct applications. Our particular task in this project is to identify and modulate biological factors to obtain the desired quality and quantity of B21st feedstock. We use state-of-art methods in plant molecular biology and biochemistry. Our studies are carried on wheat (*Triticum aestivum*) as well as *Arabidopsis thaliana* and *Brachypodium distachyon* as plant model organisms.

We specifically investigate two main factors responsible for the regulation of biomass formation which we identified by a bioinformatics approach: one is the hormonal regulation, especially exerted by brassinosteroids and the second is the role of nitrogen supply.

We are currently analyzing two sets of *Arabidopsis* and *Brachypodium* plants, the first treated with brassinosteroids and the second grown in hydroponics in presence of different nitrogen supply regimes. Cell wall will be analyzed by microscopy and by Comprehensive Microarray Polymer Profiling (CoMPPs) for component composition assessment. The levels of brassinosteroids and sterols will be analyzed by recently set-up GC-MS analytical method. To investigate the brassinosteroids effect on cell wall composition and biomass accumulation, mutant lines of wheat and *Brachypodium* over-expressing DET2 and Br6ox2, two genes involved in brassinosteroids biosynthesis, will also be generated. Several *Brachypodium* T-DNA mutants in brassinosteroid- and nitrogen assimilation- related genes were obtained from public mutant collections and will be investigated. Particular interest will be focused to study the role and distribution of mineral elements like alkali and silica which affect the overall quality of biofuel. Preliminary results from our studies will be presented and discussed.
Designing a technology platform for identifying transporter protein function and elucidating the glucosinolate transporter complement

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**Background:** Metabolites are transported both intra- and intercellularly, over short and long distances, from organelles to organs. Consequently, the metabolites cross numerous barriers to get from the point of production to the point of use. Transport is in many cases facilitated by carrier proteins and channels. We use glucosinolates, a class of defense-related secondary metabolites, to study these processes in an effort to identify the full glucosinolate transporter complement. The first glucosinolate transporters, GRT1 and GTR2, were discovered by constructing and screening a cDNA library with candidate transport genes containing 10-14 transmembrane domains\(^1\). GTR1 and GTR2 are specific proton-dependent glucosinolate importers, facilitating glucosinolate translocation via the plant vasculature. They are also essential for allocating glucosinolates into seeds. As a consequence of the apoplastic phloem loading mechanism of glucosinolates in Arabidopsis, glucosinolate exporters must exist. Moreover, intracellular importers and exporters of glucosinolates are likely to exist since glucosinolates are synthesized in the cytosol but assumed to be stored in vacuoles. These additional glucosinolate transporters have not yet been identified.

**The Experimental Task:** To identify glucosinolate exporters and novel importers, we are building an unprecedented full length cDNA transporter protein library. The library will contain more than 750-850 known and putative Arabidopsis transporter proteins. Selection criteria are as unbiased as possible where all proteins available to us with 2 or more transmembrane domains have been included. At completion, the library will contain more than half of the total number of possible transporters in Arabidopsis based on genome annotation. The library is currently being optimized for expression in Xenopus oocytes by cloning the genes into a Xenopus expression vector. Additionally, the library is continuously being expanded with protein families of special interest and transporters identified as candidates in co-expression databases. We will screen the library for glucosinolate transporter activity through heterologous gene expression in Xenopus oocytes. In vitro transcribed RNA for each gene will be injected in pools to enable high-throughput screening of the library for either glucosinolate influx or efflux by analyzing the oocytes internal glucosinolate content by LC-MS.

**Perspectives:** The technology platform generated in this project will be a unique tool with unparalleled potential for identifying plant metabolite transporters. Screening the library with plant metabolite extracts and high valued compounds will generate discoveries of both academic and commercial interest. The technology platform will play a key role in advancing transport engineering and allow us to make super crops with improved nutritional, commercial and agronomical value.

The role of starch structure in cereal seed germination and seedling establishment

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Barley and other cereal endosperms store starch and protein which are accumulated during grain development and are later degraded to provide energy and nitrogen during germination and seedling establishment. In the mature barley grain, starch accounts for about 65% while other polysaccharides make 15%, non-starchy polysaccharides usually constitute less than 10% and proteins 10% – 12% by weight of the grain [1].

The objective of this work is to evaluate the impact of stored starch composition and structure on its utilization by growing seedlings. In this study we used two transgenic barley lines which vary in their starch composition and structure, one which is a “hyperphosphorylated” starch line with a 10-fold increased amount of phosphorylation as compared to starch from wildtype barley and the other is a “amylose-only” starch line. Our previous results suggested, hyperphosphorylated starch to be easily accessible and readily degraded [2] and amylose-only starch to be highly resistant [3]. Our current work emphasizes the in planta role of these starches during germination and seedling establishment in barley. These findings can be extended to other cereal plants.

Probing the Xylan Interactome

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A growing body of evidence suggests that the complexity of plant cell wall carbohydrates is achieved through the co-operative function of closely associated glycosyltransferases (GTs). There is evidence for a glucurono(arabino)xylan synthase complex in Wheat, containing xylosyl- and arabinosyltransferases. In Arabidopsis, complexes containing GTs have been shown to be involved in the biosynthesis of homogalacturonan, and there is evidence for protein complex formation in xyloglucan biosynthesis.

Xylan is a polymer of great industrial significance offering a renewable source of carbohydrate for various industrial processes. Contributing around 30\% by mass, it is the most abundant non-cellulosic polysaccharide in hardwood secondary cell walls and also contributes to the secondary cell walls of gymnosperms and the primary cell walls of many plants, particularly the grasses. Consisting of a linear backbone of $\beta$-(1,4)-linked D-xylosyl residues, the backbone may be further elaborated by substitution with glucuronic acid, 4-O-methylglucuronic acid, arabinose; and more rarely, galactose and xylose.

In the last 5 years, numerous glycosyltransferases (GTs) mediating the production of xylan have been identified, including both backbone and substituting transferases. Of note are the six putative xylosyltransferases (XylTs) IRX9, IRX10, IRX14 and their close structural homologs, IRX9-I, IRX10-I and IRX14-I. When knocked out in Arabidopsis the degree of xylan backbone polymerisation is reduced and a reduction in XylT activity is observed. Predictive protein interaction mapping suggests that there are no direct protein-protein interactions between these XylTs, but that they may function as part of a larger complex interacting via ‘linker’ proteins.

To begin to understand the PPIs involved in xylan biosynthesis, we have applied a split \textit{Renilla} luciferase complementation assay (SLCA) for study of binary PPIs by heterologous expression in \textit{Nicotiana benthamiana}. Preliminary data indicates the Arabidopsis XylTs do not directly associate with one another. To enable the identification of proteins within the xylan interactome, cDNA libraries have been prepared from Arabidopsis tissues undergoing both primary and secondary wall biosynthesis and are being tested against bait XylTs in a yeast split ubiquitin system. The results of this screen will be presented here.
The effects of glucosinolate transport engineering on pathogen and herbivore susceptibility in Arabidopsis

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Plants utilize transport processes to allocate defense compounds strategically in organs with highest fitness values. Within these tissues defense compounds are often allocated unevenly with highest concentrations at outer perimeters or around valuable tissues. This is exemplified in Arabidopsis thaliana where intra-leaf allocation of the major defense compounds, glucosinolates, shows highest concentrations at the leaf edges and along the midvein. In a recent study, we abolished accumulation of glucosinolates in seeds by mutating the two long-distance glucosinolate transporters GTR1 and GTR2 (1). Concomitantly, this lead to several folds increases in glucosinolate accumulation in leaves. In this study, we investigate the role of the GTRs in establishing the distinct intra-leaf distribution of glucosinolates in Arabidopsis. Moreover, we investigate where in the leaf the excess glucosinolates levels accumulate and whether this affects susceptibility towards selected pathogens and herbivores. We show that the edge allocation of glucosinolates in leaves is more pronounced in the gtr1gtr2 knockout plants. Furthermore, we show an increased concentration in the apoplast. Surprisingly, mature leaves from the gtr1gtr2 mutant plant are more susceptible to specific pathogens. The effect on herbivore susceptibility will also be presented.

Oxidative stress repair enzymes recruited to specialized metabolism

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In Arabidopsis thaliana (Col-0), high levels of the glucosinolate defense compounds accumulate in seeds. There they serve an important role in providing immediate defense for germinating seedlings and can also be metabolized to provide nitrogen and sulfur under nutrient scarcity. Seeds rely on transport processes from the maternal plant to accumulate glucosinolates. Yet the composition of glucosinolates in seeds is distinct from that in the maternal plant. In particular, seeds accumulate the methionine derived methylthioalkyl (thio) glucosinolates whereas the maternal plant predominantly contains the oxidized forms, namely, methylsulfinylalkyl (sulfinyl) glucosinolates. It is not known how the seeds are able to predominantly accumulate thio glucosinolates. Using co-expression databases we identified genes which we show are able to reduce sulfinyl- to thio glucosinolates in vitro. Seed analyses of mutant plants showed reduced concentrations of thio glucosinolates in seeds. Coordinately, ectopic overexpressor plants showed increased concentrations of thio glucosinolates in leaves. Moreover, the gene is expressed in seeds with an expression pattern that appears to be coinciding with the occurrence of thio glucosinolates in seeds. The identified family is ubiquitous and its members have hitherto been shown to repair oxidatively damaged proteins. Here we assign a novel function to this family in shaping the profile of methionine derived defense compound composition.
Using NIR Spectroscopy and PLS Regression to Model Biomass Glycans Content in a Collection of Winter Barley Varieties

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Comprehensive microarray polymer profiling (CoMPP) is a well-established microarray technique to detect plant cell-wall glycans in a semi-quantitative manner. Cell wall components are extracted from plant organs and tissues then spotted on microarrays. These are probed with specific antibodies (mAbs) or carbohydrate binding modules (CBMs) and signals from each sample recorded. The increasing number of cell-wall specific mAbs and CBMs available makes CoMPP test a powerful tool to screen for a wide range of valuable cell-wall components in plants. Yet, the semi-quantitative nature of the test restricts the number of samples available for simultaneous analysis to the number of extracts that a single microarray can harbor. In fact, arrays probed with the same mAbs or CBM present different mean signal intensities which masks real biological variation, bringing misleading results. A similar issue, known as batch-effect, is still of great debate in the field of gene expression microarrays.

These technical restrictions limit the applications of CoMPP test. When large number of samples need to be screened to obtain statistically robust results (e.g. genome wide association scanning) alternative approaches are required.

Partial least squares (PLS) regression models for plant cell-wall composition are widely used to overcome the problem of expensive and time consuming laboratory analysis. Briefly; a subset of samples is screened using spectroscopy techniques (infrared, near-infrared; or IR and NIR). The same subset is then evaluated with classic wet-chemistry protocols. Spectra recorded and chemical data are then combined to obtain cross-validated PLS models. Finally, the composition of the remaining samples is predicted using spectroscopy data only.

The aim is to verify whether it is possible to overcome the CoMPP test probing-effect issue analyzing a large collections of samples using NIR spectroscopy and PLS regression. A collection of 124 winter barley varieties was field-tested in 2009 and 2010 in Fiorenzuola d’Arda (North Italy), summing to 948 straw samples. NIR spectra on ground biomass were collected. CoMPP test was performed on a subset of 120 samples for glycans related to bio-fuel production. PLS models were created and cross-validated. Glycans content of the remaining samples could then be obtained using NIR spectra only.

Results will be presented during the meeting.

A biology driven, microarray-assisted approach for production of green algae cell wall directed antibodies

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Monoclonal antibodies (mAbs) are powerful tools for the in situ detection of cell wall components. However, the overwhelming majority of mAbs are directed against epitopes occurring in the walls of angiosperms. There is increasing interest in the study of the cell walls of early divergent plant groups including the charophytic and chlorophytic algae. This interest is driven largely by the pivotal phylogenetic position of these algae in relation to plant, and plant cell wall evolution. Given the paucity of relevant mAbs we have undertaken to produce new sets of antibodies directed against algal cell walls – and to do this we are using a novel biology-driven approach. Most mAb production is based on the desire to produce probes against a specific and predefined target molecule. Although this strategy has certainly been successful in many cases, it ideally requires an extensive prior knowledge of the cell walls concerned, and risks that quantitatively minor but important components may be overlooked. A biology-driven approach is based on immunisation with crude cell wall preparations, which as far as possible represent whole cell wall glycomes. After initial ELISA screening against cell wall preparations, mAbs are selected by labeling of sections through mixtures of algae. mAbs that show interesting binding profiles (for example binding to particular cellular structures, life stages, etc.) are carried forward for further investigation to determine the epitopes recognised by the chosen mAbs. To do this we will use our recently developed microarrays populated with defined cell wall derived oligosaccharides that have proven utility for high resolution, high throughput mAb characterisation. In addition, microarray data is complemented by competitive inhibition ELISAs using small amounts of pure oligosaccharides. This less biased approach offers the potential for the identification of mAbs with novel specificities even though immuno-dominance will no doubt limit the repertoire of mAbs generated to some extent. Progress towards the development of new algal directed mAbs will be presented.
Mapping the structural diversity of red and brown macroalgal cell walls

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Red and brown algal cell walls are complex and diverse. In principle, monoclonal antibodies (mAb) and carbohydrate binding modules (CBM) can be used for their analysis, as has been the case for land plants. However, there are very few mAb and CBM probes available with specificities against red and brown algal cell wall polymers. Therefore our goal is to obtain new antibodies to be probed against these macroalgae cell walls and systematically map their structures. These analysis could elucidate unknown evolution and structural diversity, as well as novel carbohydrate products with commercial application.
Cellulose degradation by a lytic polysaccharide monooxygenase (LPMO) from S. coelicolor

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Bacterial proteins classified as family 33 carbohydrate-binding modules (CBM33) have primarily been described for chitinolytic bacteria, where they act synergistically with chitinases. Recently, it was discovered that these proteins are metalloenzymes that cleave crystalline polysaccharides using an oxidative mechanism that is boosted by an external electron donor [1,2]. In this work we describe a CBM33 protein from S.coelicolor which degrades cellulose. CBM33 enzymes are structurally similar to the fungal family 61 glycoside hydrolases (GH61) which act synergistically with cellulases [3]. The similarity includes a conserved copper binding motif that is essential for the enzymatic activity of CBM33s [4], indicating that GH61s and CBM33s employ similar mechanisms. We now collectively name these proteins lytic polysaccharide monooxygenases (LPMOs), a name representing their true activity [5]. The products released during enzymatic activity can be screened by different HPLC based methods [6].

Identification of protein-protein interactions involved in pectin biosynthesis in the Golgi apparatus

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Increasing evidence suggests that enzymes and proteins involved in cell wall biosynthesis form complexes [1-3]. An example is the homogalacturonan synthase core complex formation between GAUT1 and GAUT7. Interestingly, GAUT1 has been shown to be proteolytically cleaved from its transmembrane anchor domain and its catalytic domain is retained by GAUT7, thus ensuring biosynthesis of homogalacturonan in the Golgi apparatus [4]. We have recently identified, by heterologous expression in \textit{Nicotiana benthamiana}, that GAUT5 is also able to anchor GAUT1 in the Golgi apparatus. However, the homozygous \textit{gaut5 gaut7} double mutants in \textit{Arabidopsis thaliana} were found to be viable, which may suggest that an additional anchoring protein(s) exist. The retention mechanism of GAUT1 sets a new paradigm in the mechanism of pectin and glycan biosynthesis in the Golgi apparatus. In order to identify the additional anchor protein(s), we are currently screening an \textit{A. thaliana} cDNA library for GAUT1-interacting protein(s) by using split-ubiquitin system and the results will be presented.

In addition, we are developing a split-luciferase complementation assay (SLCA) in, to perform binary interaction screening in a mid- to high-throughput manner. We have demonstrated that SLCA successfully shows \textit{in situ} interaction between the positive controls (ARAD1) with limited background signal, suggesting that SLCA may be used to analyze \textit{in situ} protein-protein interactions between Golgi resident proteins. Detailed characterization of the system both in \textit{N. benthamiana} and yeast will be presented.

Distinct above- and belowground synthesis and vascular transport control short- and long-chained aliphatic glucosinolate distribution in vegetative Arabidopsis

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Plants often produce defense compounds in one tissue for deployment in separate organs via long-distance transport pathways. In Arabidopsis, the glucosinolate transporter proteins GTR1 and GTR2 are essential for accumulation of the defense compounds glucosinolates (gls) in seeds (1). In this study, we investigate vascular mobility of gls and source-sink relationship between above- and belowground tissues in vegetative Arabidopsis plants. We show that both the rosette and roots are able to de novo synthesize aliphatic and indole gls. In vivo feeding demonstrates that gls can move GTR1- and GTR2-dependently from the rosette to roots in the phloem and independently of GTR1 and GTR2 upwards from the roots to rosettes along the ascending xylem sap. Reciprocal grafting experiments between wildtype, biosynthesis and transport mutants indicate that the rosette and roots are primary source tissues for short-chained and long-chained aliphatic gls, respectively. GTR1 and GTR2 are shown to play a role in controlling the distribution of aliphatic, but not indole gls, through long-distance transport in both the phloem and xylem. We conclude that the specific rosette and root gls patterns of aliphatic glucosinolates in Arabidopsis are formed by integration of long-distance transport through the vasculature and specific above- and belowground de novo biosynthesis.

Plant P4-ATPases: lipid translocators with a role in membrane trafficking

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The secretory pathway is involved in several vital cellular processes, including host-pathogen interactions, nutrient and gravity sensing, and protein sorting [1-3]. In the past years, a subfamily of P-type ATPases has been suggested to be involved in vesicle formation. P-type ATPases comprise a large family of membrane proteins involved in pumping different physiologically-relevant substrates across biological membranes [4]. The members of the P4 subfamily (also known as flippases) catalyze the energy-driven translocation of lipids necessary for establishing transbilayer lipid asymmetry [5], a feature necessary for correct functioning of the cells [6,7]. Deletion of one or more P4-ATPase genes causes defects in vesicle budding in various organisms [8-10] and some members of the yeast family have been shown to interact with the vesiculation machinery [11,12]. Thus, unraveling the key features of P4-ATPase functioning is crucial to understand the mechanisms underlying the whole secretory and endocytic pathways.

In the model plant Arabidopsis, 12 members of the P4-ATPase family have been described (ALA1-ALA12, for Aminophospholipid ATPase) [4]. In the past years, we have characterized several members of this family with respect to their localization, substrate specificity, physiological role and requirement for the presence of a β-subunit [9,13-15]. At the moment we are working on understanding the mechanism of lipid transsport and the regulation of these pumps. In this context, we have recently completed the biochemical characterization of two ALA proteins: ALA2, a prevacuolar compartment-localized protein with an unusually tight specificity, and ALA10, a plasma membrane-localized protein with unforeseen broad substrate specificity. Besides providing an insight into the mechanism of lipid translocation, our results suggest that the different transport features of these proteins might be related to their physiological function at the membrane where they are located.

Mapping Lipid Specificity of P4-ATPases

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P4-ATPases are a subfamily of P-type pumps located to different biomembranes. Driven by ATP, they generate the characteristic asymmetry of the lipid bilayer by translocating or ‘flipping’ specific phospholipids across the membrane towards the cytosolic leaflet. For example, in Arabidopsis this protein family has 12 members displaying different lipid specificity profiles. The function of flipping lipids is peculiar as all other well-characterized P-type ATPases pump cations, which are much smaller substrates.

How these pumps transport lipids is not understood. Clarification and identification of key elements involved in the binding and flipping of specific phospholipids is important since these pumps serve a vital function in the eukaryotic cell.

This project focuses on identifying key transmembrane segments and amino acid residues of Arabidopsis P4-ATPases (ALAs) that determine their lipid specificity. For this transmembrane segments will be switched between different ALA proteins and the lipid specificity profile will then be investigated by heterologous expression in yeast mutants lacking endogenous P4-ATPases. Drop-tests will be performed to study the ability of chimeras to transport natural lipids and flow cytometry will be used to investigate the transport of fluorescently-labeled lipids at the plasma membrane. Furthermore, localization of the chimeras in planta will also be performed using transient expression in tobacco leaves to investigate if the switch of transmembrane segments affects the subcellular localization.
Intracellular signaling by diffusion: Can waves of hydrogen peroxide transmit intracellular information in plant cells?

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Amplitude- and frequency-modulated waves of Ca²⁺ ions transmit information inside cells. Reactive Oxygen Species (ROS), specifically hydrogen peroxide, have been proposed to have a similar role in plant cells. We consider the feasibility of such an intracellular communication system in view of the physical and biochemical conditions in plant cells. As model system, we use a H₂O₂ signal originating at the plasma membrane (PM) and spreading through the cytosol.

We consider two maximally simple types of signals, isolated pulses and harmonic oscillations. First we consider the basic limits on such signals as regards signal origin, frequency, amplitude, and distance. Then we establish the impact of ROS-removing enzymes on the ability of H₂O₂ to transmit signals. Finally, we consider to what extent cytoplasmic streaming distorts signals. This modeling allows us to predict the conditions under which diffusion-mediated signaling is possible.

We show that purely diffusive transmission of intracellular information by H₂O₂ over a distance of 1 μm (typical distance between organelles, which may function as relay stations) is possible at frequencies well above 1 Hz, which is the highest frequency observed experimentally. This allows both frequency and amplitude modulation of the signal. Signaling over a distance of 10 μm (typical distance between the PM and the nucleus) may be possible, but requires high signal amplitudes or, equivalently, a very low detection threshold. Furthermore, at this longer distance a high rate of enzymatic degradation is required to make signaling at frequencies above 0.1 Hz possible. In either case, cytoplasmic streaming does not seriously disturb signals.

We conclude that although purely diffusion-mediated signaling without relaying stations is theoretically possible, it is unlikely to work in practice, since it requires a much faster enzymatic degradation and a much lower cellular background concentration of H₂O₂ than observed experimentally.
Monitoring programmed cell death of living plant tissues in microfluidics using electrochemical and optical techniques

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Programmed cell death (PCD) in plants can influence the outcome of yield and quality of crops through its important role in seed germination and the defence process against pathogens. The main scope of the project is to apply microfluidic cell culture for the measurement of electrochemically or optically detectable events during PCD in barley aleurone layer, a cell model for living plant tissues, for a better understanding of the underlying mechanisms of PCD in plants.

Microfluidic cell culture enables in vitro experiments to approach in vivo conditions. The major advantage of electrochemical sensors and detection systems is that they can be miniaturized, multiplexed and automated without losing their performance making them suitable for integration with microfluidic devices1,2. Combining microfluidics with electrochemical and optical detection allows implementation of a wide range of assays for online, real-time, parallel analysis of important parameters such as redox activity (NADPH:NADP ratio), H2O2 concentration, oxygen consumption, extracellular pH, cell viability and release of target enzymes (α-amylase and limit dextrinase).

Probing the intracellular redox activity is of major importance, since it is known that reactive oxygen species, which are affected by changes in the redox activity of the cells3, are involved in PCD in plants, but the relationship between and mechanisms behind ROS and PCD is only poorly understood in plant cells4. Recently, it has been shown, using optical detection, that the H2O2 concentration changes depending on phytohormone activation or inactivation of aleurone layer metabolism and subsequent PCD5.

Currently, we are working on the optimization of an intracellular whole-cell redox activity (NADP:NADPH ratio) assay5 to be able to detect possible changes of the cellular redox activity in barley aleurone layer. In our initial experiments using the electrochemical mediator-assisted assay we observed changes in the redox activity with tendencies similar to those for the H2O2 concentrations presented by Ishibashi et al. Further experiments are needed in order to improve reproducibility of the measurements and to find the optimal parameters suitable for its application in the microfluidic device. Meanwhile, we successfully detected PCD induced by phytohormones in barley aleurone layer using a double-fluorescent probe-system also used by Fath et al6, and it is planned to integrate this system in the microfluidic device.

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EFFECT OF AGENTS ABLE TO DISRUPT PROTEIN SECRETION IN THE PROTEOMES OF THE BARLEY ALEURONE LAYER.

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The cereal aleurone layer (AL) plays an essential role in germination by synthesizing and secreting enzymes that hydrolyze the starchy endosperm in response to gibberelic acid produced by the embryo (Finnie et al. 2011). The barley AL can be isolated from the other seed tissues and maintained in culture, allowing the in vitro study of intracellular and secreted protein patterns.

In the present work, we analysed the effect of the antibiotic tunicamycin (as inhibitor of the first enzyme in the N-glycosylation pathway) (Luczak et al. 2008) and the heat shock treatment (as causing selective suppression of secretory protein synthesis) (Shaw et al. 2003) in the proteomes of gibberelic acid treated AL. From the 2-DE gels corresponding to the secreted proteins a decrease in the most abundant hydrolytic enzymes during germination, including α-amylases and several endopeptidases, has been noticed. Moreover, the analysis of the intracellular water-soluble proteins has led to the identification of proteins involved in protein folding and endoplasmic reticulum stress response, reporting noticeable changes in their abundance after tunicamycin and/or heat treatments.

Furthermore, knowing the importance of Reactive Oxygen Species (ROS) signaling during germination and apoptosis of the AL, the H$_2$O$_2$ production and the cell death was monitored in in vitro treated AL, determining the lipid peroxidation as indicative of oxidative damage as well.

Finally, these results will provide the basis for in-depth studies of the mechanisms involved in protein secretion and their interplay with ROS metabolism during barley seed germination.

References:
Finnie, Christine et al. (2011) *Proteomics* 11(9): 1595
Regulation of glucosinolate transport

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Transport processes are important for the reallocation of defence compounds during development and predation. A recent identification of two glucosinolate transporters provided a long-sought molecular tool to study a range of questions regarding the mechanisms underlying distribution of defence compounds using glucosinolates as a model system¹. The aim of this study was to investigate whether and in what context phosphorylation regulates the transport activity of AtGTR1 and AtGTR2.

The results indicate a role for phosphorylation in decreasing AtGTR1 and AtGTR2 transport activity. We used publicly available phosphoproteomics data to search for phosphorylation sites in AtGTR1 and AtGTR2. Phosphorylation mimics of selected sites showed decreased transport activity whereas dephosphorylation mimics had wild type transport activity. We employed co-expression analysis to search for likely kinase candidates. Preliminary oocyte co-expression experiments indicate that one kinase decreased the transport of both transporters whereas another kinase decreased the transport of AtGTR2 only.

We hypothesize that AtGTR inactivation by phosphorylation is involved in a defence response by controlling glucosinolate levels in the apoplastic space. In perspective, the research could further our understanding of the regulation of transport processes involved in distribution of defence compounds at the cellular, tissue and organ level.

References
Functional Reconstitution of Monomeric Arabidopsis thaliana Plasma Membrane P-type H⁺-ATPase into Phospholipid Bilayer Nanodiscs

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The plasma membrane H⁺-ATPases are a family of membrane proteins responsible for extruding protons out of the cell, thereby essential for establishing and maintaining the crucial proton gradient in plant and yeast. Due to its physiological importance extensive efforts have been made in attempt to elucidate the detailed physiological role and biochemical characteristics of plasma membrane H⁺-ATPases. The studies involves both in vivo and in vitro approaches, with the latter employing either solubilisation by detergent micelles, or reconstitution into lipid vesicles. Despite resulting in a large body of information on structure, function, and regulation of H⁺-ATPases, key questions, in particular concerning the detailed interaction of regulator proteins with the H⁺-ATPases, remains answering that may require the use of new approaches. Here, we report the reconstitution of the proton pump Arabidopsis thaliana plasma membrane H⁺-ATPases isoform 2 (AHA2) into soluble nanoscale lipid bilayers, also termed nanodiscs. Through extensive analysis we confirm the correct assembly and reconstitution of active aha2Δ73 into nanodiscs. The pump inserts as a monomer and we thereby provide evidence that the plasma membrane H⁺-ATPase can function as a monomer. Insertion of the H⁺-pump into nanodiscs has the potential to enable structural and functional characterization using various techniques, not normally applicable for membrane proteins, as we exemplify by the specific immobilization of reconstituted proton pump using surface plasmon resonance.
NAC transcription factors in senescence

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NAC (NAM, ATAF1, CUC1,2) transcription factors (TFs) are plant specific TFs involved in both development and biotic and abiotic stress responses. The focus of this Ph. D. project is on a sub-group of NAC TF involved in plant senescence and thereby of importance to nutrient quality. We are especially interested in NAP, from the model plant Arabidopsis thaliana, and the barley ortholog, HvNAC005, due to the significant induction of the expression of their genes in response to both senescence and the abiotic stress hormone abscisic acid. To understand the NAP/HvNAC005 network, recombinant versions of their DNA-binding domains were produced and purified, and the DNA-binding specificity determined using the novel large scale platform DNA binding microarray. This revealed very similar binding sequences, although with some variation in the flanking regions, which could be of importance for determining the physical interactions between NAP/HvNAC005 and their direct target genes. This allows identification of potential direct target genes from the rapidly accumulating DNA microarray data, thereby mapping NAP/HvANAC005 centered gene regulatory networks. The transcriptional regulatory domains of NAP/HvNAC005 are also analyzed. Biophysical analyses have shown that they are characterized by a large degree of intrinsic disorder. Yeast based one and two hybrid assays and bioinformatic analyses are used to determine regions and sequence motifs of importance to activation activity and interactions and are related to the functionality of protein intrinsic disorder.
Investigation of the signal transduction cascade that involves the putative Ser/Thr kinase PmgA in the regulation of photosynthetic apparatus under light stress in Synechocystis sp. PCC 6803.

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Cyanobacteria are alternative green platforms for the production of valuable organic compounds. The elucidation of the transcriptional pathways involved in the carbon fixation and light harvesting systems represent a crucial step towards the possibility of tweaking the system in order to enhance the robustness of the production. PmgA is a putative Ser/Thr kinase that has been correlated with the regulation of both photosystems stoichiometry under light stress and photomixotrophic growth and sugar accumulation in Synechocystis sp. PCC 6803 [1-4]. The project aims at elucidating the components of the PmgA signal transduction cascade, particularly focusing on how PmgA carries its role as regulator and which are the other gene products interacting with it. Two different approaches are used to address these questions. Global transcriptomic profiling of the pmgA mutant and the wild type was carried out in order to identify downstream components that are regulated by PmgA. Preliminary results show that photosystem I and phycobilisome related genes are down regulated in the pmgA mutant as compared to the wild type with concomitant increase of SyR1 (Synechocystis ncRNA 1), a 130 nt long non-coding transcript. It has been shown that overexpression of SyR1 causes a severe reduction of pigments [5-6]. Therefore we speculate the possibility that SyR1 may be involved in the PmgA-mediated regulation of the photosynthetic apparatus. In the second approach, a modified Y2H system (DUALhunter system) is used to identify protein-protein interaction partners of PmgA in order to elucidate the components involved in the signal transduction cascade. The screening for the possible interacting partners is currently performed on both chosen proteins and genomic library and the results will be presented.

Searching for interaction partners of plant phospholipid flippases

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Background
Type IV P-type ATPases (P4-ATPases) play significant roles in various cellular functions, including membrane biogenesis and maintenance of phospholipid asymmetry in cellular membranes. They are putative phospholipid translocators and presumably act by flipping phospholipids from the exoplasmic to the cytoplasmic leaflet of cellular membranes thereby creating asymmetry between the two leaflets of the bilayer. This asymmetry is necessary for vesicle formation and indicates an important role for phospholipid translocators in the initiation of vesicle budding. P4-ATPases are as a consequence an essential part of intracellular protein trafficking involving various membranous organelles; i.e. the secretory and endocytic pathways.

Phospholipid flippases of the P4-ATPase class are eukaryotic membrane pumps consisting of ten transmembrane domains, with the N- and C-termini present in the cytosol. They belong to the P-type ATPase family which is driven by ATP-dependent autophosphorylation of a conserved residue. These proteins have been studied to the greatest extent in Saccharomyces cerevisiae and most topological and functional characterization relies on data from studies in this organism. In Arabidopsis thaliana 12 P4-ATPases have been identified but have only in recent years been subject to in-depth analyses.

Characterizing P4-ATPases in terms of function and by the identification of interaction partners will give essential knowledge to how these membrane pumps work in a coordinated manner with the rest of the machinery involved in vesicle formation from cellular membranes. This knowledge is useful for in-depth understanding of the mechanisms underlying the secretory and endocytic pathways.

Aim of project
The aim of this study is to investigate possible interaction partners for phospholipid flippases from a multicellular organism. Two A. thaliana P4-ATPases, ALA2 and ALA10, will be used as models for flippases in the pre-vacuolar compartment and the plasma membrane, respectively. Our goal is to identify both regulatory interaction partners (kinases, phosphatases, etc.) as well as elements of the vesiculation machinery (small GTPases, SNAREs, etc.) that might act together with the two flippases in question.

Strategy for project
For this purpose, we have cloned the fragments corresponding to the N- and C-terminal ends of ALA2 and ALA10 in plasmids that will allow us to express them as fusions to one of the two halves of the Gal4 yeast transcription factor. These constructs will be used in a classical yeast two-hybrid screening against an A.thaliana cDNA library. Positive clones found in this screening will be verified and characterized using complementary in vitro protein-protein interaction techniques, e.g. co-immunoprecipitation and pull-down assays. In case of identifying any putative regulatory kinases, in vitro phosphorylation assays will be performed and mass spectrometry will be used to identify the amino acid residue target for phosphorylation.
Two secreted peptides regulate plasma membrane proton pump during cell elongation

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Acidification of the apoplastic space followed by loosening of the cell wall is a key step in cell expansion. This study describes the interplay of two secreted peptides in controlling the acidification of the apoplastic space. PSY1 is a secreted tyrosine-sulphated glycopeptide that promotes cellular expansion and elongation1. PSY1 binds to the extracellular domain of a Leucine rich repeat receptor kinase (LRR-RK) leading to activation plasma membrane H+-ATPases with the result of lowering of the apoplastic pH3.

A microarray study was conducted in order to identify components of the PSY1 peptide-signaling cascade. Here the effects of PSY1 treatment on wild type as well as on receptor knock out plants were examined. Among genes that were differentially expressed upon PSY1 treatment, three belong to the family of “Rapid ALkalinization Factor” (RALF)2.

RALF is a large family of genes containing 40 isoforms in Arabidopsis thaliana. The identified RALF isoforms have not previously been characterized. We have investigated their effect on morphology and additionally their biochemically effect on plasma membrane proton pumping activity. Exogenous application of RALF peptides severely affected root morphology such as reduced root length, loss of apical root growth, increased lateral roots and root hair formation. RALF peptides stimulated alkalinisation measured in vivo through genetically encoded pH sensor.

Based on our results, it is concluded that up-regulation of certain RALF peptides ensures regulation of apoplastic pH through regulation of plasma membrane H+-ATPases. This feedback mechanism by two different kinds of peptide signals provides a tight regulation of H+-ATPases and may play a vital role in pH homeostasis inside the cell.

References
Molecular characterization of glycosyltransferases involved in the biosynthesis of major proteoglycan on plant cell-surface, arabinogalactan protein

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Arabinogalactan proteins (AGPs) are a class of abundant and highly diverse cell-surface proteoglycans found in diverse plant species. AGPs are thought to play as signaling molecule on cell surface and involved in many aspects of growth and development (for reviews, Seifert and Roberts, 2007, Ellis et al., 2010). The major part of AGPs (>90%) consists of complex and heterogeneous glycan moieties. Numerous studies using monoclonal antibodies against glycan epitopes of AGP showed well-defined temporal and spatial expression patterns during development, indicative of more or less subtle changes of AGP glycans and its involvement in organ development. However, since the precise epitope structure for those antibodies is not known, the critical features for the molecular function of the AGPs remain to be determined.

One major bottle neck of AGP research is the heterogeneity of the glycans. We have approached the issue by studying biosynthetic enzymes which build up the glycans on AGPs.

AGP glycans are synthesized by post-translational modifications on the protein core in the endomembrane systems catalyzed by glycosyltransferases (GTs). The AGP glycans are heterogeneous, but commonly composed of a beta-1,3-linked galactan backbone substituted at O6 with variable side chains rich in galactose and arabinose (type II arabinogalactan). More than 10 GTs are required to synthesize the various linkages identified in AGP glycans. So far, only one GT activity (represented by two fucosyltransferases from Arabidopsis, FUT4 and FUT6) has been biochemically characterized (Wu et al., 2010).

We identified four novel Arabidopsis GTs (AtGlcAT14A, AtGlcAT14B, AtGalT29A, AtGalT31A) which appeared to be involved in the biosynthesis of AGP glycans. We characterized them by i) heterologous expression and biochemical enzyme assay in vitro; ii) protein-protein interactions and its significance on enzyme activity, iii) mutant analyses. We found AtGlcAT14A works alone, but AtGalT29A interacts with AtGlcAT14B and AtGalT31A. The recombinant AtGalT29A alone showed an GT activity for elongating both beta-1,3- and beta-1,6-galactan at low level, but the protein complex AtGalT29A/AtGalT31A showed much higher activity towards beta-1,6-galactan elongation. When AtGalT29A is in a complex with AtGalT31A, it did not show the elongation activity for beta-1,3-galactan, thus the complex formation seems to determine the direction of GT activity encoded by AtGalT29A.

We believe these analyses on GTs offer molecular tools towards understanding molecular function of AGP glycans during plant growth and development.

References:
A barley NAC transcription factor involved in abscisic acid signalling

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Members of the plant specific NAC family of transcription factors have been shown to regulate several important aspects of plant stress tolerance and development in both monocot and dicot species. The NAC family is the second largest family of transcription factors in plants, comprising more than a hundred members. Global gene expression studies in both Arabidopsis and rice, have shown that many of the NAC genes are associated with hormone signalling. Studies on mutant and transgenic plants have so far confirmed direct regulatory roles of a few NAC transcription factors in abscisic acid (ABA), jasmonic acid and ethylene signalling, with various effects on drought tolerance, defense against pathogens and fruit ripening, respectively.

We have studied the barley HvNAC005 transcription factor and have shown that it is significantly induced by abscisic acid, which was supported by the identification of ABA-responsive elements in the HvNAC005 promoter. Transgenic barley plants were generated, constitutively overexpressing HvNAC005. The phenotype of these plants seems to mimic a typical ABA-induced drought response, i.e. short tillers, early senescence and poor seed set. The expression levels of a series of stress and ABA responsive genes were investigated by qRT-PCR, both in HvNAC005 overexpressing plants and WT plants treated with ABA. A high degree of correlation was found between the two experiments, suggesting HvNAC005 may have a prominent role in regulating ABA-induced drought signalling in barley.
Small RNAs involved in bacterial root symbiosis and its regulation in Lotus japonicus

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Legume plants are able to form symbiosis with rhizobial bacteria leading to the formation of nitrogen-fixing nodules. The symbiotic interactions and nodule organogenesis require complex cellular signaling and reprogramming of root cells, in which miRNAs and other small RNAs are emerging as regulatory components.

Colleagues and I have identified small RNAs potentially involved in Lotus japonicus root symbiosis with nitrogen fixing bacteria through analysis of a large-scale ILLUMINA small RNA sequencing dataset. Candidates are selected based on their expression pattern in the context of symbiosis, as well as in silico analysis involving prediction of potential mRNA targets, analysis of target expression data and degradome data from different plant species. Small RNA expression patterns are confirmed by Stem-Loop qRT-PCR and/or Northern Blotting. If symbiosis-dependent regulation can be confirmed, small RNA and target transcript abundances and integrity are manipulated by transgene expression to investigate a possible role in symbiosis. We are currently performing functional analysis on two symbiosis-upregulated miRNA candidates and associated target mRNAs, which we confirmed to be subject to endonucleolytic cleavage using RACE analysis.

This work is funded by the Danish National Research Foundation and the Oticon Foundation.
Studies of the role of *Lotus japonicus* LysM receptor kinases in plant root symbiosis

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In the model legume *Lotus japonicus*, a large family of LysM receptor like kinases has been identified. Two of these are already known to be the Nod-factor receptors (NFR1 and NFR5 [1, 2]), were as the remaining 15 are yet to be functionally characterized. However their expression pattern has been analyzed in various conditions and plant organs [3]. One of these LysM-kinases (LYS11) is a very close homologue to NFR5. All though these two proteins are very similar, there are significant differences, especially in the LysM II domain, which has been shown to contain the Nod factor-binding site [4]. This correlates nicely with the expression pattern, which does not show any induction upon rhizobial inoculation.

To get a deeper insight in the role of *Lys11* in symbiosis, we have obtained TILLING mutants in the *Lys11* gene, in the Gifu and MG20 background. Furthermore we have LORE1 (Lotus japonicus retrotransposon 1) insertions mutants in the *Lys11* promoter region.

The status of the mutant analyses regarding their phenotype in the presence of rhizobia and arbuscular mycorrhiza will be presented.

Characterization of chloroplast proteins controlling manganese use efficiency by quantitative proteomics using 2D-LC-MS/MS

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Manganese is important for molecular functions in plants, i.e. as a co-factor in enzymes and in the oxygen evolving complex of photosystem II. Soils that lack plant available micronutrients such as manganese are not generally suitable for crop production. Fortunately, various plant genotypes differ in their ability to grow in soil with low amounts of micronutrients, providing an opportunity to identify strains that can tolerate low manganese levels. In order to identify and characterize proteins involved in manganese use efficiency, focusing on the role of manganese, preparations from two barley genotypes, manganese efficient (Vanessa) and inefficient (Antonia) genotype, were studied using advanced quantitative proteomics technology. Protein samples were digested using a modified spin filter-based protocol, fractionated by an automated 2D (RP-RP) UPLC system and analyzed by electrospray ionization tandem mass spectrometry (2D LC-MS/MS). The resulting MS/MS data was subjected to computer analysis for unique protein identification and quantification. With approximately 1000 proteins identified and quantified including several proteins from Photosystem I and II, we demonstrate the speed, robustness and reproducibility of this online 2D LC-MS/MS approach for a relative complex thylakoid preparation. Finding relevant protein markers using advanced proteomics techniques opens up new avenues for selecting and crossing resource efficient genotypes.
Establishing and screening a *Lotus japonicus* LORE1 mutant population

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*Lotus japonicus* is a model legume plant, which is able to fix atmospheric nitrogen through symbiosis with *Mesorhizobium loti*. Up to now, a number of genes that are important for symbiosis have been discovered. To facilitate progress in this field, we developed a new reverse and forward genetic tool based on insertional mutagenesis – the Lotus retrotransponson 1 (LORE1) resource. LORE1 is a 5kbp long Gypsy-type retrotransponson that can be derepressed during tissue culture. This leads to new insertions in the following generations (Fukai et al., 2010) and it was shown that LORE1 has an exonic insertion preference (Urbanski et al. 2012). The LORE1 resource is freely available to the community through [http://carb.au.dk/resources/](http://carb.au.dk/resources/). At present we have obtained seeds and LORE1 sequence insertion data of app. 40,000 lines. The LORE1 insertion data are available online for these lines. In 2012 we have been growing 27,000 additional lines. That will result in mutants for ca. 80% of all *L. japonicus* genes. In 2013 a similar amount of lines will be grown again.

We hope to find novel components in the nodulation signal transduction pathway with the help of the LORE1 population. During our ongoing screening we found flower, leaf, dwarf and nodulation mutants, and we were able to find LORE1 insertions in known symbiotic genes including *CCaMK, SymRK* and *Nin* resulting in the expected phenotypes. One focus is currently a fix- mutant identified by screening the LORE1 population. Instead of fully developed nodules, only small black bumps are observed on the mutant roots and the mutants die within 8-9 weeks. Microscopic characterization showed that the mutant is able to form infection threads.

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Methods development to study Zn binding proteins in barley

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Cereals grains have lower concentration of Zn than the seeds of legumes [1]. In barley (Hordeum vulgare) grain Zn is localized in hull (2% of the total), embryo (13% of the total), endosperm (25% of the total) and testa-aleurone-endosperm gradients (58% of the total) [2]. The cells of sub aleurone layers of barley contain mainly S-rich and S-poor prolamins (principally B- and C- hordeins) [3]; and also quantity of proteins among different barley cultivars are determined by the proteins localized in sub-aleurone layers [4]. Barley grain aleurone and embryo count for 12.8 % of total dry weight having 52.6 % of total protein, whereas 87.2% of total dry weight count for starchy endosperm having 47.4% of extractable proteins [5]. Therefore, it is very important to understand the binding / storage capabilities of major abundant proteins (especially prolamins) of the grain to be able to increase Zn content in cereals (especially in the endosperm).

We have developed a radioactive Zinc (65ZnCl₂) blotting technique to detect zinc binding in the alcohol soluble protein fraction of the barley whole grain flour. This fraction contains mostly prolamins. The method was further developed for an easy to follow non-radioactive colourimetric zinc blotting method using dithizone (DTZ). The detected major zinc binding protein in alcohol soluble fractions was identified as B1-hordein by Western blotting experiment. We also conducted tissue enriched (e.g., embryo, de-embryonated endosperm; and seed coat) Osborne fractionations with 30 DAP grain from barley (Hordeum vulgare cv Golden Promise) and with our developed DTZ staining we were able to detect the most abundant zinc binding proteins in different tissues. The major Zn binding proteins found as albumins, globulins and glutelins in embryo; S-rich prolamins in de-embryonated endosperm; and albumins in the seed coat. For molecular weight fractionation with liquid phase recovery we used GelFree8100 fractionation system (Expedeon) and identification of those proteins with nanoLC-ESI-Q-TOF are ongoing. We also conducted experiments with three different Zn treatments in the greenhouse and major zinc binding proteins (which are up/down regulated) will be detected and identified with these methods.

Reference List

Effect of recombinant *Hordeum vulgare* endoprotease B2 (HvEPB2) on protein degradation in a barley based liquid feed

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Inefficient nitrogen utilization in intensive animal production results in environmental problems, therefore it is of great interest to obtain new methods for improving protein digestibility in the feed. Assuming that protein digestibility is depended on solubility and molecular size, it is likely that the digestibility can be optimized by a proteolytic “pre-digestion”.

Here we report on the effect of adding a potentially new proteolytic candidate for feed optimisation; the recombinant barley endoprotease HvEPB2. This endoprotease was added to liquid feed composed of grinded barley cultivars, resulting in comprehensive degradation of the hordeins into low molecular weight peptides. HvEPB2 was produced recombinant in *Pichia pastoris* and 2.25 Units were added to 250 mg of fine grinded barley. The level of protease was chosen after initial titration experiments with recHvEP-B2 that revealed the level of endogenous protease inhibitor of the barley grain extract. Two different feed cultivars, Finlissa and Zephyr were subjected to this treatment. Prior to the addition, the cultivars were soaked at 20°C in 2.5 mL 100mM acetic acid, pH 4.5, for 8 hours which allowed also the endogenous proteases to hydrolyze endogenous substrates. After 8 hours, no major proteolysis occurs in liquid feed. Hence recHvEPB2 was added to the liquid feed followed by incubation for additional 4 hours. After centrifugation, the soluble fraction and the pellet sample were used for monitoring the proteolysis. The proteins were extracted with urea and analyzed by SDS-PAGE.

Based on the level of hordein degradation, the recHvEP-B2 represents a good candidate for improving the protein digestibility of barley proteins in feed.
Polyphosphate Metabolising Enzyme Activities in Moss

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In bacteria, fungi and lower plants, phosphate can be stored as polyphosphate, a linear polymer of phosphate residues. Polyphosphate has not been reported in higher plants. Polyphosphate may serve as P-storage, as energy reservoir, and will chelate metal ions. Our aim is to reveal potential roles of polyphosphate in plants, with Physcomitrella patens (moss) as model organism. Polyphosphate kinase (PPK1) from bacteria catalyses the reversible synthesis of polyphosphate from ATP. Physcomitrella has two genes with homology to PPK1. We have isolated two PPK1 cDNAs. We have expressed the moss proteins in E.coli. Soluble preparation purified by affinity chromatography are now available and PPK activity has been confirmed. We also analyzed the expression of PPK in Physcomitrella in relation to P starvation. To create a new form of P storage in higher plants, we have engineered Arabidopsis to express Physcomitrella PPKs. These plants will be analyzed for polyphosphate content, phosphate and carbon metabolism.
Glyphosate hormesis – does it only take place when photosynthesis is sink limited?

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Hormesis is a dose-response phenomenon characterized by low dose stimulation and high dose inhibition by a stressor to a biological system (Calabrese et al. 2007). In plant science this phenomenon has been observed in many species (e.g. maize, barley, duckweed) with a number of stressors (pesticides, radiation, metals etc.). In this study the focus has been on understanding the mechanism behind the growth stimulating phenomenon by low doses of glyphosate treatment to Lemna minor (lessor duckweed). Glyphosate is the most used herbicide worldwide and its mode of action is by binding to the enzyme EPSP synthase (5-enolpyruvylshikimate-3-phosphate synthase) of the shikimate pathway, thus blocking the synthesis of aromatic amino acids. Continuous trials to get a reproducible and stable plant system showing glyphosate hormesis lead to an observation that hormesis was easier obtainable, when the plant system was exposed to a certain level of nutrient stress and only occurred at sufficient light and CO₂ regimes (Cedergreen & Olesen 2010).

We hypothesize that glyphosate hormesis only takes place when photosynthesis is sink limited and the plants do not grow at their maximum capacity.


Transformation approaches to manipulate the activity of Pi transport pathways in *Brachypodium distachyon*

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Phosphorus (P) is an essential nutrient for plant growth. Since P is also one of the least plant-available nutrients in the soil, it is often growth limiting. Uptake and transport of inorganic phosphate (Pi) through plant membranes is mediated by a number of families of transporter proteins. In arbuscular mycorrhizal (AM) plants two Pi uptake routes are present in plants: the mycorrhizal pathway and the direct pathway. The mycorrhizal pathway can be dominating even when Pi uptake and plant growth is unaffected and this implies that the direct Pi uptake is reduced. The non-responsive model grass, *Brachypodium distachyon* is used to investigate the activity of the two pathways under different growth conditions.

Thirteen phosphate transporter genes have been identified in *B. distachyon* and their expression pattern analyzed in non-mycorrhizal and mycorrhizal plants grown at different phosphate levels. Three putative direct transporter genes were down-regulated at high phosphate levels in mycorrhizal plants and two of these have been selected for further investigation. A transformation approach is used to manipulate the activity of the Pi transport pathways, by generating over-expression and knock-down (RNAi) lines of the selected phosphate transporters. The transgenic *B. distachyon* lines will be used to investigate the specific roles of the phosphate transporters in Pi uptake. Furthermore, the potential for increasing Pi uptake efficiency in plants will be evaluated. The Pi uptake efficiency of crops might be improved if a high activity of the direct uptake pathway in mycorrhizal plants is maintained, thereby making the two pathways additive instead of complementary.
Increasing the zinc content of seeds by biofortification - the role of heavy metal ATPases

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Affecting more than one-fourth of the world’s population, zinc deficiency is one of the leading causes of premature deaths in developing countries. Zinc deficiency is especially pronounced in areas where people rely on a plant-based diet, as edible parts of grains generally contain a low amount of bio-available zinc. A promising way to combat zinc deficiency is biofortification, where the zinc content in the edible part of the grain (the endosperm) is naturally enhanced. However, biofortification requires an in-depth understanding of the mechanisms involved in the translocation of zinc in the plant, from the roots through the leaves to the edible endosperm. In Arabidopsis the two plasma membrane localised heavy metal ATPases HMA2 and HMA4 are known to be involved in zinc xylem loading in roots for root-to-shoot translocation of zinc. I have been investigating the role of HMA2 and HMA4 in the export of zinc from the seed coat. This active export is needed for further import of zinc into the endosperm. I studied this by looking at the gene expression pattern of HMA2 and HMA4 in different compartments of the developing seed. This showed that HMA2 and HMA4 are indeed expressed in the seed coat, a prerequisite for their proposed function in the seed. Also, the zinc accumulation pattern in the hma2 and hma4 single mutants as well as in the hma2,hma4 double mutant was investigated using the fluorescent zinc probe Zinpyr-1 as well as multi-elemental bioimaging. Compared to wild-type seeds zinc was found to accumulate in the seed coat of both developing and mature mutant seeds, indicating that HMA2 and HMA4 play a predominant role in export of zinc from the seed coat. The observation that HMA2 and HMA4, in addition to their involvement in zinc xylem loading in roots is involved in export of zinc from the seed coat makes them prime candidates for the purpose of biofortification. Presently, I am engaged in altering the expression of HMA2 and HMA4 in the cells of the mother plant adjacent to the endosperm. The goal is to enhance the translocation of zinc to the edible endosperm.
Individual functions of cytosolic glutamine synthetase isogenes in nitrogen metabolism of Arabidopsis

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Plants have a fundamental dependence on nitrogen (N), which is usually taken up as nitrate and ammonium. Unfortunately, a substantial portion of the applied N in agriculture is lost from the soil and leached into the groundwater and waterways. Several approaches have been followed to improve plant N use efficiency (NUE), but it seems a more thorough understanding of N metabolism is necessary to further this work. Cytosolic glutamine synthetase (GS1) is central for plant N metabolism as it assimilates ammonium into glutamine following uptake of inorganic N into roots and re-assimilates ammonium formed in metabolism, especially during senescence (Martin et al. 2006).

The focus of this project is a reverse genetics approach to characterize the individual GS1 isogenes of which there are 5 in Arabidopsis. We selected the two isogenes, Gln1;1 and Gln1;2, as they are the highest expressed in shoot tissue in Arabidopsis. We used single and double knockout mutants to study how the different GS1 isogenes contribute to plant N metabolism and growth. The project is divided into the following three aspects.

Firstly, in order to study which gene contributes the most to maintain ammonium homeostasis in plants, we studied the response of the mutants to high levels of ammonium. The result shows that the Gln1;2 gene plays an important role in coping with high ammonium. Also, it seems that Gln1;2 is upregulated in the gln1;1 mutant and as such they might have a complimentary function.

Secondly, $^{15}$N is being used to monitor N remobilization during senescence. Previous results showed that the Gln1;2 gene is not involved in N remobilization (Lothier et al. 2011). However, we think that the poor N uptake of these plants is hampering the measurement of N remobilization. Therefore, we are building reciprocal grafted plants with gln1;2 mutant shoots and the wild type roots. A comparison to Wt/Wt grafted plants will allow us to assess the role of Gln1;2 in remobilization. And it is possible to only focus on the Gln1;2 gene shoot function in N remobilization to seeds.

Furthermore, with the use of promoter-reporter constructs we aim to draw a map of the expression patterns of the individual isogenes in terms of their organ- and cell type- specific expression in shoots during the life cycle of the plant and in response to different regimes of N supply.

Lothier J et al. (2011) The cytosolic glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium homeostasis in Arabidopsis rosettes when nitrate supply is not limiting. Journal of Experimental Botany 62: 1375-1390
Martin A et al. (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18: 3252-3274
Cell wall composition in wheat straw: Interactions with nitrogen status

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This study is part of the B21st, Biomass for the 21st century project. This project is a Danish initiative for the integrated development of biomass and conversion technologies for biobased fuels and chemicals. The partners involved are A.P. Moller Maersk, DONG Energy, Haldor Topsoe, MAN Diesel & Turbo, Novozymes, Technical University of Denmark and University of Copenhagen.

The aim of this work is to reveal and overcome bottlenecks in biomass productivity, quality and resource-use efficiency. For that, wheat has to be developed into a dual purpose crop with and increased straw biomass, an increased density of plant cell wall and a reduced content of problematic alkali element and silicates. The improvement of straw yield and quality must be achieved without compromising grain yield. The supply of nitrogen has a decisive influence on the yields of both grain and straw biomass. However, very little information is available on how the composition and structure of the cell walls are affected by increasing N status. The final aim of this work is to develop nitrogen strategies to ensure high straw quality and productivity.

For the first year of the study, winter wheat was grown in field in different conditions. Three levels of nitrogen and the influence of growth regulator application were tested. Different harvest times were chosen along the plant life cycle. These samples are used to make a first characterization of plant cell wall composition and structure according to the nitrogen level. Nitrogen and carbon levels are quantified by IR-MS. Cell wall components structure profile are characterized with a carbohydrate microarray technique, the CoMPP. Cell wall structure will also be understood by microscopy studies with specific cell wall antibodies and staining. The analyses will be complemented by cellulose quantification, lignin analysis, protein content and saccharification performance.
Seed priming with micronutrients improves seedling development and increases grain yield of maize exposed to low root zone temperatures during early growth

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Low root zone temperatures (RZT) at early spring is a major constraint for maize production and other crops in Central and Northern Europe. Nutrient acquisition, nutrient uptake and particularly root growth are severely reduced at low RZT and consequences of these growth depressions are frequently not completely compensated until final harvest. Perspectives to overcome these limitations by seed priming treatments with different micronutrients (Fe Zn, Mn) were studied with maize seedlings exposed to low RZT (12°C). Model experiments were performed in nutrient solution and soil culture using rhizo-boxes with root observation windows under green house conditions. To observe effects on final grain yield, additionally two field experiments were conducted in 2010 and 2011.

Nutrient seed priming resulted in a significant increase in seed contents of the respective mineral nutrients i.e. Fe (25 %), Zn (500%) and Mn (800%). At low RZT, biomass production and total root length of maize plants were significantly increased after Fe and Zn+Mn priming treatments both in nutrient solution and in rhizobox culture. There was no prominent difference in shoot Fe, Zn and Mn concentrations but total shoot contents per plant were significantly increased after nutrient seed priming. Plant growth promotion and an improved micronutrient status was detectable also under field conditions at 5 weeks after sowing and finally resulted in a 10- 15% increase in grain yield. Effects of the additional micronutrient supply on photosynthesis and detoxification of reactive oxygen species are investigated as putative causes for growth promotion under low RZT stress.

The priming effects were detectable in seeds sown instantaneously after the priming treatments but persisted also after drying back to initial seed moisture and storage for several weeks. This offers perspectives for combination with conventional seed dressing strategies for farmers practice.
Barley Endoprotease B2 as Feed Enzyme Candidate.

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During germination of barley grains, a complement of specialized proteases degrades the cereal storage protein complexes and one of the key enzymes is the cysteine endoprotease *Hordeum vulgare* Endoprotease B2 (HvEPB2). Based on both the pH regulated activity and the specificity, HvEPB2 could be a desirable candidate for feed applications and two different protein production strategies were pursued.

In the first strategy, a C-terminal truncated version (HvEPB2ΔC) was heterologous expressed and secreted from the yeast *Pichia pastoris*. By fusing a new proteolytic resistant His6 tag to the C-terminal of r-HvEPB2ΔC, the protein was isolated from the induction medium by a single step Nickel affinity chromatography after maximum expression. A purification yield of 4.26 mg r-HvEPB2ΔC per l supernatant was obtained. By testing r-HvEPB2ΔC activity against native and recombinant barley storage proteins (hordeins) an efficient degradation was observed.

In the second method, the HordD promotor was used to over express HvEPB2 in the endosperm of developing *Golden promise* grains. A transformation efficiency of 13 % was achieved. Over expression levels in T1 and T2 developing endosperms were evaluated by RT-PCR showing up to 5.6 (T1) fold and 4.8 (T2) fold increase in mRNA levels compared to the wild type. Screenings of mature T1 and T2 grains with the endoprotease-specific substrate Z-Phe-Arg-pNA-HCl showed up to 13 times more activity than the wild type. Protein extract from an over expression line was capable of partially degrading native hordeins and faster than extract from the wild type cultivar.
Characterization of photosynthetic parameters in barley (*Hordeum vulgare*) genotypes suffering from manganese deficiency

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Future sustainable improvement in the productivity of cropping systems depends on novel resource-efficient plant genotypes designed to match site-specific soil and climatic conditions. Deficiency in essential mineral micronutrients such as manganese (Mn), iron, zinc, copper and boron is a significant problem for crop productivity in major parts of the world.

Mn is activating more than 30 enzymes and an essential constituent of the oxygen evolving complex in photosystem II (PSII). Therefore, Mn deficiency is a serious plant nutritional disorder causing alterations in the photosynthetic capacity and thereby growth, occurring mostly on alkaline soils that reduce the bioavailability of this metal. In addition, barley (*Hordeum vulgare*) genotypes display a marked difference in their ability to tolerate growth at low Mn concentrations, a phenomenon designated as differential Mn use-efficiency. Previous studies have shown that the functional active amount of Mn can enable differentiating contrasting barley genotypes in contrast to analyzing total Mn concentrations. The objective of the current study is therefore to describe the role of Mn in stabilizing and protecting the photosynthetic apparatus in contrasting barley genotypes and examine the potential for developing novel biomarkers for characterizing the Mn status of plants.

Employing various fluorescence-based and biochemical techniques the photosynthetic apparatus was characterized in two contrasting barley genotypes titrated to different Mn levels. Mn deficiency reduces the PSII yield as well as the ability to dissipate excess energy. Key protein subunits stabilizing the oxygen evolving complex were also affected by Mn deficiency. Data from fluorescence-based and biochemical analysis will be presented at the conference.
Improving nitrogen utilization efficiency in barley

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Nitrogen (N) is often regarded as the main nutrient limiting plant production. However, the use of nitrogenous fertilizers often results in significant amounts of N being lost to the environment. Increasing the crop N utilization efficiency (NutE, grain biomass/shoot N) would allow less fertilizer to be applied without sacrificing yield (McAllister et al, 2012). A key enzyme of N assimilation and remobilization is glutamine synthetase (GS) which catalyses the fixation of inorganic N (NH₄⁺) to organic N (glutamine). GS is found as multiple isoenzymes in the cytosol (GS1) and chloroplast (GS2). GS1 has a crucial role in primary N assimilation in root and in the remobilization of N from senescing tissue whereas GS2 is important in the assimilation of NH₄⁺ from NO₃⁻ reduction in photosynthetic tissue as well as from photorespiration (Swarbreck et al, 2011).

In our project, we have introduced one or more extra copies of the endogenous cytosolic GS1 gene together with its promoter in the barley variety Golden Promise (Kichey et al, 2009). We are expecting a “gene dosis” effect, resulting in improved efficiency with which GS1 remobilizes NH₄⁺ at senescence and thus a higher NutE.

An initial experiment with three transformed T3 lines (#12, 83 and 103) grown together with wildtype (wt) barley at low (0.5 mM) or high (5 mM) N supply and harvested at 48 days after germination (DAG) demonstrated an increased GS1 gene expression in root and shoot of all three transformed lines at low and high N supply. A significant increase in GS1 enzymatic activity was measured in roots at high N, but not at low N, in all lines. GS1 is normally found also in photosynthetic tissue and initial experiments with HPLC separation of the GS isoforms in shoot protein extracts indicate a higher GS1 enzymatic activity also in this tissue. An experiment with two transformed T3 lines (#12 and 83) grown to maturity together with wt barley at low (1 mM), high (5 mM), or high:low (5 mM with transfer to 1 mM at ear emergence) N supply demonstrated a significantly higher shoot and grain biomass in both transformed lines at low N supply, but not at high nor high:low N supply. Shoot N content will be measured to calculate the NutE. A field trial in 2012 with ten transformed lines and wt barley at low (40 kg/ha) or high (120 kg/ha) N supply demonstrated an increase in NutE at high N supply but not at low N supply in three lines (#12, 35 and 101) as compared to wt. However, another line (#103) had a higher-than-wt shoot biomass at low N supply. These results indicate that there is potential for improving the NutE in barley under certain growth conditions by increasing the expression of endogenous GS1. Further experiments with selected transformed GS1 lines grown at elevated atmospheric CO2 will evaluate the possibility to further increase the NutE by providing higher level of limiting carbon skeletons for the N metabolism.

References:
Salvia hispanica seed phytases

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Chia (Salvia hispanica L., Lamiaceae), herbaceous summer annual, produces highly nutritious seeds for human and animal nutrition. During the pre-Columbian times, millions of Central Americans cultivated chia as one of the main staple foods. Chia seed contains protein (29%), lipids (32%), ash (5%), fiber (27%) and carbohydrates (7%) by weight. It is also an important source of essential polyunsaturated fatty acids (omega (ω)-3 (n-3) and omega (ω)-6 (n-6)), antioxidants, vitamins and minerals, vital for human and animal health. Its high content and promising ratio (n-3/n-6) of these essential polyunsaturated fatty acids with low amount of saturated fatty acids increases their use for human and animal consumption. However, in addition to this, recent studies in our laboratory have found that the mature chia seed also has a substantial activity of the enzyme phytase. Plant seeds store the majority of their phosphate in the form of phytic acid, and phytase activity is important for making phosphate bio-available in food and feed. Furthermore, non-hydrolyzed phytic acid chelates and reduces the bio-availability of nutritionally important minerals such as Fe and Zn. High phytase activity in oil crop seeds is a most valuable trait, because after pressing the oil out, the remaining cake is often used in feed. Unfortunately main oil crops like rape, sunflower and soy bean has basically not phytase activity in the mature seed. It is the aim of the current study to undertaken the characterization of the chia seed phytases.
The structure and evolution of barley powdery mildew effector candidates

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The powdery mildew fungus, Blumeria graminis f.sp. hordei (Bgh) is a recently sequenced obligate biotrophic pathogen of barley. In addition to being a significant agricultural pathogen, it also serves as a model for studies on powdery mildew fungi and other obligate biotrophic pathogens. Here, I present a comprehensive survey of 491 Candidates for Secreted Effector Proteins (CSEPs) representing more than 7% of the protein coding genes found in the Bgh genome. Based on sequence homologies, we clustered the CSEPs into families of paralogs and show that CSEP genes have duplicated in the genome, most likely due to unequal crossing over during evolution, which has caused them to cluster in the genome. Within many of these families, we find strong evidence for positive selection for diversity. When we mapped the amino acid residues under positive selection on 3D structural models, they were usually predicted to be exposed on the protein surface, and thus possibly involved in protein interactions. Expression studies show that the CSEPs preferentially are expressed in haustoria. Many CSEPs across different families appear to be related to microbial RNases, and we propose that a large proportion of the CSEPs have evolved from an ancestral microbial RNase. We speculate that these RNases may have been an ideal starting material for building up an effector arsenal. Our data fit well with a model for CSEP evolution driven by selection for gene duplications and for amino acid chances resulting in a large diversity allowing the fungus to yield a highly diverse palette of effector functions.
The haustorium – Where fungi and plants meet

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The haustorium is a feeding organ developed by certain fungi, including biotrophic plant pathogens and symbiotic mycorrhiza. Haustorial are placed inside the plant cells, but surrounded by an extra haustorial matrix and a plant-derived extrahaustorial membrane. This specialized structure is thus creating a unique host–pathogen interface, central for nutrient uptake. It the same time is is a battleground for attack and defence reactions between the host and the pathogen. The haustorium is therefore of special interest for studies of biotrophic pathogens, and isolation of haustoria for subsequent extraction of proteins or mRNA is a key technique in many studies.

We are interested in basic questions regarding the formation of the extrahaustorial membrane and in understanding the interplay between pathogen effectors and defence mechanisms. In a study on the barley powdery mildew interaction, we aim to isolate haustoria, including the extrahaustorial membrane, for proteome analysis. Our previous work (Godfrey et al., 2009) focused more on the proteins expressed in and/or secreted from haustoria, and did not require the extrahaustorial membrane. So in our current project, we will adapt the method to enrich for haustoria containing the intact membrane.

In a study on yellow rust on wheat, caused by *Puccinia striiformis* f. *sp. tritici*, we isolate haustoria for subsequent RNA-seq in a comparison between new aggressive and less aggressive isolates. For this purpose, we use haustoria without the extrahaustorial membrane, and exploit surface-exposed D-mannose within the haustorial cell wall for affinity purification on the lectin, Concanavalin A (Hahn and Mendgen, 1992). Results from both projects will be presented.


FMO1 and ALD1 mediate a common NPR1-dependent and SA-independent defence signal

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In Arabidopsis, the lesion-mimic double mutant pen1 syp122 exhibits multiple activated defence signalling pathways in the absence of pathogens. In a previous suppressor mutant screen, ALD1 (AGD2-LIKE DEFENSE RESPONSE PROTEIN 1) and FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE 1) were discovered to play important roles for lesion formation and plant growth retardation (Zhang et al., 2008). FMO1 and ALD1 have previously been identified to have important roles in pathogen defence. Although it is known that FMO proteins in general catalyse the transfer of hydroxyl groups to nucleophilic heteroatoms such as sulphur, nitrogen, selenium, or iodine, the specific substrate and product of FMO1 are yet unidentified. Furthermore, there are examples that FMOs can change the cellular redox state through the production of reactive oxygen species. ALD1 has aminotransferase activity with substrate specificity for lysine, and is involved in the biosynthesis of pipecolic acid (Návarová et al., 2012).

In the present study, rosette leave size analysis of triple, quadruple and quintuple mutants in the pen1 syp122 background suggests that ALD1 and FMO1 act on the same defence signalling pathway, which is independent of SA-signalling, but dependent on the SA-downstream component, NPR1.


The role of ABA and the *HvNAC6* transcription factor in response to abiotic and biotic stress in barley

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Transcription factors have been shown to be involved in the crosstalk between abiotic and biotic stress-signalling networks. In an attempt to investigate the *in vivo* functions of the barley NAC transcription factor, *HvNAC6*, we generated transgenic barley plants by an RNA interference (RNAi)-based method. Our results indicate that *HvNAC6* participates in an abscisic acid (ABA)-dependent stress-signalling pathway and is a central component of barley penetration resistance towards *Blumeria graminis* f. sp. *hordei* (*Bgh*) attack. We have shown that exogenous ABA stimulates resistance to attempted *Bgh* penetration, suggesting that ABA is a positive regulator of penetration resistance. This, in turn, implicates *HvNAC6* as a positive regulator of ABA signalling that is required for maintenance of effective penetration resistance towards *Bgh*. Furthermore, we are currently measuring ABA levels and expression of a positive regulator of ABA signalling, *HvPKABA1*, known to correlate with ABA levels, during *Bgh* infection. We have also demonstrated that alteration of *HvNAC6* expression modulates ABA responsiveness in barley *i.e.* *HvNAC6* RNAi plants were less sensitive to ABA. Following drought treatment, *HvNAC6* RNAi plants showed a higher rate of water loss than WT. A significant reduction of ABA marker transcript levels for *HvPKABA1* was also observed in *HvNAC6* RNAi plants under drought stress. We therefore suggest that ABA-mediated responses to both abiotic and biotic stress are regulated by *HvNAC6* in barley.
RNA-seq and metabolite studies of the barley/powdery mildew interaction during climate change

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The predicted changes in the world’s climate are believed to affect the physiology of plants, and their interaction with pathogens. It is generally hypothesized that crop plants may become more prone to diseases in the future, but it is difficult to generalize, and not much information is available from experiments using multi climatic factors. Furthermore the molecular and biochemical mechanisms behind the observed and predicted changes in plant disease susceptibility are not fully understood.

Previously, we have examined how climatic factors are affecting disease severity in spring barley (Hordeum vulgare) towards fungal pathogens with opposite lifestyles: the biotrophic Blumeria graminis f.sp. hordei (powdery mildew) and the hemibiotrophic Bipolaris sorokiniana (spot blotch), and observed that development of the diseases was affected differentially by the climatic factors. Disease severity of powdery mildew was decreased when plants were grown in a phytotron with increased levels of temperature, [CO₂] and [O₃] as single factors. However, combinations of the factors lead to increased disease severity.

To learn about the molecular and biochemical mechanisms involved, we have chosen to dig deeper into the well-known barley-powdery mildew interaction and have made RNA-seq analysis (whole transcriptome) and metabolite studies, to elucidate how barley plants acclimates to the climatic factors introduced, and how this can explain the observed changes in disease severity. We are focusing on metabolites and genes from the phenylpropanoid pathway, which is known to be affected by both abiotic and biotic stresses. Preliminary results from these analyses will be presented.
R gene specificity in bread wheat

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An important family of plant resistance genes (R genes) encodes NB-LRR proteins consisting of a nucleotide binding (NB) and a leucine rich repeat (LRR) domain, the former holding a p-loop essential for function. NB-LRR genes harboring a specific p-loop mutation act dominant negatively when introduced into Arabidopsis and Nicotiana. This confers susceptibility to pathogens normally controlled by the corresponding wildtype R gene. We aim to introduce this principle in cereals in a strategy that has been designed to match novel R gene candidates in bread wheat (T. aestivum) to specific pathogens. The wheat - yellow rust (P. striiformis) system will be used for proof-of-concept, where the R gene Yr10 holding the p-loop mutation will be transformed into the wheat cultivar ‘Avocet-Yr10’ harboring the wildtype gene. Transgenic lines expressing the mutation are expected to exhibit susceptibility to an appropriate avirulent P. striiformis isolate harboring the AvrYr10 gene. Gold particle bombardment of immature wheat embryos is employed for gene transfer. For the subsequent screening strategy the wheat cultivar ‘Bobwhite S-26’ will be used. From deep transcriptome sequencing a cultivar-specific library of expressed NB-LRR type R gene candidates will be established. Transgenic ‘Bobwhite S-26’ lines expressing p-loop mutated forms of candidates will be inoculated with a selection of avirulent isolates of P. striiformis. Obtained susceptibility to a particular isolate will reveal the specificity of the wildtype R gene corresponding to the mutant in question. Identified R genes will be an important new resource in disease resistance breeding.
Association Mapping for Common Bunt Resistance in Wheat

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Key words: Common bunt (Tilletia caries), Wheat (Triticum aestivum), Association mapping, Organic farming

Common bunt, caused by Tilletia caries and T. foetida, is a fungal disease of wheat found worldwide. Infection, occurring via seed borne teliospores, is generally controlled by the application of seed treatments prior to sowing. Farming systems like organic agriculture with a very limited range of organic seed treatments available, rely heavily on common bunt resistance genes within wheat and could benefit from improved knowledge of the molecular background of common bunt resistance in wheat. In the framework of the BIOBREED project an association study in winter wheat was conducted, aiming at the identification of genetic marker linked to resistance towards common bunt in wheat.

152 European wheat cultivars were phenotyped for their resistance reaction in 2011 and 2012 at Agrologica research station in Mariager. Infection was scored as percent infected ears. The scorings were log-transformed to fit a disease scoring scale ranging from 1 to 9. A genome-wide association analysis was performed for each year separately as well as for the mean scoring of the two years. The wheat cultivars were genotyped with DArT markers, yielding 1832 polymorphic marker on 1151 loci.

Thirteen out of the total of 1832 marker in our study were linked to common bunt resistance in wheat (-log10(P) > 3). These marker were located on 8 of the 21 wheat chromosomes. Comparisons of these findings with other published results are difficult since only very little is known about the precise location of common bunt resistance genes/QTL in wheat. Chromosome 2B was previously reported to carry gene(s) for common bunt resistance. In accordance with these results, four markers on chromosome 2B showed tight linkage to common bunt resistance in our study. Two markers on chromosome 2D – also previously reported to carry common bunt resistance genes – were associated with common bunt resistance. We can expect that we marked hitherto not localized bunt resistance genes by the other seven associations found in our study.

Our results demonstrate the possibility to find makers linked to common bunt resistance in wheat by genome-wide association studies. Markers for marker assisted selection as well as genotypes carrying these resistance can be identified and used to breed for wheat cultivars tailored for the needs of organic agriculture.

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References
Searching for fungal effectors shaping the extrahaustorial membrane

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The causal agent of barley powdery mildew, Blumeria graminis f.sp. hordei (Bgh), is an intracellular biotroph that depends on the formation of a host-derived membrane around its feeding structure, the haustorium. Therefore, Bgh must be able to use effectors to highjack its host’s endomembrane traffic in order to establish disease. The mechanism of such an effector-mediated endovesicle sequestration is still elusive and the pool of vesicles that determine the nature of extrahaustorial membranes (EHMs) is yet to be shown. Our present research focuses on understanding how the barley powdery mildew fungus induces formation of the EHM. A Bgh cDNA library was generated 16 hours after inoculation, which is a critical time point for EHM formation. This library was ectopically expressed in yeast and used for the Pathogenic Effector Protein Screen in Yeast (PEPSY). Twelve candidate genes were identified as presenting disrupted secretion phenotypes. The importance of these candidate genes for virulence is currently being tested by RNAi using Host Induced Gene Silencing (HIGS). Identification of effector-mediated vesicle sequestration and their interacting targets in the host will bring new insights into the host origin of the EHM and the molecular processes governing the establishment of the Bgh intracellular interface within its host cell.
Impact of Climate change on emerging plant diseases and their threat to food security

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Climate change is influencing our agro-eco system and the consequences related to plant diseases are expected to include:

- changed crop physiology, growth and quality
- new crops/ cropping systems and new emerging diseases
- changed severity of already established diseases.

Increased temperature and elevated CO2 and ozone gases are predicted to have a progressively negative effect on yield and biomass production, but possible effects on plant disease are less clear. We are investigating the interaction of the climate change factors temperature, CO2 and ozone with cereals and their diseases.

Spot blotch caused by the fungus Bipolaris sorokiniana is used as model disease. In some areas B. sorokiniana has become one of the most devastating pathogens in barley and wheat production. Spot blotch disease outbreaks have increased severely in areas that have become more humid and warmer within the last years due to climate change. After a symptomless early biotrophic stage this hemi-biotrophic fungus kills the mesophyll cells with the help of phytotoxins which leads to necrotic leaf spots. These phytotoxins are demonstrated to be responsible for the development of symptoms on the leaf of infected plants (Berestetskiy, 2007).

We are investigating how environmental conditions are linked to the aggressiveness of the fungus and if production of phytotoxins is directly related with disease severity. Normally symptom development and the number of necrotic lesions is an indicator of disease severity and plants with fewer spots are expected to have mechanisms that restrict fungal growth in the leaf. However, our experiments indicate that there is more fungal biomass in plants when there is no characteristic symptom development which is in contrast to the expected necrotrophic growth strategy of B. sorokiniana. Moreover, different genotypes seem to vary in symptom development, but there is no clear correlation with the amount of fungal biomass in the plant.

In another experiment, barley genotypes were grown under different treatments of climate change factors and the leaves were exposed to the toxic compounds that are produced by B. sorokiniana. Some genotypes developed the clearest necroses under elevated CO2, while others were more sensible under elevated temperature or did not show differences in symptom occurrence at all.

Our results show that symptom development and disease severity are driven by several factors like environmental conditions and the genetic predisposition of the plant. These results will be useful to assist in finding disease resistant cultivars under varying climatic conditions.

Barley: The correlation between powdery mildew infection and leucine-derived hydroxy nitrile glucosides

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Cyanogenic glucosides are well-known defence compounds in plants. Barley produces five hydroxy nitrile glucosides (HNGs), which accumulate in the epidermis and account for 90% of the soluble carbohydrates in this tissue. Only one of them, Epiheterodendrin, is a cyanogenic glucoside, hence having the potential to release cyanide upon β-glucosidase degradation. Barley powdery mildew (Bgh) is an obligate biotrophic fungus specialised to infect and feed on barley epidermal cells. It is speculated that the fungus exploits the HNGs as a glucose and nitrogen source. The relationship between Bgh susceptibility and leaf cyanide potential is unclear. Cyanide is not liberated during powdery mildew infection, and no β-glucosidase able to cleave Epiheterodendrin is known in barley leaves. Epiheterodendrin is also known to stimulate fungal appressoria formation in vitro, suggesting that Bgh uses Epiheterodendrin in host recognition¹.

To investigate the proposed link between Bgh susceptibility and HNGs, we are currently measuring HNG levels +/-Bgh. Preliminary results indicate that after fungal infection HNG levels and the relative HNG distribution changes in susceptible barley leaves. Additionally, we found that HNGs are transported to the wax layer. The route of transport has not been elucidated. Apart from functioning as a structural barrier between the plant and the environment, the wax layer may be more dynamic taking direct, chemical action in defence. Also, the bioactive compounds encountered in the wax can, upon degradation, take part in plant-to-plant signalling and/or be used as fungal host recognition factors.
Endophytic bacteria reside inside plant tissue and can have beneficial effects on plants. The underlying mechanisms regulating interactions between plant and microbe remain unclear. There are long-standing fundamental questions regarding the true diversity of endophytic bacterial populations and how they colonize and co-exist with plants in peace while exerting beneficial effects on the hosts. Using culture-independent approaches including 16S rRNA clone-library sequencing and the 16S rRNA microarray technology known as Phylochip, we found diverse bacterial communities associated with healthy Arabidopsis roots and leaves. This diversity encompassed 1877 bacterial taxa belonging to 42 phyla. To further investigate the interaction between host and microbes, a culture-dependent approach was undertaken and bacteria were isolated from surface-sterilized *A. thaliana* growing in a variety of soils. This non-exhaustive isolation yielded a total of 56 bacterial species. Regardless of the soil in which plants were grown, some species were consistently found associated with roots; the most common isolates included Methylobacterium and *Microbacterium ginsengisoli*. Current studies are focusing on molecular interactions between *A. thaliana* and the isolated bacteria. We are particularly interested in the role of the plant cell wall and secondary metabolites in colonization and co-existence as well as the interactions between diverse bacteria in planta.
The role of plant cell wall acetylation on plant fitness

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Acetylation of plant cell wall polysaccharides is an important target in the optimization of bioenergy crops since the acetyl groups inhibit key processes in the biofuel production pipeline (Selig et al. 2009 and Helle et al. 2003). Therefore, it is very important to elucidate the biological function of polysaccharide acetylation and determine the consequences of altering the cell wall acetylation pattern. We are investigating a range of cell wall acetylation mutants using different strategies. Firstly biotic and abiotic stress responses of the mutants were analyzed. Secondly the effect of the mutations on the overall cell wall structure is being investigated. The mutants analyzed are rwa2, axy4, tbl10, tbl32, tbl38 and tbl40, each of which affects the pattern of cell wall acetylation differently. Two mutants showed increased resistance to the necrotic fungal pathogen Botrytis cinerea: the reduced wall acetylation 2 (rwa2) mutants with a 20% reduction in the acetylation of pectins and hemicelluloses (Manabe et al. 2011) and the axy4 mutants with complete elimination of the acetylation of xyloglucan (Gille et al. 2012). We showed that the rwa2 mutants exhibit increased leaf surface permeability and a faster perception of pathogen- and host-derived elicitors, which explain the increased resistance of the rwa2 mutants to B. cinerea as compared to WT. In contrast, in the axy4 mutants, these characteristics are not observed suggesting unique resistance mechanisms between these mutants. Possibly, the reduced level of acetylation in axy4 leads to other secondary effects responsible for the increased resistance. This could be changes in the immune response or in the cell wall structure. In regards to the latter, preliminary results from CoMMP analyses indicate that cell wall structures other than xyloglucan acetylation are also altered in the axy4 mutants as compared to WT. Further investigation of the cell wall alteration and the analysis of the transcript levels of genes connected to the general immune responses in the axy4 mutants are in progress.

ABC transporters are potentially evolutionary evolved to provide resistance to xenobiotics in the mycoparasitic fungus Clonostachys rosea IK726

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Clonostachys rosea is a parasitic fungus effective in suppressing mycotoxigenic Fusarium species that are known to produce a variety of toxins. Previously, we used genome-wide transcriptome analysis to firstly show that ABC transporters were associated in providing resistance to the mycotoxin zearalenone in C. rosea. The transporters identified were of subfamily G of fungal ABC transporters, where several members of this group were identified as plietropic drug resistance transporters in other fungal species. This suggests a potential role of ABC transporters in parasitism of the fungus. To address whether resistance to xenobiotic in mycoparasitic fungi is connected to their parasitic lifestyle, we conducted evolutionary analysis of ABC transporters in C. rosea IK726 and 4 additional Trichoderma spp. A primary analysis of the Illumina- and SoLid-based draft genome of C. rosea IK726 revealed an expansion of ABC transporters in which a total of 85 putative ABC transporters were identified. The analysis of gene birth and death showed gene birth in C. rosea and ancestor to T. virens/T.harzianum. Further investigation suggested that the expansion was occurred in subfamily G of C. rosea and subfamily C of ancestor to T. virens/T.harzianum. On the other hand, gene loss was identified in T. reesei and T. atroviride, specifically in the subgroup C. It is likely that the expansion and contraction of ABC transporters are evolutionarily shaped by mycoparasitic lifestyle. For example, the expansion of ABC transporters observed in C. rosea, T. virens and T. harzianum is to withstand ranges of toxic metabolites produced for antagonism in these species, while the parasitism of T. reesei and T. atroviridae that relies on direct parasitism results in the contraction of the transporters.
Barley powdery mildew effector candidate CSEP0443 interacts with a RING finger E3-ligase-type protein

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The barley powdery mildew fungus (Blumeria graminis f. sp. hordei, Bgh) is an obligate biotrophic pathogen, which has a negative effect on crop yield. Pathogens in general and in our case Bgh produce effector proteins. These are transferred to the plant cell by unknown mechanisms to re-direct nutrients or suppress plant defence mechanisms. So far, about 500 effector candidates have been identified from Bgh. However, their roles and how they perform their functions remain unsolved.

Here, we selected to silence effector candidates with high transcription, which hopefully could change the pathogenicity. The “host-induced gene silencing” showed interesting results. In case of CSEP0443 (Candidate for Secreted Effector Proteins), it significantly lowered the fungus’ pathogenicity. Further studies by yeast-2-hybrid (Y2H, an in vivo protein-protein interaction method) showed that CSEP0443 interacted with a RING finger E3-ligase protein. Down-regulation of this barley gene in epidermal cells increased susceptibility to Bgh, supporting its role in regulating the host immune response. The RING finger E3-ligase protein seems to be a cytosolic protein, suggesting the interaction with CSEP0443 occurs in the plant cytosol. E3-ligases are part of the ubiquitin-proteasome system, which attach ubiquitin to target proteins, therefore function to determine the specificity of the substrate. We are currently trying to screen for targets of the RING finger E3-ligase protein from a barley cDNA library via Y2H. We also suggested some putative interactors, based on literature studies, which will be tested for true interactions. The aim is to understand why and how Bgh’s pathogenicity is increased by CSEP0443. To investigate if the RING finger E3-ligase protein is an important hub in plant defence, we use the homologues XBAT34 and XBAT35 in Arabidopsis thaliana, which have been shown to be linked to the ethylene pathway. Therefore, we also prepare to test powdery mildew fungal pathogenicity in T-DNA mutant lines of Arabidopsis thaliana.
Abiotic stress tolerance in 138 barley accessions tested in the future Nordic climate

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Climate change progress fast and consequently development of high yielding cultivars suitable for the future growth environment is vital. We have screened 138 spring barley accessions in the RERAF phytotron under scenarios representing the climate in Northern Europe around year 2075. The abiotic factors; temperature, [CO₂] and [O₃] were constantly elevated in single factor treatments, and in a more realistic doublefactor treatment with temperature and [CO₂]. Temperature was elevated by 5°C to 24°C/17°C (night/day), [CO₂] was almost doubled to 700 ppm and [O₃] was 100-150 ppb; as control was present Danish summer conditions (19°C/12°C (night/day), 385 ppm [CO₂]. All climate scenarios were provided the same amount of water. The material screened included landraces, old and modern Northern cultivars in addition to lines from Nordic breeding companies. The +5°C treatment reduced the yield by about 50% in average and the elevated [CO₂] treatment increased yield by about 15%. No change was found when spring barley was grown under elevated [O₃]. In the doublefactor treatment, the yield stimulating effect of elevated [CO₂] was not able to counteract the reductions in yield caused by the +5°C, as the grain yield in the doublefactor treatment was reduced by about 30%. In general, the reduced yield was caused by decrease in seed number and not amount of ears. The same trends were found for biomass as for yield, but the biomass was generally less affected than the yield. Accessions with static and dynamic yield stability were also identified. Based on the results an array of accessions has been selected for test in extreme event scenarios, where we e.g. explore the phenotypic and molecular response to a heat wave.

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Knowledge on Linkage Disequilibrium (LD) is important when considering genotyping strategies for genomic selection. A rapid decay of LD necessitates the need for a higher marker density to enable markers to capture the entire phenotypic variation in the population. Outbreeding species with large effective population sizes are expected to have very low levels of LD. We have utilised a draft assembly of the perennial ryegrass genome to assess the extent of genome wide LD in a perennial ryegrass association population. The population consists of 174 single individuals originating from 72 varieties and 22 ecotypes, and there is a 92, 82 split between forage and turf types. The population was genotyped using a genotype by sequencing approach with the restriction enzyme APEKI (5bp cutter). Sequence tags were mapped onto scaffolds from the draft genome assembly that were greater than 10Kb in size, in order to identify SNPs. The genotypes were determined for each individual at these SNP sites, and used to calculate the squared correlation of allele frequencies between SNP pairs falling within the same genomic scaffold. The LD decayed rapidly between inter-SNP distances of 1-4Kb. The population was then used to find alleles differentiated between forage and turf types. This identified variation in a gene involved in sterol synthesis, a precursor in brassinosteroid hormone synthesis.
The genome and transcriptome of perennial ryegrass mitochondria

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Perennial ryegrass (\textit{Lolium perenne} L.) is one of the most important forage and turf grass species of temperate regions worldwide. Its mitochondrial genome is inherited maternally and contains genes that can influence traits of agricultural importance. The DNA sequence of mitochondrial genomes has been established and compared for a large number of species in order to characterize evolutionary relationships. Therefore, it is crucial to understand the organization of the mitochondrial genome and how it varies between and within species. Here, we report the first \textit{de novo} assembly and annotation of the complete mitochondrial genome from perennial ryegrass.

Intact mitochondria from perennial ryegrass leaves were isolated and used for mtDNA extraction. The mitochondrial genome was sequenced to a 167-fold coverage using the Roche 454 GS-FLX Titanium platform, and assembled into a circular master molecule of 678,580 bp. A total of 34 proteins, 14 tRNAs and 3 rRNAs are encoded by the mitochondrial genome, giving a total gene space of 48,723 bp (7.2\%). Moreover, we identified 149 open reading frames larger than 300bp and covering 67,410 bp (9.93\%), 250 microsatellites, 29 tandem repeats, 5 pairs of large repeats, and 96 pairs of inverted repeats. The genes encoding subunits of the respiratory complexes – \textit{nad}1 to \textit{nad}9, \textit{cob}, \textit{cox}1 to \textit{cox}3 and \textit{atp}1 to \textit{atp}9 – all showed high expression levels both in absolute numbers and after normalization.

The circular master molecule of the mitochondrial genome from perennial ryegrass presented here constitutes an important tool for future attempts to compare mitochondrial genomes within and between grass species. Our results also demonstrate that mitochondria of perennial ryegrass contain genes crucial for energy production that are well conserved in the mitochondrial genome of monocotyledonous species. The expression analysis gave us first insights into the transcriptome of these mitochondrial genes in perennial ryegrass.

Reference

Genotyping by sequencing of a training population for genomic selection in *L. perenne*

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There has been limited success to date in directly applying molecular markers in traditional breeding programs. A major factor in this was the paucity of molecular markers to adequately cover the genome. While finding marker-trait associations on within family populations has been possible with limited markers, the cost and time to apply such markers has meant they have only been used on a limited number of experimental crosses, and thus any identified markers may be of little use in breeding material. Finding more useful marker-trait associations in populations (e.g. breeding material) is only possible with dense marker coverage to overcome the low linkage disequilibrium present in outbreeding species. However, the rapid development of sequencing technologies means marker density is no longer a bottleneck. These advances are now enabling plant breeders to consider genomic selection as a tool to increase genetic gain in breeding programs, in a manner similar to that seen in animal breeding. We are involved in a large project (ForageSelect) that is tasked with implementing genomic selection in a traditional breeding program. The genomic selection models will be developed on a training population consisting of 1000 perennial ryegrass families. Initial work focused on selecting an appropriate strategy for genotyping of 1000 families, and the results of pilot experiments favored a genotype by sequencing approach using a frequent cutting enzyme for genome complexity reduction. This has now been applied to the genotyping of the complete training population and the results will be discussed in terms of (1) number of molecular markers discovered at different minor allele frequency thresholds, (2) coverage at each marker across populations and the level of missing genotype data, and (3) the frequency of marker discovery in protein coding sequences.
An RNA-Seq approach to study vernalization and the induction of flowering in perennial ryegrass

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Perennial ryegrass (\textit{Lolium perenne} L.) is the main forage grass species utilized in temperate agricultural regions due to its high digestibility and feed quality. However, the onset of flowering leads to a decrease in forage quality, and feed quality would therefore be enhanced by prolonging vegetative growth. This has led to a research effort to understand the underlying genetic components affecting variation for flowering time in perennial ryegrass, with the long term goal of developing molecular tools to assist breeding programs in developing varieties with enhanced agricultural value. Vernalization is a key factor in the induction of flowering in perennial ryegrass and was the focus of this study. The transcriptome of two genotypes with contrasting vernalization requirements was studied during both primary (vernalization and short day conditions), and secondary induction (higher temperature and long day conditions) using an RNA-Seq approach. This revealed transcripts with expression profiles indicative of a role to play in floral induction. The expression patterns were validated by quantitative RT-PCR and candidate genes were converted to molecular markers. These genes were mapped in an experimental mapping family segregating for the vernalization requirement and were compared to the location of previously identified vernalization QTLs.
**De novo transcriptome analysis in perennial ryegrass**

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Perennial ryegrass (*Lolium perenne* L.) is an important grass species for both forage and amenity purposes for temperate regions worldwide. It is envisaged that breeding efforts may be enhanced with the assistance of new breeding technologies such as genomic selection. A major step towards genomic selection will be the availability of a reference genome, and efforts are underway within our group to deliver this. An important step in *de novo* assembly will be defining the gene set, and the availability of transcriptome sequencing data will greatly aid gene prediction and validation, and the development of functional markers for improved ryegrass breeding. Therefore, the goal of this study is to analyze a *de novo* assembly of the perennial ryegrass transcriptome from the same inbred genotype being used for *de novo* genome assembly. Furthermore, we also conducted *de novo* transcriptome assembly with other heterozygous genotypes to enable SNP discovery for marker-assisted selection (MAS) and to determine the number of synonymous and non-synonymous SNPs. In this study we have performed RNA-seq analysis of leaf, stem, inflorescence, leaf sheath, root and meristem from the inbred and heterozygous genotypes; to get a good representation of the transcriptome.
Genetic Modification of Oilseed Rape to Create New Quality and Flower Characteristics

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Keywords: Oilseed rape, Agrobacterium rhizogenes, transformation, rol-genes

Oilseed rape (Brassica napus L.) is among the most important oilseed crops in the world. Genetically, oilseed rape is a close relative to the model plant Arabidopsis thaliana, both found in the Brassicaceae family. A translational science approach, where molecular knowledge from Arabidopsis within floral development and hormonal signalling is combined with practical know-how from ornamental breeding, flower and senescence physiology, holds great prospects for crop improvement. Potentially, new plant cultivars with changed characteristics affecting crop yield and performance could be generated. In the present study, we transform oilseed rape with a wild type strain of Agrobacterium rhizogenes. During transformation, A. rhizogenes root-loci-genes (rol-genes) are integrated into the rape genome resulting in the generation of hairy roots. After selection of putative rol-transformed roots followed by regeneration of plants in vitro, plants with a changed morphology can be generated. The rape transformation method comprises expression optimization of bacterial virulence-genes in A. rhizogenes, explant type and regeneration experiments. Positive transformants in rape will be validated on DNA and RNA levels using polymerase chain reaction (PCR) and by quantitative real time PCR. In ornamentals, the presence of rol-genes results in plant compactness, increased rooting and changed flower characteristics, however, on the molecular level rol-gene induced changes are not well characterised. Via the hormonal signalling pathways characterised in Arabidopsis and the insertion of rol-genes into oilseed rape, we aim to broaden the understanding of the functions of rol-genes on phenotypes related to flowering and crop yield.
To feed the world's growing population, and support a societal shift from fossil fuel-based to bio-based precursors for the production of energy and fine chemicals, agriculture has to increase space and nutrient efficiency. At the same time it is necessary to diminish the environmental footprint of farming. Crops with storage organs in the soil, like potato, produce twice the amount of calories per area unit with similar or less input of nutrients and water and are thus attractive alternatives to cereals in the future. However, further crop improvements are necessary not the least with regard to disease resistance. Unfortunately potato breeding suffers from acute inbreeding depression, and a slow breeding cycle which is largely done in a traditional "mate and phenotype" approach. In contrast, in cereals Marker Assisted Selection (MAS) platforms have accelerated breeding tremendously. With the recent release of the reference potato genome sequence(1) it is now possible to implement MAS on a grand scale in potato as well. Furthermore, to bypass the slow MAS-marker discovery cycle, we propose to utilize the ultra-high throughput of Next Generation Sequencing (NGS) technology to develop de novo markers and candidate genes using a combination of multi-parent crosses and bulk segregant analyses linked to 6 commercially relevant phenotypic traits for potato: earliness, yield, dry matter, morphology, chipping quality, and late blight resistance. These "true markers" can be employed directly in subsequent MAS development of improved potato varieties. Additionally the dataset will also be mined for null alleles to unravel the genetic loci in potato that suffer from inbreeding depression to facilitate selective outbreeding of inbred alleles in future breeding programs. Here we will present our novel methodology for NGS driven bulk segregant analysis together with the status of our preliminary analyses of the parent germplasm and the anticipated outcomes of the project.

The influence of laccases on cell wall recalcitrance

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Laccases, EC 1.10.3.2 or p-diphenol : dioxygen oxidoreductases, have been proposed to be involved in the oxidative polymerization of monolignols into lignins in plants. As part of the strategic research centre Bio4Bio, the present project deals with laccase functions in the cell wall biosynthesis of ryegrass (Lolium perenne L.). Plant laccases are multigene families with e.g. 17 and 14 members in Arabidopsis thaliana and maize (Zea mays L.) respectively. The functional assignment of individual laccases in relation to cell wall biosynthesis is therefore a major challenge, but also an opportunity. Thus, laccases may offer a route to modulate cell wall recalcitrance while maintaining agronomical performance. RNAi constructs have been generated that specifically target four different ryegrass laccases. Ryegrass has been transformed with the constructs by biolistics. Nine to thirteen independent transformant lines for each construct were identified and among these are lines with stunned growth or curled up leafs. Control plants transformed with empty vector also display some phenotypic variation, but they do not have the distinct phenotypes of the RNAi lines. Real time qPCR have been deployed to examine the correlation between phenotypes and altered laccase expression. Transformant lines with strong knock down of one of the laccases have been identified and they also display stunned growth. Repetitions are ongoing in order to confidently identify lines with intermediate knock down and less severe phenotypes. Severe and intermediate phenotypes will be characterized further with respect to metabolic profiling, leaf mechanical properties and cell wall morphology.
Isolation of *FLOWERING LOCUS T* in oil palm

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The oil palm (*Elaeis guineensis* Jacq.) is the most important source of vegetable oil. There are three varieties based on quantity of oil and fruit characteristics; thick shelled Dura, thin shelled Pisifera and a hybrid between Dura and Pisifera named Tenera. Oil palm displays a cyclic flowering habit where male and female reproduction organs alternate on the same plant. Each year, the ratio of male inflorescence is higher than female in an individual palm tree. Consequently, the flowering time has an impact on field performance in terms of overall biomass, quality, and seed production. Genetic control of flowering genes in oil palm is unclear. Therefore, isolation of *FLOWERING LOCUS T* (*FT*) gene was undertaken. The *FT* gene from three varieties of oil palm leaves was isolated by PCR with primers based on date palm sequences. The results showed that the gene was highly conserved among varieties and compared to date palm. The *FT* gene was 1,727 bp in length and contained a coding sequence of 531 bp encoding a putative protein of 176 amino acids. Sequence analysis using the deduced amino acid sequence revealed homology to the *FT* genes of *Cymbidium goeringii* (ADI58462.1) and *Ananas comosus* (ADU15498.1) with 87 and 85% identities, respectively and 81% identity to the *FT* gene of *Aegilops tauschii* (ABI34864.1), *Triticum aestivum* (AAW23034.1), *Lolium perenne* (CBN73215.1) and *Hordeum vulgare* (ADW82821.1). These results suggest that the *FT* gene in oil palm could function to promote flowering. Further experiments by RT-qPCR will investigate how *FT* gene expression is regulated in oil palm.
Induction and identification of mutations in barley seedlings after the transformation with target specific TAL effector nucleases.

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Recent advances in sequencing technologies have led to the availability of comprehensive sequence information of several major commodity crops. However, utilizing this information by targeting and manipulating specific genes remains a major challenge in crop breeding. Site directed mutagenesis using custom nucleases such as Meganucleases and Zinc Finger Nucleases has been reported for several organisms. These nucleases can be designed to create double stranded breaks (DSBs) at specific sites in a genome and thereby allow for the generation of mutations at the location. However, so far the implementation of these nucleases has been limited due to the high cost and the expertise required.

Recently a novel nuclease, a transcription activator-like effector nuclease (TALEN), was developed (Christian et al., 2010). The advantage of custom TALENs lies in the ease of assembly and the cost effectiveness. In plants the technology is still at its infancy but recently TALENs have been shown to induce DSBs in rice, which led to mutations affecting the susceptibility to a pathogen (Li et al., 2012).

We have previously presented our work in establishing the TALEN technology as a genome editing tool for barley gene modification (Wendt et al., 2012). Now we have assembled several TALENs and tested their ability to cleave their target in vivo in a modified yeast-2-hybrid assay. One TALEN was selected for genetic transformation studies in immature barley embryos. Transgenic barley plants were regenerated from callus and using an enrichment assay we were able to show that the TALEN frequently induced mutations at the selected location in the tested seedlings. The identified mutations were deletions in the range between 1 and 20bp. Further analysis showed that different somatic cells in individual plants contained different mutations, indicating that mutation events occurred (i) during plant development following transformation and (ii) independently in different cells. The necessity for enrichment indicates that only a fraction of the total cells of one individual carry mutations. Present research efforts focus on establishing whether these mutations are heritable in successive generations. In parallel, new TALEN constructs are developed to control the temporal and spatial activity of the TALEN constructs after their introduction into barley.

References:
Phytic acid (PA, InSp₆) is the main storage form of phosphorus in cereal seeds. PA is considered an anti-nutritional factor as it is a strong chelator of iron and other minerals, reducing their bioavailability. This phenomenon may contribute to "hidden hunger" in human populations where cereals are the primary source of nutrition. In husbandry the main problem is that monogastric animals cannot digest and utilize PA, which might result in environmental phosphate pollution due to excretion of PA. Reduced content of PA in the grain is thus a target for molecular breeding of wheat in order to improve quality of the grain for both food and feed.

Genome wide association study (GWAS) is a method to identify significant associations between markers and traits of interest in large collections of e.g. wheat varieties, and can be used to dissect complex quantitative traits and identify candidate genes for the trait(s) of interest. In the present study we have evaluated a collection of 169 hexaploid wheat varieties grown under ecological conditions at Agrologica, Denmark as part of the BIOBREED project. Content of phytic acid phosphorous (PA-P) and Pi in the grain was quantified using colorimetric assays and were found to range from 3.9-5.5 mg PA-P/g seed and 0.27-1.26 mg Pi/g seed, respectively. Neither PA-P nor Pi content was correlated to grain weight. These phenotypic data will be combined with genotypic data from approximately 2500 polymorphic DArT (Diversity Arrays Technology) markers distributed over the wheat genome to identify markers associated with the two traits. This may increase our understanding on the genetic regulation of PA and Pi content in the wheat grain and may in the future lead to development of markers useful for breeding.

1http://molbreed.life.ku.dk:8080/biobreed
The Perennial Ryegrass GenomeZipper – Targeted Use of Genome Resources for Comparative Grass Genomics

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Whole-genome sequences established for model and major crop species constitute a key resource for advanced genomic research. For outbreeding forage and turf grass species like ryegrasses (*Lolium* spp.), such resources are yet to be developed. Here, we present a model of the perennial ryegrass (*Lolium perenne* L.) genome on the basis of conserved synteny to barley (*Hordeum vulgare* L.) and the model grass genome *Brachypodium* (*Brachypodium distachyon* L.), as well as rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor*). A transcriptome-based genetic linkage map of perennial ryegrass served as a scaffold to establish the chromosomal arrangement of syntenic genes from model grass species. This scaffold revealed a high degree of synteny and macro-collinearity, and was then utilised to anchor a collection of perennial ryegrass genes *in silico* to their predicted genome position. This resulted in the unambiguous assignment of 3,315 out of 8,876 previously unmapped genes to the respective chromosomes. In total, the GenomeZipper incorporates 4,035 conserved grass gene loci which were used for the first genome-wide sequence divergence analysis between perennial ryegrass, barley, *Brachypodium*, rice, and sorghum. The perennial ryegrass GenomeZipper is an ordered, information-rich genome scaffold, facilitating map-based cloning and genome assembly in perennial ryegrass and closely related Poaceae species. It also represents a milestone in describing synteny between perennial ryegrass and fully sequenced model grass genomes, thereby increasing our understanding of genome organization and evolution in the most important temperate forage and turf grass species.
The potato tuber mitochondrial proteome

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Mitochondria fulfill some basic roles in all plant cells. They supply the cell with energy in the form of ATP and reducing equivalents (NAD(P)H) and contribute to a number of specialized functions and processes depending on the tissue and cell type, as well as environmental conditions. Bioinformatic analysis of the Arabidopsis genome, using primarily prediction algorithms, have estimated that mitochondria may contain as many as 2000-3000 unique proteins (Millar et al., 2005;Millar et al., 2006;Cui et al., 2011). Surprisingly, 20 years into the proteomics era no more than 500 unique proteins have been experimentally confirmed from any plant mitochondrial source! (Taylor et al., 2011). To further interrogate this system, we have performed an in-depth proteomic study of mitochondria from potato tubers. Mitochondria were isolated using Percoll gradients and 0.3 mg of protein was pre-fractionated using 1D gel electrophoresis. Three replicates of resolved mitochondrial protein was sectioned into 40 gel slices for in-gel digestion with trypsin followed by LC-MS. Data mining was performed against the translated potato genome (Potato Genome Sequencing et al., 2011) using four different search algorithms. In total, 1060 proteins were identified with a false discovery rate less than 1%. Using five different localization prediction tools, 65% of MS-detected proteins were predicted to be mitochondrial, although many known mitochondrial proteins were not properly targeted using these programs. Compared to previous studies many of the identified proteins were small and hydrophilic. The functional categories most enhanced were RNA processing (including 71 PPR proteins) and protein synthesis, but we also found many membrane transporters. Lastly, a large number of posttranslational modifications were identified ranging from acetylation and methylation to phosphorylation and different forms of oxidation. We estimate that we have achieved a coverage of the potato tuber mitochondrial proteome approaching 80%, discovered new functions, and identified hitherto unknown components of known metabolic pathways.

References:
Identification of cis-elements in abscisic acid inducible promoters by a new approach to sequence analysis

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Identification of cis-regulatory elements in DNA promoters by computational approaches are a great help in genome-wide elucidation of signal transduction, transcriptional regulation and gene expression. However, to accomplish this has proven a difficult problem in computational genomics in part due to the redundant nature of cis-regulatory elements1. Moreover, promoter context and chromatin structure have an important influence on the function of cis-elements and does further complicate the identification of functional cis-elements2,3.

A related computational approach to promoter studies is to use the sequences of known cis-elements for promoter prediction4. This is a computational alternative to genome-wide DNA array analysis of gene expression and can be used to confirm the importance of known promoter elements on a genome level. Unfortunately, this approach does not yield information about new cis-regulatory elements.

We have developed a method for identifying short, conserved motifs in biological sequences such as proteins, DNA and RNA5.

This method was used for analysis of approximately 2000 Arabidopsis thaliana promoters that have been shown by DNA array analysis to be induced by abscisic acid6. These promoters were compared to 28000 promoters that are not induced by abscisic acid. The analysis identified previously described ABA-inducible promoter elements such as ABRE, CE3 and CRT1 but also new cis-elements were found. Furthermore, the list of DNA elements could be used to predict ABA-inducible promoter with more than 70 % accuracy for the top 40 predictions, which is better than previously described methods4.

In conclusion, identification of short, conserved motifs is a useful computational approach for identification of cis-elements in DNA promoters and for promoter prediction in genome analysis.

References:
Protein processing the in endomembrane pathway of Arabidopsis

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Free flow electrophoresis (FFE) is a powerful and flexible technique used for the separation of peptides, organelles and cells according to surface charge. Using FFE, techniques have been developed for isolation of Golgi vesicles from plant cell suspension cultures at approximately 80% purity levels (Parsons et al. 2012), an unprecedented improvement on previous purity levels. This allowed in-depth proteomic characterization of the plant Golgi (Parsons et al. 2012). Many of the post-translational modifications in proteins occur in the ER (endoplasmic reticulum) and Golgi. Understanding these modifications is key to understanding protein interactions, protein trafficking and enzyme activity. In the better-characterised examples of protein post-translational modification in plants, sequential modifications are thought to occur in the Golgi with levels of complexity increasing in later Golgi compartments. A gradient of surface charge is proposed to exist across the compartments of the endomembrane pathway. As a result it has been possible to partially separate ER vesicles, and Golgi sub-compartments (cis-, medial- and trans-cisternae) using FFE. However, these isolation and separation techniques were developed in Arabidopsis cell suspension cultures. These cultures are very difficult to transform and so, despite this partial separation of subcompartments, analysis of progressive protein modifications has been limited. At Copenhagen University these techniques are being extended to liquid-grown Arabidopsis plantlets. Once separation has been demonstrated in plantlets, this will allow detailed analysis of proteins in the secretory pathway in plants transformed with tagged proteins that are known to undergo glycosylation and other forms of protein processing. This will allow specific hypothesis concerning protein fate in the secretory pathway to be tested and greatly improve our understanding of protein processing in plants.

Understanding cell wall diversity and evolution: An integrated glycomic and genomic approach

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Cell walls are a defining feature of extant plants and there is increasing evidence that they were vitally important during the colonisation of land around 450 million years ago, and during the subsequent global radiation of plant life. The study of cell wall diversity underpins our attempts to understand the evolution of cell wall components and is also crucial for the optimal utilisation of the largest source of biomass on earth. The comprehensive survey of plant cell wall compositions and architectures across the plant kingdom presents considerable difficulties. Even if a large collection of suitable plant species can be assembled, one is faced with the challenges inherent in sampling all cell wall types and their subsequent high-throughput analysis. Moreover, a survey of polysaccharides per se often prompts further questions relating to their genetic origins and the relative contributions of conserved versus convergent evolution and lateral gene transfer.

We have implemented an integrated omics approach for understanding cell wall diversity and evolution. Plant species are collected that as far as possible represent the most significant taxa or morphotypes in the plant kingdom – from the chlorophytes to angiosperms. High throughout cell wall profiling is performed using immuno-glycoarrays which provide information about the polysaccharide content of the cell walls in each plant type. In parallel, we are mining genome and transcriptome data sets in order to identify cell wall related glycosyl transferases in phylogenetically diverse species. The integration of these two data sets provides insight into the timing and mechanisms of the emergence of cell wall components. Initial findings will be presented with particular focus on green algae and the origins (1→4)-β-D-mannan.
Cell wall evolution and diversity of green algae

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Plant cell walls are highly sophisticated fibre composite structures that have evolved to fulfill a wide range of biological roles that are central to plant life. Some cell wall components have ancient prokaryotic origins and others are present in extant descendents of the algal ancestors of land plants. Using carbohydrate microarray technology we have found that many polysaccharides previously thought to have arisen after the colonization of land, are present in Charophycean green algae (CGA). This may imply that the ability to produce particular cell wall components was an aspect of pre-adaptation that enabled the ancestors of the CGA to make the transition to land. However, our current understanding of the genetic mechanisms underling the early evolution of plant cell walls is limited.

We have developed a multi-disciplinary, multi-layered strategy for sampling cell wall polysaccharides and genetic diversity across the plant kingdom (including Chlorophyte and Charophyte algae). The first layer consists of primary screens for polysaccharides based on carbohydrate microarrays, and for cell wall biosynthetic genes (glycosyl transferases, GTs) based on established bioinformatics techniques. A second layer of analysis seeks to obtain more detailed information about certain polysaccharides and genes from subsets of plants and algae. This second layer is based mostly on established biochemical methods for polysaccharide analysis whilst elected genes are cloned and their sequences confirmed. In some cases, genes are expressed and biochemical activities of GTs determined through heterologous expression. Furthermore, we are also investigating functional conservation of some early GTs from Chlorophytes, CGA and Physcomitrella patens by knocking them into Arabidopsis thaliana mutants that are defective in putative orthologues of those genes.
PROFILING OF THE COMPOSITION AND SECRETOMES OF FUNGAL COMMUNITY OF BARLEY GRAINS AND DETERMINATION OF THE XYLANOLYTIC AND XYLANASE INHIBITOR ACTIVITIES IN DIFFERENT BARLEY CULTIVARS

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Xylanolytic enzymes (e.g. xylanases, arabinofuranosidases, etc.), widely produced by fungi, actinomycetes and some bacteria, have various biotechnological applications including the improvement of animal feed, the quality of baked products, etc. [1,2]. Endo-β-1,4-xylanases (EC 3.2.1.8) hydrolyze internal β-1,4-glycosidic linkages between xylose units in the arabinoxylan backbone of cell walls. Cereals like barley produce xylanases for remodeling of the cell walls during seed development and germination [3]. In addition, cereals also contain xylanases produced by microorganisms residing on surface/outer kernel layers of the grains. Accumulating evidence show that microbial xylanases are highly sensitive to inhibition by known proteinaceous xylanase inhibitors (i.e. TAXI, XIP and TLXI) present in cereal grains, whereas the plant xylanases are uninhibited [4].

In the current study, the variability of xylanase (plant vs. microbial) and xylanase inhibition activities in different barley cultivars was investigated with application of different techniques including activity assays, zymograms and gel-based proteomics analysis (2DE and MALDI-TOF MS, MS/MS) coupled with immunoblotting. The indigenous fungal community and proteins present on the surface/outer layers of different barley cultivars were profiled using 2DE and MS, together with microscopic evaluation and metabolite profiling of fungi isolated from grain surfaces.

Activity measurements show that there is considerable inter-cultivar variability in the level of both microbial and endogenous xylanase activities, as well as at xylanase inhibitor levels. 2D-immunoblots probed with anti-TAXI/XIP/LXI polyclonal antibodies enabled detection of the occurrence, variability and different isoforms of the different xylanase inhibitors. Additional experiments will reveal whether the observed trends are a function of genetic, environmental factors and/or their interaction. Moreover, xylanase activity levels were highly dependent on the composition of the fungal community. The cereal storage fungi, e.g. Aspergilli and Penicillia, were found to be prominent producers of xylanases compared to the field fungi. Moreover, the secretomes of Aspergillus and Fusarium upon growth on wheat arabinoxylan and barley flour were also profiled and the protein identified.

References:

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.
A bioinformatics approach to study repression of gametophyte specific genes in the sporophyte

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Among the most highly expressed genes in pollen are four isoforms of the Arabidopsis plasma membrane H+-ATPase family (AHA). Remarkably, these four isoforms (AHA3, AHA6, AHA8, AHA9) are expressed with strong spatial constrains in the different stages of developing pollen and the growing pollen tube, but not in any tissue of the sporophyte. Until now little is known about the signals and players leading to such a tissue specific and strong expression of genes. Drawing on this lack of knowledge, our aim is to understand the transcriptional regulation during pollen and sporophyte development, using the plasma membrane H+-ATPase family as a model.

To investigate how genes coding for H+-ATPases expressed in the male gametophyte are repressed in the sporophyte, we first performed in silico studies of epigenetic marks in Arabidopsis seedlings. Interestingly, while many pollen specific genes carry a specific histone modification, trimethylation of histone H3 lysine 27 (H3K27me3) in seedlings, AHA3, AHA6, AHA8 and AHA9 do not. We then aimed to find out whether known transcription factors play a role in the strong pollen specificity of H+-ATPases. In the promoter sequences of all four isoforms we identified the target motifs for AtMIKC* transcription factors, namely CArG boxes and MEF2 binding sites. We are currently characterizing Arabidopsis aha8 and aha9 knock out lines and analyze the promoter regions of AHA8 and AHA9 by complementation studies using mutated promoter regions (deletions, insertions or base changes). Arabidopsis plants carrying these inserts are studied by real time PCR to measure differences in AHA8 and AHA9 transcript levels, in both organs of the sporophyte and pollen at different developmental stages. Results of our analysis will provide knowledge relevant for understanding the duality of transcriptional repression in the sporophyte followed by activation in the male gametophyte.
Redirecting photosynthetic reducing power towards bioactive natural product synthesis

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Besides the products of photosynthesis, the chloroplast provides the energy and carbon building blocks required for synthesis of a wealth of bioactive natural products of which many have potential uses as pharmaceuticals. In the course of plant evolution, energy generation and biosynthetic capacities have been compartmentalized. Chloroplast photosynthesis provides ATP and NADPH as well as carbon sources for primary metabolism. Cytochrome P450 monooxygenases (P450s) in the endoplasmic reticulum (ER) synthesize a wide spectrum of bioactive natural products, powered by single electron transfers from NADPH. P450s are present in low amounts and the reactions proceed relatively slowly due to limiting concentrations of NADPH. Here we demonstrate that it is possible to break the evolutionary compartmentalization of energy generation and P450-catalysed biosynthesis, by relocating an entire P450 dependent pathway to the chloroplast and driving the pathway by direct use of the reducing power generated by photosystem I in a light-dependent manner. The study demonstrates the potential of transferring pathways for structurally complex high-value natural products to the chloroplast and directly tapping into the reducing power generated by photosynthesis to drive the P450s using water as the primary electron donor. We analysed plants transiently expressing the entire dhurrin pathway and stable transformants expressing CYP79A1. Current work is directed towards establishing stable tobacco transformants with multi-enzyme pathways expressed in the chloroplast able to produce interesting bioactive compounds directly driven by light in significantly larger quantities than can be achieved in their natural hosts.
Eliminating bottle necks in metabolic engineering of the cancer-preventive glucosinolate - Glucoraphanin

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Glucosinolates (GLs) are specialized bioactive compounds characteristic to plants of the Brassicaceae family, including the model plant Arabidopsis thaliana. GLs are important key players in the plant’s natural defense system against herbivores and microorganisms. Additional biological functions were assigned to GLs ranging from flavor compounds to bio-pesticides. Particularly, glucoraphanin, the major glucosinolate in broccoli has been associated with broccoli’s cancer-preventive properties.

Glucoraphanin is derived from the essential amino acid methionine that first undergoes a series of reactions to form the chain-elongated dihomomethionine. Methionine chain-elongation requires activities of at least four enzymes (BCAT4, MAM1, IPMI and IPMDH); the latter three are localized to the chloroplast.

Recently, we have demonstrated that it is feasible to engineer the pathway of glucoraphanin biosynthesis from A. thaliana into Nicotiana benthamiana by transient expression[1]. One major bottleneck is the low availability of free methionine in plants (~5-15 nmol/g fresh weight). This is especially due to a very tight regulation of the first committed step in the de novo methionine biosynthesis catalyzed by cystathionine gamma-synthase (CGS). By removing regulatory motifs from CGS we succeeded in supplying the chain-elongation machinery with amounts comparable to external application of methionine in our transient expression system. An alternative approach is targeted to highjack the regeneration system of methionine from S-adenosylmethionine which is a universal methyl donor in plant cells[2]. The transient expression system in tobacco serves as a platform to test and optimize the individual parts of glucoraphanin biosynthesis. The goal is to ultimately transfer all enzymes of the complex pathway into yeast using our stable integration yeast expression platform. Using this platform, we have successfully produced simple GLs derived from protein amino acids[3].

Cyanogenic glucosides are amino-acid derived plant chemical defense compounds against generalist herbivores. The subtropical crop plant *Sorghum bicolor* synthesizes the L-tyrosine derived cyanogenic glucoside dhurrin through the activity of two multifunctional cytochrome P450s (CYPs), CYP79A1 and CYP71E1 and an UDP-glucosyltransferase UGT85B1. The two CYPs are dependent on electrons donated from the cytochrome P450 oxidoreductase (POR) for activity. Upon tissue disruption dhurrin is deglucosylated to an α-hydroxynitrile by specific β-glucosidases. The α-hydroxynitrile is unstable and will dissociate into hydrogen cyanide and a keto compound.

The biosynthesis of dhurrin is highly channeled as only trace amounts of intermediates are detected *in planta*, therefore it is thought that the biosynthetic enzymes form transient enzyme complexes, metabolons (1,2). The CYP79A1, CYP71E1 and POR are all ER localized by an N-terminal anchor, whereas the UGT85B1 is thought to be cytosolic with tight association to the ER. Fluorescence resonance energy transfer (FRET) studies have shown that the soluble UGT is associated close to the ER localized CYPs, which enables efficient glycosylation and stabilization of the labile α-hydroxynitrile produced by the CYPs (3). Protein homology modeling has revealed that a hydrophobic patch in the loop B region of the sorghum UGT85B1 might be involved in the interaction with the membrane-bound CYPs.

This project aims to understand the role of loop B in the coupling of the UGT with the dhurrin metabolon. Different UGT constructs have been made with alternative versions of the loop B region to investigate the importance of the loop structure for mediating interaction. Seven constructs have been made in total to highlight whether or not loop B is the site of interaction between the cytosolic UGT and the membrane-bound CYPs. These UGT versions are analyzed by coupled activity assays with the CYPs from microsome preparations or purified liposome reconstituted enzymes. Furthermore, interactions between CYP79A1 or CYP71E1 reconstituted in nanoscale lipid bilayer discs, nanodiscs, with the different UGT versions are analyzed by quartz crystal microbalance (QCM). Upon formation of a nanodisc monolayer at the quartz crystal, containing either CYP79A1 or CYP71E1, the binding of the UGT is monitored by a shift in the density of the immobilized layer.

We believe that these studies will provide a detailed understanding of the formation of metabolons in the biosynthesis of plant natural products. Furthermore, the synthetic biology approach might enable the modification of UGTs in order to design desirable glycosylation patterns of bio-active compounds.

Glycoproteome analysis of wheat albumins by ZIC-cotton HILIC

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Hydrophilic liquid chromatography (HILIC) has been used extensively for glycopeptides enrichment by several different types of HILIC material. Here, a quick and easy strategy to enrich glycopeptides was pursued using a combination of two HILIC materials: ZIC-HILIC (Zwitterionic Hydrophilic Interaction Liquid Chromatography) and Cotton for enrichment in both ion-pairing and non ion-pairing mode. With this approach together with site-specific glycosylation labeling and LC-MS/MS, 46 different glycosylation sites were assigned for 40 albumin proteins from wheat grains. Most of identified glycoproteins from wheat albumins are homologous to defense related proteins in plant and food allergens. This global glycosylation analysis provided more information on wheat flour albumin components for future study to improve understanding on protein structures and properties related to the defense mechanism and immunogenicity. Moreover, this study may also provide targets for further study on wheat beer quality.
Engineering the MEV pathway in Physcomitrella patens for efficient terpenoid production

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Terpenoids are the oldest and structurally most diverse class of specialized metabolites in all plants and they are widely used as flavors, fragrances, pharmaceuticals and nutraceuticals. Chemical synthesis of terpenoids is often not industrially feasible due to the complexity of the structures, especially >C15 terpenoids including sesquiter- and diterpenoids. Therefore, biochemical synthesis is an attractive alternative due to the increasing availability of terpenoid synthase genes and knowledge of the terpenoid biosynthetic pathways in different organisms in recent years, including the two pathways supplying the ubiquitous C5 building blocks, the MEV (mevalonate) pathway and the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway.

Here we report production in high yield of a valuable sesquiterpenoid in a novel plant platform Physcomitrella patens (moss) using only light and atmospheric CO2 without any external carbon source. The results open up a promising industrial application of moss for production of high-value terpenoid products.

To boost terpenoid production, a truncated version of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) has been overexpressed in moss. HMGR is generally regarded as the enzyme responsible for the major rate-limiting step of the MEV pathway and the truncation is expected to result in an enzyme insensitive to feedback regulation, ultimately increasing the metabolic flux towards desired terpenoid products.
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